The Integrative Physiology of the Plasma Membrane Calcium ATPase in Platelets

Antonia Bibiana Solomon

December 2011

A thesis submitted for the degree of
Doctor of Philosophy of Imperial College London

Investigations described in this thesis were performed at the:
Platelet Biology Group
National Heart and Lung Institute
Molecular Medicine Section
Sir Alexander Fleming Building
Imperial College London
Exhibition Road
London, SW7 2AZ
United Kingdom

Imperial College London

DECLARATION

I, Antonia Bibiana Solomon confirm that this work submitted for assessment is my own and is expressed in my own words. Any uses made within it of the works of other authors in any form (e.g. ideas, equations, figures, text, tables, programmes) are properly acknowledged at the point of their use. A full list of the references employed has been included.

Signed…….

Date…….

A promise once made, is my debt now paid.

For Mabs

Who always believed in me...

"One's mind, once stretched by a new idea, never regains its original dimensions."

Oliver Wendell Holmes, Sr. (1809-1894

ABSTRACT

Plasma Membrane Calcium-ATPases (PMCA's) extrude calcium from a variety of cell types and have recently been shown to regulate signalling events and function in the cardiovascular system. PMCA (isoform 4) is expressed in platelets, however its functional role remains undefined.

The aims of this project were to investigate the roles of PMCA in regulating calcium homeostasis and human platelet function during the various stages of platelet activation by evaluating the effects of a pharmacological inhibitor of PMCA, carboxyeosin, upon a range of *in vitro* and *in vivo* assays. Additional experiments were performed to determine the functional consequences of PMCA4 ablation using platelets from PMCA4 knock-out mice and the roles of PMCA were contrasted with other calcium transporters. Finally, the mechanism of action of PMCA in platelets was investigated by integrating molecular studies with the functional assays.

The findings presented in this thesis suggest that PMCA plays an important role in regulating calcium both basally and during platelet activation. PMCA positively regulates platelet aggregation through the modulation of negative signalling pathways but negatively regulates the earlier and later stages of activation measured as adhesion and clot retraction respectively. PMCA also regulates platelet function *in vivo*. Experiments in PMCA4 knock-out mice implicated the PMCA4 isoform in the regulation of platelet function. Comparison of PMCA, SERCA (sarco-endoplasmic reticulum ATPase) and NCX (Na^+/Ca^{2+}-exchanger) pumps indicated that all three calcium transporters play pivotal but contrasting roles in platelet function and calcium homeostasis.

In conclusion, PMCA is a key regulator of calcium homeostasis and differentially regulates the various stages of platelet activation. PMCA regulates intracellular signalling events in platelets although the mechanisms remain to be fully determined. PMCA is therefore a key modulator of haemostasis and thrombosis and may be a potential target in the treatment of conditions such as arterial thrombosis and stroke.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Dr. Mike Emerson who has supported me throughout this project with his patience and knowledge whilst allowing me the room to work in my own way. I attribute the level of my work to his encouragement and effort and without him this thesis, too, would not have been completed or written. Secondly I'd like to thank my secondary supervisor, Prof. Jane Mitchell for continuously being there during the early (more daunting) years of my project and mentor Dr. Birgit Leitinger for all her advice and one to one coffee trips we shared.

I would also like to thank all my colleagues in the lab, past and present, for their support and helpful advice, especially Sarah for 'showing me the ropes' when I first joined.
A big thank you goes to David for being humorous and always making me smile. Your expertise in the field of cardiovascular physiology was a great help. Thank you to Harry for all the fun times sitting in 'cattle class' and introducing me to all the *in vivo* techniques (how you managed three years in a windowless room, I'll never know). To Chris for his continuing help with the mice, listening to Ricky Gervais podcasts will always remind me of you. And finally Lisa Holbrook, for her support and advice during my final year, I really will miss 'our little trips to stores together'.

I would also like to thank Prof. Ludwig Neyses, Florence and Ellie at Manchester University for the mice and help genotyping and Daniel in the CBS for looking after the litters. I would also like to thank Paul Kemp for spending much time giving me encouraging and helpful advice. I also owe a big thank you to all the blood donors who have been bled throughout this project and the National Heart and Lung Foundation for funding this study.

I would like to thank my lovely friends, Sara (Yorkshire pudding) and Biljana (Sweden) for the fun times outside the lab, Ellie, Emma and Eleanor for all their kind words and Jeannie for her support. I would also like to thank Musole, for putting up with me and sharing the good times with a bottle of wine great nights out in London.

Finally, I would like to say a special thank you to Tomasz, whose love and continual support in the past few months did not go unnoticed. Without the freshly made coffee that was so expertly made in the mornings, patience, encouragement and shoulder to lean on, I would not have finished this thesis. Last but certainly not least I'd like to thank my mum and Neil for their love and unfailing support throughout this project and my cat, Leah, for being the cutest but fussiest best friend in the world.

PUBLICATIONS, PRESENTATIONS & AWARDS

Publication: Jones A[#]., **Solomon A**[#]., Sanz-Rosa D., Moore C., Holbrook L., Cartwright EJ., Neyses L & Emerson M. 'The Plasma Membrane Calcium ATPase (PMCA) modulates calcium homeostasis, intracellular signalling events and function in platelets.' Journal of Thrombosis and Haemostasis. December 2010, Vol 8, Pages 2766-2774.

[#] *Made an equal contribution*

Publication: Tymvios C., Moore C., Jones S., **Solomon A**., Sanz-Rosa D & Emerson M. 'Platelet aggregation responses are critically regulated in vivo by endogenous nitric oxide but not by endothelial nitric oxide synthase'. British Journal of Pharmacology. December 2009;158(7):1735-42.

Abstract: **Solomon A**., Jones J., Sanz-Rosa D., Holbrook L., Moore C & Emerson M.
'The Plasma Membrane Calcium ATPase Regulates Homeostasis, Function and Negative Signalling in Platelets.' Circulation.Vol 122, Issue 21, Supplement; November 23, 2010/Abstracts from the American Heart Association Scientific Sessions, Chicago, USA.

Abstract: **Solomon A**., Jones S., Sanz-Rosa D., Tymvios C., Moore C & Emerson M.
'De La Membrana Plasmática Calcio ATPasa: Una novedosa diana terapéutica en el tratamiento de la trombosis asociada hypertension.' Congress: Hipertensión y Riesgo Cardiovascular. Vol 27. Feburary 2010. Poster and Oral Presentation

Abstract: **Solomon A**., Jones S., Sanz-Rosa D & Emerson M.
'A comparative study of calcium homeostasis and function following inhibition of calcium channels in human platelets.' Published on PA2 Online British Pharmacological Society, BPS Winter Meeting. Catalogue, 1, Vol 7, Issue 4. December 2009. Poster.

Abstract: **Solomon A**., Jones S., Sanz-Rosa D & Emerson M.
'The Plasma Membrane Calcium ATPase regulates calcium homeostasis and platelet function.' Journal of Thrombosis and Haemostasis ISHT Supplement Addition. Vol 7, Issue Supplement. July 2009. Poster

Abstract: **Solomon A**., Jones S., & Emerson M.
'Functional roles of the Plasma Membrane Calcium ATPase in platelets.' Fundamental and Clinical Pharmacology. Vol 22:73-73. August 2008. Poster

---

Award: International Society of Thrombosis and Haemostasis: Awarded a commendation prize for having an abstract rated in the top third within the platelet physiology category. Boston, USA, 2009.

LIST OF ABBREVIATIONS

-/-	Homozygous (Knock Out)
[^{3}H]-5HT	Tritiated 5-hydroxytryptamine (serotonin)
[Ca^{2+}]$_i$	Intracellular calcium concentration
+/-	Heterozygous
+/+	Wild Type
µ	Micro
µm	Micrometer
µm^3	Micrometer cubed
^{111}In	^{111}Indium Oxine
5-HT	5-hydroxytryptamine (serotonin)
AA	Arachidonic Acid
AC	Adenylate cyclase
ACD	Acidic-citric acid-dextrose
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BHQ	2, 5-di-(tert-butyl)-1,4-benzohydroquinone
bp	Base pair
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CaM	Calmodulin
cAMP	Cyclic adenosine 3'-5'-phosphate

CE	Carboxyeosin
CMFB	Calcium and Magnesium free Modified Tyrodes HEPES Buffer
COX	Cyclooxygenase
DAG	1,2- diacylglycerol
ddH_20	Double distilled H_20 (water)
DNA	Dexoyribonucleic acid
DTS	Dense tubular system
ECL	Enhanced chemiluminescence system
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
eNOS	Endothelial nitric oxide synthase or NOSIII
EPO	Erythropoietin
ER	Endoplasmic reticulum
FcRγ	FC receptor gamma chain
Fg	Fibrinogen
FITC	Fluorescein isothiocyanate
g	Gravitational force (centrifugal force)
GDP	Guanosine diphosphate
GP	Glycoprotein
GPCR	G-protein coupled receptor
GPCRK	G-protein coupled receptor kinase

GPIb-V-IX	Glycoprotein Ib-V-IX
G-protein	GTP binding protein
GPVI	Glycoprotein VI
GTP	Guanosine triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HETE	Hydroxy-eicosatetranoic acid
HRP	Horseradish peroxidise
HUS	Haemolytic-uremic syndrome
Ig	Immunoglobulin
iNOS	Inducible nitric oxide synthase or NOSII
IP_3	Inositol-1,4,5-trisphosphate
IP_3R	Inositol 1.4.5 triphosphate receptor
ITAM	Immunoreceptor tyrosine-based activation motif
K^+	Potassium
kDa	Kilodaltons
l/L	Litres
LRR	Leucine-rich-repeat
m	Milli
M	Molar
mA	Milliamps
mAb	Monoclonal antibody
MBq	Mega Becquerel
Mg^{2+}	Magnesium

MLCK	Myosin light chain kinase
mTHB	Modified Tyrodes HEPES buffer
MW	Molecular weight
n	Nano
NCX	Sodium (Na^+)-Calcium (Ca^{2+}) Exchanger
ng	Nanograms
NKA	Sodium (Na^+)-Potassium (K^+) adenosine triphosphatase
nM	Nanomolar
nm	Nanometre
nNOS	Neuronal nitric oxide synthase or NOSI
NO	Nitric oxide
OCS	Open canalicular system
Orai1	Ca^{2+} release-activated Ca^{2+}channel protein 1
P2	Purinergic 2
PAF	Platelet activating factor
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PECAM 1	Platelet endothelial cell adhesion molecule 1
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin

PI3K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidyl inositol 4,5 -bisphosphate
PIP_3	Phosphatidyl inositol 3,4,5 - trisphosphate
PKA/C/G	Protein kinase A/C/G
PLA_2	Phospholipase A_2
PLC_β	Phospholipase C_β
$PLC_{\gamma 2}$	Phospholipase $C_{\gamma 2}$
PM	Plasma membrane
PMCA	Plasma membrane calcium adenosine triphosphatase
p-NPP	Para-nitrophenyl-phosphate
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PVDF	Polyvinylidene difluoride
RASSF1	Ras association domain-containing protein 1
RBC	Red blood cell
rcf	Relative centrifugal force
rpm	Revolutions per minute
RT-PCR	Reverse Transcription-Polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
Ser	Serine
SERCA	Sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphatase

SERT	Serotonin (5-HT) transporter
sGS	Soluble Guanylate cyclase
SOCE	Store-operated calcium entry
SPCA	Secretory pathway calcium adenosine triphosphatase
Src	Sarcoma (family of receptor kinases)
STIM1	Stromal interaction molecule 1
TAE	Tris acetate EDTA buffer
TBS-T	Tris-buffered saline-Tween
TEMED	*N,N,N',N*- tetramethylethylene-diamine
TF	Thrombokinase (Tissue Factor)
TM	Transmembrane
TP	Thromboxane receptors
TPO	Thrombopoeitin
TPS	Thrombospondin-1
Tris	Tris-hydroxymethyl aminomethane
TxA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
TXS	Thromboxane synthase
UV	Ultraviolet
v/v	Volume to volume
VASP	Vasodilator-stimulated phosphoprotein
VMAT	Vesicular monoamine transporter
vWF	von Willebrand factor

w/v	Weight to volume
α	Alpha
β	Beta
γ	Gamma
λ	Lamda

LIST OF FIGURES & TABLES

CONTENTS

CHAPTER ONE

1.0. Introduction

1.1. Platelets

1.1.1. Platelets and haemostasis

Platelets are small discoid, anuclear cells that circulate freely within the bloodstream. They play an important role in primary haemostasis i.e. the initial interaction of platelets when in contact with a damaged blood vessel and also in secondary haemostasis initiating factors with the coagulation cascade.

Under normal conditions, platelets will circulate within blood vessels without directly coming into contact with the vasculature walls. However, when a blood vessel is severed, the interface between vessel and blood is altered promoting platelet adhesion between cell surface glycoprotein (GP) receptors and newly exposed extra cellular matrix (ECM) substances such as collagen. Platelets rapidly tether to these proteins, adhere to the exposed area, and spread to form a monolayer of cells. This, combined with the initial vasoconstriction of blood vessels, minimises both the flow of blood to the wound and prevents any further blood loss.

The initial interaction of platelets to collagen promotes platelet shape change and activation through tyrosine kinase based signalling pathways , causing the release of biologically active substances, some of which are released from storage granules and some synthesised *de novo.* Soluble pro-thrombotic factors such as adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2) act as a positive feedback as well as up-regulating cell surface receptors of nearby or approaching platelets flowing past the site of injury.

Platelet aggregation then follows, leading to the activation and a conformational change of the fibrinogen integrin αIIbβ3 and the formation of platelet aggregates through divalent cross linkage of platelets to one another. Stabilisation of the platelet aggregate is promoted through endogenous factors within the coagulation cascade which can become active when damaged tissues release thrombokinase (TF, tissue factor). This promotes the important catalytic conversion of Factor X to Xa and zymogen prothrombin to thrombin, an enzymatic component of the coagulation system and also a potent platelet agonist. Thrombin in turn cleaves circulating and platelet derived fibrinogen to yield fibrin. The deposition of fibrin to the site of injury is stabilised by Factor XIIIa (Falati et al. 2002), which provides a stable mesh encapsulating passing blood cells and compositing them into the assembling thrombus. The integrity of a vessel and blood flow is normalised as a result of connective tissue formation once the underlying vascular injury has healed sufficiently and final dissolution of the clot has occurred.

1.1.2. Platelet production

It is now widely accepted that platelets are formed from megakaryocytes derived from megakaryoblasts found in bone marrow. This is achieved by a process of haematopoietic differentiation from haematopoietic stem cells under the regulation of thrombopoeitin (TPO) and its receptor c-Mpl (Italiano & Shivdasani 2003; Kaushansky 1995). TPO drives endomitosis in which the megakaryocytic DNA is replicated many times but cytokinesis does not occur (Patel et al. 2005) (S. R. Patel et al. 2005)(S. R. Patel et al. 2005)enabling the megakaryocyte to generate multiple copies of the proteins and receptors to be packaged into the platelet progeny. Megakaryocytes constitute less than 1% of all haematopoietic cells within the bone marrow (Patel et al. 2005), they can also be found to a much lesser extent in the lung (Sharma & Talbot 1986) and peripheral blood (Hume et al. 1964).

Although the origin of blood platelets was recognised over a century ago (Brodie & Russell 1897), the mechanisms by which megakaryocytes release platelets into the circulation was elusive and it is only recently that more detailed theories have surfaced to clarify this. Most theories of platelet production focus on the hypothesis that pro-platelets bud from pseudopodial extensions of the megakaryocyte (Becker & De Bruyn 1976; Italiano & Shivdasani 2003). Italiano and colleagues described that platelets were released *via* the reorganisation of the megakaryocyte cytoplasm into an extensive pseudopodial network containing immature platelets. They observed this using video microscopy to monitor the structure of the megakaryocyte

cytoskeleton at specific stages of pro-platelet morphogenesis (Italiano et al. 1999), hypothesising that platelets are released only from the ends of pro-platelets and branching observed in these pseudopodia serves to increase platelet production. However, the mechanisms by which pro-platelets enter the blood remain unclear. It has been suggested that pro-platelet extensions push between endothelial cells and protrude into sinusoidal blood vessels where pro-platelets or platelets are shed from the tips (Hartwig & Italiano 2006).

1.1.3. Platelet ultrastructure

Platelets measure between 1-3.5 μm in diameter and have a mean volume of around 7.6 μm^3. The normal human circulating platelet count ranges from 150-400 x 10^9 cells/L blood (Martin et al. 1982). In their inactive state, platelets remain disc shaped and circulate within the plasma for around 8-10 days (Stuart 1975), but their lack of nuclei, prevents them from regenerating themselves should they become damaged. Once aged, platelets are thought to be removed primarily via phagocytosis within the spleen (Hartley 2007). However, the exact physiological mechanisms controlling the removal and clearance of platelets are not fully understood.

The outer phospholipid bilayer (plasma membrane, PM) is supported by a layer of microtubules and actin filaments (submembrane specialised filaments and microfilaments) which maintain the discoid shape of resting platelets and depolymerise when aggregation occurs (Fox 2001). Furthermore, contractile proteins can operate to initiate shape change and protrusion of pseudopodia at the onset of spreading (Werner & Morgenstern 1980).

The membrane contains a number of proteins, integrins and G-protein coupled receptors (GPCR) which act as receptors for soluble agonists that are important for platelet functional responses. There are at least nine cell surface glycoproteins, some of which are important in facilitating the adhesion of platelets to damaged endothelium and also mediating cell signalling events that result in platelet aggregation (Bussel et al. 2000).

There are two types of channels expressed in platelets (Behnke 1967), a unique surface-connected open canalicular system (OCS), which comprises a series of membrane invaginations that extend from the plasma membrane into the platelet cytoplasm and serves as a transport system for substances entering or exiting the platelet (White & Escolar 1991), and a second network of channels found within platelets termed the dense tubular system (DTS), which is believed to be the remnants of the megakaryocytic endoplasmic reticulum (ER) and is widely distributed throughout the cytoplasmic region of the platelet (Cutler et al. 1978). The DTS is also a site for cyclooxygenase (COX) which is an enzyme involved in the conversion of membrane derived arachidonic acid (AA), important for TXA_2 and prostaglandin synthesis. The DTS plays an important role in platelet responses by acting as a receptor-mediated Ca^{2+} store, which undergoes rapid morphological changes that are characteristic of Ca^{2+} release in response to platelet activation (Ebbeling et al. 1992). The DTS is also a store of ATPases which regulate the activity of adenylate cyclase (AC) which is important for the regulation of signalling downstream of GPCR's (Teijeiro et al. 1999). Due to the anuclear nature of platelets, the majority of platelet protein content is determined by the parental megakaryocyte, although evidence of protein translation has been reported (Denis et al. 2005; Evangelista et al. 2006).

Platelets contain three types of secretory granules within their cytoplasm, these are known as alpha (α), dense and lysosomal granules which form stores for adhesive proteins and other small molecule agonists. Alpha granules contain proteins

involved in platelet adhesion and aggregation which include fibrinogen, fibronectin, von Willebrand factor (vWF), fibrinolysis (e.g. α-antiplasmin, plasminogen), P-selectin (Harrison & Cramer 1993; Holmsen 1994), various growth factors such as platelet derived growth factor (PDGF), epidermal growth factor (EGF) and a number of transmembrane receptor molecules including platelet endothelial cell adhesion molecule-1 (PECAM-1) and integrin αIIbβ3 (King & Reed 2002). The dense granules are a store for low molecular weight compounds involved in platelet activation such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), Ca^{2+}, magnesium (Mg^{2+}), serotonin ((5-HT), 5-hydroxytryptamine) and catecholamines. The contents of these granules are released into the peripheral blood (*via* the OCS) upon cell activation and act in either a paracrine fashion on other platelets or in an autocrine manner stimulating cell surface receptors of the secreting cell and endothelial cells (Gibbins 2004). The platelet cytoplasm also contains stored glycogen particles and mitochondria for energy generation purposes (see Figure 1.1. for a diagrammatical representation of a platelet structure). Lysosomal granules contain acidic hydrolytic enzymes, which upon secretion are thought to participate in the resolution of the haemostatic plug. Platelets also generate and release prostacyclin (a lipid ecosanoid), plasminogen activator and anti-coagulant substances heparin, heparin sulphate and nitric oxide (NO).

However, the generation of NO in platelets has been recently challenged (Gambaryan et al. 2008; Tymvios et al. 2009).

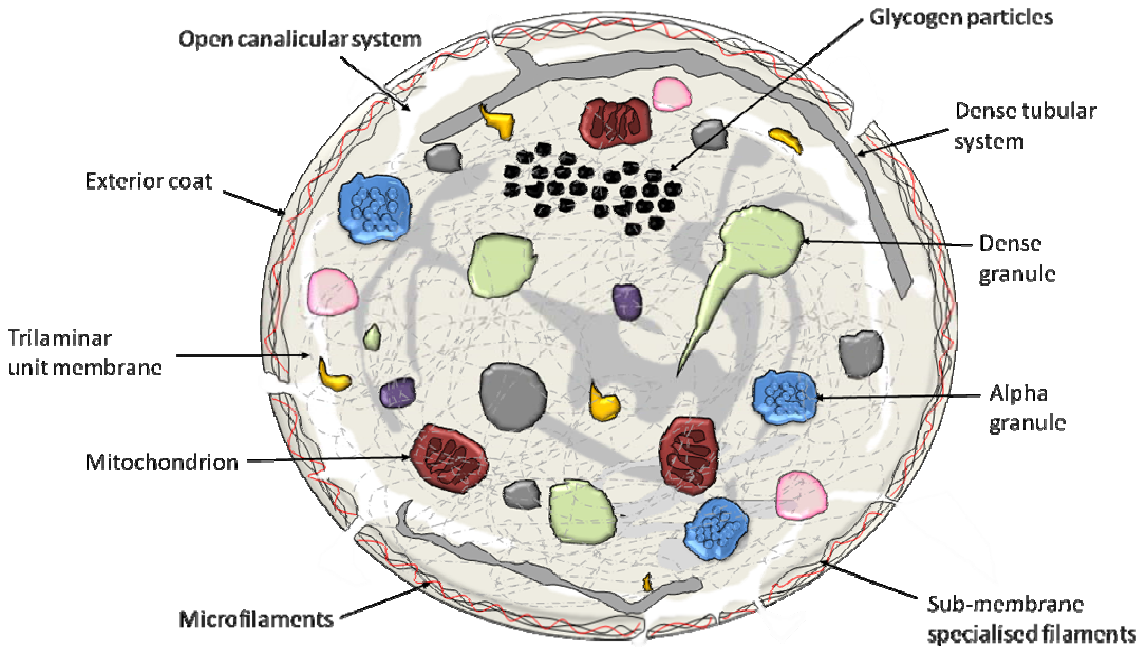


Figure 1.1. Diagram representing the ultra-structural features of a human platelet (equatorial plane)

The peripheral zone compartments include the exterior coat, trilaminar unit membrane and sub-membrane area containing specialised filaments (e.g. actin). This forms the platelet wall and channels of the surface-connected (open) canalicular system (OCS). The external membranes comprise a circumferential band of microtubules and microfilaments which are important for the maintenance of the resting platelet shape and changes to this shape upon cellular activation. Two distinct membrane systems are found in platelets, the surface-connected OCS and the dense tubular system (DTS), both of which are important for the transport of substances into or out of the cytoplasm. The platelet cytoplasm contains many organelles which are important for platelet function, including the alpha and dense granules, lysosomes, mitochondria and stored glycogen particles (White 1979).

(Modified from White J G: Am. J. Clinical Pathology 71: 363, 1979)

1.1.4. Platelets and disease

Several physiological mechanisms exist to prevent the inappropriate activation of platelets in the circulation; however these mechanisms are not infallible. Clot formation in the absence of vascular bleeding is known as thrombosis and is a common cause of mortality in developed society (Coronary Heart Disease Statistics 2008/2009, *British Heart Foundation*). Thrombosis is routinely linked with other medical conditions such as atherosclerosis, coronary heart disease, stroke and also occasionally arises as a complication during pregnancy (Davis & Branch 2010).

1.1.4.1. Atherosclerosis

Atherosclerosis for example, selectively affects medium to large size arteries and can be attributed to many factors from genetic modification to environmental factors and lifestyle. The coronary localisation of atherosclerosis results in its major clinical manifestation, coronary artery disease (Libby 2000). Atherosclerosis can propagate uniformly across the arterial tree, usually starting within the distal abdominal aorta, or can evolve in a mosaic fashion, with predilection for certain locations, such as coronary and extra-cranial vessels. Biological markers have evolved to assist with predicting an individual's risk of developing the thromboembolic complications of atherosclerosis. The levels of C-reactive protein, low density lipoprotein (LDL), high density lipoprotein (HDL) and glycaemia have all been associated either directly or inversely with the likelihood of a coronary thromboembolic event. Toxins

and reactive oxygen species (ROS) generated by tobacco smoke, modified LDL particles, diabetes and resulting glycated products, homeocysteine and infectious organisms such as herpes viruses can cause endothelial injury and, in a susceptible host, can trigger the progression of atherosclerosis (Morel et al. 1983; Libby et al. 1997; Eberhardt et al. 2000).

1.1.4.2. Platelets Interactions

Platelets represent an important link between inflammation and atherogenesis. They are able to interact with inflammatory cells including leukocytes and the endothelium where these interactions lead to leukocyte recruitment towards the vascular wall, initiating extra-vasation of circulating mononuclear cells and foam cell generation. During the adhesion process, platelets become activated and release an array of potent inflammatory and mitogenic substances into the local microenvironment, thereby altering chemotactic, adhesive, and proteolytic properties of endothelial cells. These platelet-induced alterations of the endothelial phenotype support chemotaxis, adhesion, and transmigration of monocytes to the site of inflammation. Released from dense granules, α-granules, lysosomes, the canalicular system, or the cytosol, platelets secrete or expose adhesion proteins (e.g., fibrinogen, fibronectin, vWF, thrombospondin, vitronectin, P-selectin, GPIIb/IIIa), growth factors (e.g., PDGF and TGF-β), chemokines (e.g., RANTES, platelet factor 4 (CXC chemokine ligand 4), epithelial neutrophil-activating protein (CXC chemokine ligand 5), cytokine-like factors (e.g., IL-1β, CD40 ligand, β-

thromboglobulin), and coagulation factors (e.g., factor V, factor XI, PAI-1 and plasminogen). These proteins act in a concerted and finely regulated manner to influence widely differing biological functions such as cell adhesion, cell aggregation, chemotaxis, cell survival and proliferation, coagulation, and proteolysis, all of which accelerate inflammatory processes and cell recruitment. For example, IL-1β has been identified as a major mediator of platelet-induced activation of endothelial cells (Hawrylowicz et al. 1991). The IL-1β activity expressed by platelets appears to be associated with the platelet surface, and co-incubation of endothelial cells with thrombin-activated platelets induces IL-1β–dependent secretion of IL-6 and IL-8 from nearby endothelial cells (Kaplanski et al. 1994). Furthermore, incubation of cultured endothelial cells with thrombin-stimulated platelets significantly enhances the secretion of endothelial monocyte chemo-attractant protein-1 (MCP-1) in an IL-1β–dependent manner (Gawaz et al. 2000). MCP-1 belongs to the CC family of chemokines and is thought to play a key role in the regulation of monocyte recruitment to inflamed tissue and in atherosclerosis (Boring et al. 1998; Lu et al. 1998).

1.1.4.3. Platelets and Inflammation

At sites of inflammation, recruitment of circulating leukocytes by platelets to the vascular wall requires multistep adhesive and signalling events that result in the infiltration of inflammatory cells into the blood vessel wall, including selectin-mediated attachment and rolling, leukocyte activation, integrin-mediated firm adhesion, and diapedesis. Leukocytes tether to adherent platelets *via* PSGL-1–P-selectin interactions and, subsequently, firmly adhere *via* binding of Mac-1 (CD11b/CD18, αMβ2) to GPIbα and/or other receptors of the platelet membrane, including bridging proteins such as fibrinogen (bound to αIIbβ3) or high–molecular weight kininogen (bound to GPIbα). During this adhesive process, receptor engagement of PSGL-1 and Mac-1 together with platelet-derived inflammatory compounds induces inflammatory cascades in monocytes (Weyrich et al. 1996; Neumann et al. 1997). In addition, engagement of PSGL-1 by P-selectin also drives translationally regulated expression of proteins such as uPAR, a critical surface protease receptor and regulator of integrin-mediated leukocyte adhesion *in vivo* (May et al. 2002). Because atherothrombotic diseases are a major cause of morbidity and mortality in developed countries, understanding the role of platelets in vascular inflammation and atherosclerosis is an important challenge. Major achievements have been made in elucidating the molecular mechanisms of platelet interaction with endothelial cells of the arterial wall. Defining the specific requirements for platelets adhering to endothelium may lead to the development of novel therapeutic strategies. The era of genomics and proteomics has recently been introduced in

platelet research and will continue to offer major tools to help understand platelet pathology in the course of atherosclerosis.

It has become clear that, besides their role in haemostasis and thrombosis, platelets regulate a variety of inflammatory responses. Because inflammation plays an important role in the progression of cardiovascular diseases, recent studies have focused on the connection between inflammation and epoxyeicosatrienoic acids (EET's). EET's have shown to exhibit anti-inflammatory properties in the vasculature, Kesser and colleagues demonstrated that pro-inflammatory mediators like cytokines and lipopolysaccharide decrease the formation of EET's and endothelial epoxygenase enzyme expression (Kessler et al. 1999). Activated nuclear factor-κB (NF-κB) is a critical cell-signalling step for the induction of numerous inflammatory mediators in the cardiovascular system. NF-κB activity is essential for the up-regulation of genes encoding vascular cell adhesion molecule (VCAM), inter-cellular adhesion molecule and E-selectin which contribute to the progression of cardiovascular diseases (Collins et al. 1995). EET's or CYP2J2 over-expression was found to be anti-inflammatory and decreased tumor necrosis factor (TNF)-α induced endothelial VCAM-1 expression, and EET's prevented leukocyte adhesion to the vascular wall (Node et al. 1999). Several studies demonstrated that inhibiting NF-κB activation is the primary signalling pathway by which epoxides exert their anti-inflammatory effects (Falck et al. 2003). Additionally, endothelial cell shear stress can increase EET production and EET's can subsequently bind and activate the peroxisome proliferator-activated receptor α (PPARα) to provide anti-inflammatory

effects on blood vessels (Liu et al. 2005). Studies have also demonstrated that epoxides have anti-aggregatory properties in platelets (Fitzpatrick et al. 1986; Krötz et al. 2004). Fitzpatrick and colleagues showed that 11,12-EET or 14,15-EET inhibits arachidonic acid stimulation of platelet aggregation. 11,12-EET, 8,9-EET and 14,15-EET cause membrane hyperpolarisation of human platelets through activation of BK_{Ca} (big potassium and calcium) channels that subsequently inhibits platelet adhesion to endothelial cells (Fitzpatrick et al. 1986). Vascular thrombosis is regulated in part by the tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor 1 (Collen 1980). Node and colleagues also demonstrated that 11,12-EET increases the expression and activity of t-PA in human endothelial cells through activation of the G-protein, $G_{\alpha S}$ and PKA. Therefore, EET's decrease platelet aggregation through a membrane hyperpolarisation and enhanced expression of endothelial fibrolytic enzymes (Node et al. 2001). Overall these findings suggest that epoxygenase metabolites or EET agonists have the potential to decrease thrombolytic events associated with cardiovascular diseases.

Taken together these experimental findings suggest that EET's or EET agonists could be effective as anti-inflammatory agents.

1.1.4.4. Anti-platelet therapy

Platelets also play a central role in myocardial infarction and stroke and targeting platelet signalling mechanisms and receptors as well as coagulation factors may well become an important therapeutic strategy for treatment of such acute ischemic

events (Michelson 2010; Stoll et al. 2008) It must be noted however, that targeting platelets, particularly in ischemic stroke may provide limitations, as antithrombotic therapy may induce intracranial haemorrhage (Adams et al. 2008; Kleinschnitz et al. 2007; Stoll et al. 2008). It is therefore is vital to investigate new therapeutic targets in platelets to regulate the fine balance between an anti-thrombotic effect in the vasculature without affecting haemostasis.

In terms of anti-platelet therapy, acetylsalicylic acid (or aspirin) which was introduced into the commercial market in the late 1800's is a synthetic compound, with anti-pyretic, analgesic, anti-inflammatory and anti-platelet properties and has since become one of the most versatile pharmaceutical agents known in the treatment of cardiovascular disease (identified by Friedrich Bayer 1825). The pharmacological effects of aspirin are mediated primarily through its interference with prostaglandin biosynthesis (Section 1.4.2.) by its covalent modification of COX through the irreversible acetylation of a specific serine moiety on the active site (Antman et al. 2005). This ultimately prevents the formation of platelet-generated TXA_2 and subsequent inhibition of platelet aggregates (Mitchell et al. 2006). The long term use of aspirin is also accompanied by side effects. The inhibition of COX interferes with the production of homeostatic prostanoids, thereby potentially resulting in serious toxicities, including renal failure, gastric mucosal ulceration and impaired haemostasis (Batlouni 2010; Yamamoto et al. 2010; Bartfai & Conti 2010). Furthermore, aspirin's platelet inhibitory effects predispose to both bleeding and major haemorrhage.

Other commercially available compounds are Prasugrel, Ticagrelor and Clopidogrel which act as ADP receptor antagonists. These compounds are structurally similar; however, Clopidogrel differs by possessing an additional carboxymethyl side group. Clopidogrel irreversibly inhibits binding of ADP to its receptor, thereby preventing ADP-induced activation of the αIIbβ3 receptor during platelet activation. It also prevents further release of granule stored ADP (Quinn & Fitzgerald 1999; Quinn et al. 2002). In general, Clopidogrel is known as the more tolerated and safer of these compounds, however, bone marrow toxicity, aplastic anaemia and haemolytic-uremic syndrome (HUS) associated with Clopidogrel have been reported (Andersohn et al. 2004). This may be fatal and usually occurs within weeks to months following therapy initiation (McCarthy & Kockler 2003; Meyer et al. 2001; Trivier et al. 2001).

1.1.4.4.1. Aspirin and Clopidorel resistance

Aspirin and Clopidogrel have emerged as critical therapies in the treatment of cardiovascular disease. Despite their efficacy, patients on these medications continue to suffer complications. Millions of patients are currently on low-dose antiplatelet therapy but it is unknown how many of these patients are under-treated or on the wrong medication. Aspirin and clopidogrel resistance are emerging clinical entities with potentially severe consequences such as recurrent myocardial infarction, stroke, or death. The mechanism of resistance remains incompletely defined, but there are specific clinical, cellular, and genetic factors that influence therapeutic failure. These

factors range from physicians who fail to prescribe these medications despite appropriate indications to polymorphisms of platelet membrane glycoproteins.

As mentioned previously aspirin works by irreversibly acetylating the COX-1 enzyme, thus suppressing thromboxane A_2. Thromboxane A_2 serves as a potent agonist of platelet aggregation, and aspirin prevents thrombus formation *via* this mechanism. However, its antiplatelet effect is not uniform in all patients (Patrono et al. 2001) and its inhibition of platelet aggregation is subject to inter-individual and intra-individual variability (Homoncik et al. 2000). This unpredictable response to aspirin can be attributed to clinical, cellular, and genetic factors (Bhatt 2004).

Clinical causes of aspirin resistance can range from patient non-compliance to physicians who display aspirin resistance, that is, physicians who fail to prescribe aspirin appropriately. Alternatively, patients may take aspirin but not absorb it, or may have interactions because of other medications. Ibuprofen, for example, can adhere to the COX-1 binding site of aspirin, and may *via* steric hindrance prevent aspirin from exerting its antiplatelet effect and limit its cardio-protective function (Catella-Lawson et al. 2001). The dose of aspirin may contribute to uninhibited platelet activity depending on the weight and age of patients (Maree et al. 2005).

Cellular factors influencing aspirin efficacy include inadequate suppression of platelet COX-1. In addition, aspirin resistance has been attributed to COX-2 mRNA over-expression by platelets and endothelial cells, though this remains controversial (Weber et al. 1999; Zimmermann et al. 2003). Resolvins, a family of bioactive omega-3 fatty acid metabolites, mediate the inflammatory response and are

generated *via* COX-2 acetylation by aspirin (Serhan et al. 2002). A deficiency of these products could also influence therapeutic failure.

Genetics also play a role in patient response to aspirin as polymorphisms of platelet membrane glycoproteins such as P1 (A1/A2) have been associated with an attenuated response to aspirin (Macchi et al. 2003). Polymorphisms of vWF or the collagen receptor gene have also been postulated to cause aspirin resistance (Pontiggia et al. 2002). Single nucleotide polymorphisms of the $P2Y_1$ gene also can affect response to aspirin (Jefferson et al. 2005). Despite these findings, the impact of polymorphisms on aspirin response remains controversial (Bray 2000).

Clopidogrel resistance may also be because of clinical, cellular, and genetic factors. It is converted in the liver by cytochrome P450 3A4 (CYP3A4) into its active metabolite. It inhibits platelet aggregation by irreversibly binding to the $P2Y_{12}$ ADP receptor on the platelet surface. Similar to aspirin, clopidogrel therapy may fail owing to patient non-compliance or because physicians may not prescribe it. In addition, there may be variability in absorption (Taubert et al. 2004) with associated under-dosing in patients and possible drug–drug interactions (Lau et al. 2004). One study correlated the level of angina class with platelet inhibition by clopidogrel and found that patients with higher Braunwald angina class had lower inhibition of platelet aggregation (Soffer et al. 2003). Cellular mechanisms of clopidogrel resistance may be because of inter-individual differences in $P2Y_{12}$ receptors and the number of receptors an individual possesses, varying the levels of ADP release, or platelet activation *via* alternative pathways.

It is therefore justified to state that the continuous search for anti-platelet compounds or targeting particular mediators in the treatment of platelet associated diseases is paramount, with the hope of one day establishing an effective treatment regime that balances drug efficacy with minimal toxicity.

1.1.4.5. Platelets and Cancer Metastasis

Platelets have long been associated with tumour metastasis and metastasis to distant organs is dependent on interactions between tumour cells and the host microenvironment within the circulation, lymphatic vessels and target tissues. This involves blood cells, components of the coagulation system, stromal cells and the extracellular matrix. Cells within the bloodstream that contribute to metastasis are endothelial cells, platelets, lymphocytes, macrophages, mast cells, fibroblasts and bone marrow-derived progenitor cells (Joyce & Pollard 2009). The link between platelets and cancer was first documented in the mid-nineteenth century by Trousseau, who diagnosed himself and his patients with excessive blood clotting, which was caused by occult carcinoma that led to the inflammation of blood vessels. He observed an association between thrombosis and cancer, and proposed that changes to the haemostatic system support the progression of the malignancy (Khorana 2003). These initial findings suggested that tumours that are capable of activating platelets and forming clots are likely to spread and have a poor outcome. It is now well recognised that in cancer patients, tumour growth is accompanied by the development of an increased tendency towards blood clotting (hypercoagulable

state), platelet abnormalities and an increased risk of thromboembolic disease (Khorana & Connolly 2009). Moreover, thromboembolism is an early diagnostic feature and a major cause of death (Sierko & Wojtukiewicz 2007). Platelet activation is amplified in many cancers and found within the tumour vasculature (Sierko & Wojtukiewicz 2004; Blann et al. 2001; Verheul et al. 2000). Platelet counts commonly vary during cancer progression and may predict the clinical outcome. Thrombocytosis (high platelet count) associates with poor prognosis in colon, breast, lung, gastric, renal, cervical, pancreatic, endometrial, brain and ovarian cancers (Sierko & Wojtukiewicz 2004; Erdemir et al. 2007; Costantini et al. 1990; Ayhan et al. 2006; Taucher et al. 2003; Brown et al. 2005; Bensalah et al. 2006; Brockmann et al. 2007). Thrombocytopenia (low platelet count) also occurs, but is mostly associated with chemotherapy that suppresses platelet production in the bone marrow. The changing requirements of disseminating tumour cells during metastasis provide challenging questions to address and understand the complexity of platelet involvement in different stages of cancer progression. Transgenic mouse models with defined aberrations in platelet and other host cell-type functions will help to further elucidate crucial molecular mechanisms. For example, one such model that has not been explored in the context of metastasis is a model of Glanzmann's thrombasthenia (Fang et al. 2005). This model recapitulates a human disease in which platelets lack or have aberrant expression of integrin αIIbβ3. Models like this offer the opportunity to explore the contribution of plateletreceptors and platelet functions that are key to understanding tumour metastasis in disease.

1.2. Platelet receptors

1.2.1. Cell adhesion receptors

Vascular damage results in denudation of endothelial cells exposing the underlying ECM proteins of which collagen is considered to be the most thrombogenic. At least 25 different varieties of human collagen are known to exist (Hashimoto et al. 2002), of these, types I, III, IV, V, VI, VIII, XII, XIII and XIV are present within the walls of human blood vessels (Barnes & Farndale 1999). Apart from collagen, the ECM also contains a large number of adhesive macromolecules, such as laminin, fibronectin and vWF (Ordinas et al. 1990). Under conditions of high shear, such as those found in small arteries and arterioles, the initial tethering of platelets to the ECM is meditated by the interaction between the platelet receptor glycoprotein (GP)Ib-V-IX and vWF bound to collagen. However, due to their fast off-rate, this interaction is insufficient to mediate stable adhesion but rather maintains the platelet in close contact with the ECM surface by continuous translocation in the direction of blood flow (Savage et al. 1999). It is the role of an immunoglobulin superfamily receptor GPVI to establish contact with the thrombogenic collagen proteins within the ECM. While GPVI binds to collagen with low affinity, it triggers intracellular signals that shift platelet integrins, namely integrin α2β1, to a high affinity state and induces the release of secondary mediators, ADP and TXA_2.

1.2.2. Glycoprotein (GP)Ib-V-IX complex.

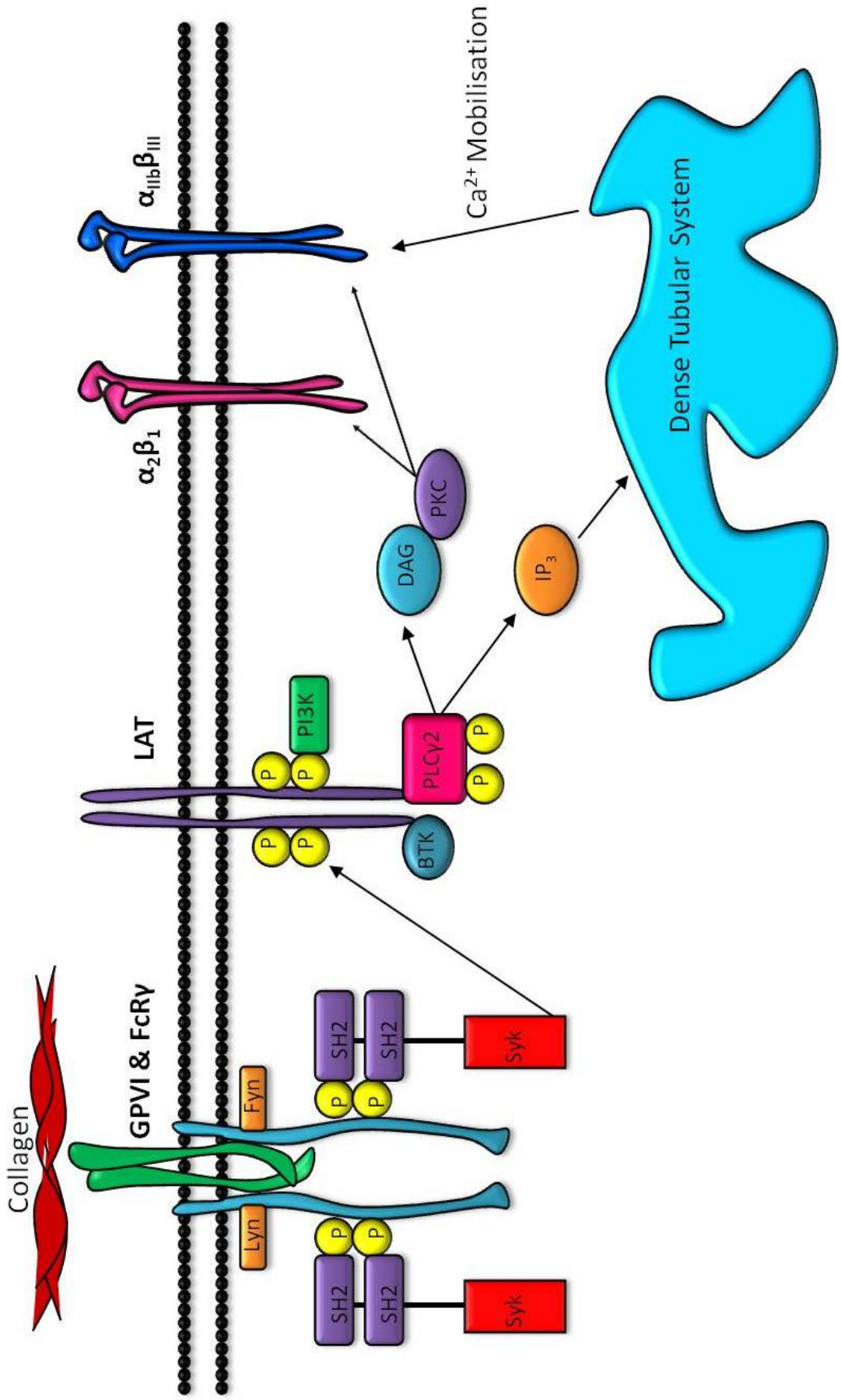
This receptor belongs to the leucine-rich-repeat (LRR) family and its multimeric adhesive ligand vWF, is essential for platelet adhesion under high shear stress. vWF has the ability to facilitate indirect interactions between the platelet surface GPIb-V-IX complex and collagen fibres through its A_3 domain present in the extracellular matrices (Alevriadou et al. 1993). This causes short lived tethering in which platelets slow down and roll along the surface of the damaged vascular tissue allowing more stable direct interactions with ECM (Moroi et al. 1997; Savage et al. 1998). GPIb-V-IX is then able to bind the vessel wall-immobilised vWF through a second binding site found in the A_1 domain of vWF (Andrews & Berndt 2004). In addition, GPIb-V-IX is also a major receptor for not only vWF, but thrombospondin-1 (TSP), the leukocyte integrin αMβ2 and P-selectin expressed on activated endothelial cells and platelets. It also acts as a signalling receptor to propagate downstream events that activate integrins α2β1 and αIIbβ3. Furthermore, GPIb-V-IX can promote procoagulant activity on platelets by binding α-thrombin, Factors XI and XII and kininogen.

1.2.3. Glycoprotein-VI (GPVI)

The GPVI receptor belongs to the immunoglobulin (Ig) superfamily and is one of the major Ig receptors for collagen (Clemetson et al. 1999). It is non-covalently associated with the Fc receptor γ-chain (FcRγ) (Gibbins et al. 1996; Gibbins et al. 1997) which is critical for platelet signalling events. When the GPVI receptor binds to collagen, it stimulates receptor clustering with the FcR γ-chain acting as the signalling component of the complex (Clemetson et al. 1999; Gibbins et al. 1997). The cytoplasmic tail of FcR γ-chain contains an immunoreceptor tyrosine-based activation motif (ITAM) which becomes phosphorylated on its tyrosine residues upon GPVI clustering (Gibbins et al. 1997; Gibbins 2002) by the Src (sarcoma) family kinases - Fyn and Lyn (Briddon & Watson 1999; Quek et al. 2000; Inoue et al. 2002) leading to the activation of multiple signalling cascades and consequent platelet activation (Nieswandt & Watson 2003; Watson et al. 2001). The importance of this interaction in platelet responses to collagen has been demonstrated using mouse models lacking the FcRγ-chain, where tyrosine phosphorylation of spleen tyrosine kinase (Syk) and phospholipase $C_{\gamma 2}$ ($PLC_{\gamma 2}$) is absent (Poole et al. 1997). (For schematic diagram of GPVI signalling see Figure 1.2.)

Figure 1.2. A schematic diagram of the GPVI signalling pathway

Collagen binding to GPVI leads to clustering of the receptor and stimulates rapid phosphorylation of the tyrosine residues in the immunoreceptor tyrosine-based activatory motif (ITAM) of the associated FCRγ-chain by the Src-family kinases Lyn and Fyn. Syk then binds to the FcRγ-chain via its tandem Src-homology (SH2) domains and becomes phosphorylated. This in turn phosphorylates linker for the activation of T-cells (LAT) which acts as a docking site for phosphatidylinositol 3-kinase (PI3K) and recruits phospholipase Cγ2 (PLCγ2) to the membrane. Downstream of this inositol 1,4,5 trisphosphate (IP_3) and diacyl-glycerol (DAG) are generated through the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) which lead to the release of calcium from intracellular stores and protein kinase C (PKC) isoform activation respectively. Ultimately this impacts on the activation state of the integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$.


Collagen
GPVI & FcRγ
Fyn
Lyn
SH2
SH2
SH2
SH2
Syk
Syk
P
LAT
PI3K
PLCγ2
BTK
DAG
PKC
IP3
α2β1
αIIbβIII
Ca2+ Mobilisation
Dense Tubular System

1.2.4. Integrin α2β1

The integrin α2β1 (also known as GPIa/IIa, VLA-2 on lymphocytes, or CD49b/CD29) was one of the first platelet collagen receptors to be identified, and serves mainly as an adhesion receptor (Farndale et al. 2004) where it is able to bind collagen in an Mg^{2+} dependant fashion (Kunicki et al. 1988). Integrin α2β1 is present on the surface of a number of different cell types (Zutter & Santoro 1990) which unlike αIIbβ3 is expressed exclusively on megakaryocytes and platelets (Wagner et al. 1996). Upon stimulation, platelets undergo inside-out signalling which induces a conformational change in both α2β1 and αIIbβ3. This change converts α2β1 into an activated form with a high affinity for collagen (Jung & Moroi 1998). The proposal of α2β1 participating in outside-in signalling or not is a somewhat controversial issue. Hers and colleagues disputed that stimulation of α2β1 does not induce tyrosine phosphorylation downstream of this receptor (Hers et al. 2000) whereas other studies using selective ligands indicate that the phosphorylation of tyrosine based signalling does occur (Inoue et al. 2003; Stevens et al. 2004). The direct contribution of α2β1 to activate platelet signalling is poorly understood; nevertheless the essential role of this receptor in mediating initial responses to vascular damage is well established.

1.3. Soluble platelet agonists

1.3.1. Introduction

After the initial capture of platelets by adhesion receptors, outside-in signalling stimulates platelet activation and the release of a variety of soluble platelet agonists, which play an important role in the assembly of a thrombus, in particular, thrombin, ADP and TXA_2. Other soluble platelet agonists include platelet activating factor (PAF) and 5-HT.

The majority of these released agonists mediate their effects through binding to specific G-protein coupled receptors (GPCR's). GPCR's are a superfamily of cell membrane receptors composed of seven putative transmembrane (TM) domains, an extracellular N-terminus involved in ligand binding and an intracellular C-terminus which together, with the third intracellular loop, interact with a guanine nucleotide binding protein (G-protein) (Lerner et al. 1996; Verrall et al. 1997).

G -proteins are heterotrimeric composing of an α, beta (β) and gamma (γ) -subunit. During their resting state the α-subunit has guanosine diphosphate (GDP) bound, which is exchanged for guanosine triphosphate (GTP) when the receptor is stimulated by an agonist. The subunits then change their conformation to form an active α-subunit and a βγ dimer. Activation of the subunits is terminated by the hydrolysis of GTP to GDP leading to the restoration of the heterologous G-protein and re-association with the receptor. Furthermore, some platelet receptors, such as

those for ADP, become desensitised by G-protein coupled receptor kinases (GPCRK) and are internalised following stimulation (Hardy et al. 2005).

1.3.2. Thrombin

Thrombin is a multifunctional serine protease that is distinctly unique among coagulation proteins. It possesses both procoagulant and anticoagulant properties (Leung & Gibbs 1997). The precursor protein prothrombin is synthesised exclusively in the liver as an inactive zymogen. The conversion of prothrombin to thrombin requires the assembly of the prothrombinase complex Factor Xa found in the coagulation cascade which plays an important role in this biological amplification system.

Thrombin generated by the coagulation cascade converts soluble fibrinogen into fibrin monomers, which polymerise to form a stable fibrin clot. It is also a potent platelet agonist which induces platelet signalling, secretion and aggregation through G-protein coupled protease activated receptor (PAR) isoforms (Kahn et al. 1998). Thrombin activates PAR's by cleaving the N-terminal region of the receptor, which in turn acts as a tethered ligand. In the cleaved state, part of the receptor itself acts as the agonist leading to downstream signalling events within platelets. (For a schematic representation of PAR activation by thrombin, see Figure 1.3.)

The first PAR's to be identified was PAR1 (Vu et al. 1991), this was then followed by the identification of PAR2 (Nystedt et al. 1994), PAR3 (Ishihara et al. 1997) followed by PAR4 (Kahn et al. 1998; Xu et al. 1998). Human platelets express PAR1 ($G\alpha_{12/13}$, $G\alpha_q$ and $G\alpha_{i/z}$ coupled) and PAR4 (coupled to $G\alpha_q$ and possibly the $G\alpha_{12/13}$ pathways) on their surface (Kahn et al. 1998; Kahn et al. 1999).

PAR receptors form a heterodimer which enhances the signalling response to thrombin (Leger et al. 2006). Signalling through the $G\alpha_q$ pathway downstream of PAR1 activation leads to increased phospholipase C_β (PLC_β) generation and phosphatidylinositol 4,5-bisphosphate (PIP_2) phosphorylation by $PI_3K\gamma$ isoforms (Lian et al. 2005). PLC_β hydrolyses PIP_2 to generate the second messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2- diacylglycerol (DAG) (Lian et al. 2005; Rhee & Bae 1997). This mediates Ca^{2+} mobilisation from intracellular stores (Brass & Joseph 1985) which is important for integrin activation *via* inside-out signalling, essential for platelet activation.

Murine platelets do not possess PAR1 receptors, instead they express PAR4 and the least understood of the protease receptors PAR3 (Leger et al. 2006). PAR3 enhances thrombin cleavage of the lower-affinity PAR4 receptor possibly by delivering thrombin to adjacent PAR4 (Nakanishi-Matsui et al. 2000).

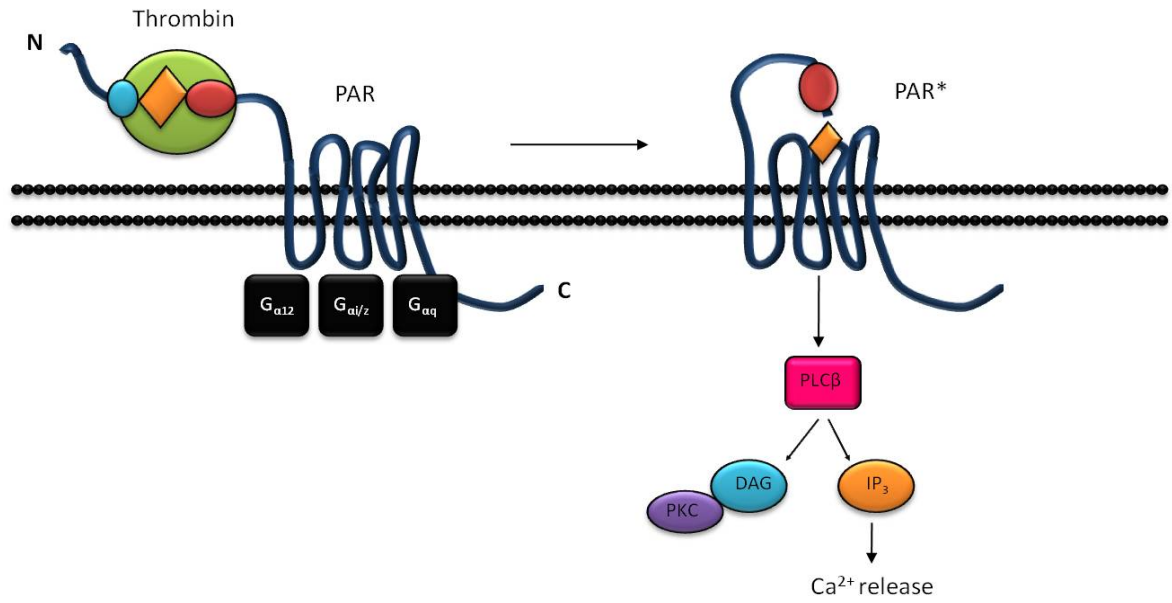


Figure 1.3. Schematic representation of PAR activation by thrombin

There are 4 known isoforms of protease-activated receptors (PARs), numbered from one to four, and platelets express 1 and 4. These receptors are members of the seven transmembrane G-protein-coupled receptor super-family. PAR's contain a thrombin cleavage site at the N-terminal exodomain of the receptor which is cleaved by thrombin to reveal a new N-terminal region which contains a recognition sequence for the receptor. Binding to the newly exposed 'tethered ligand' activates the receptor (PAR*) and initiates signalling through the $G\alpha_q$ pathway. This leads to increased PLCβ generation and PIP_2 phosphorylation by PI3Kγ isoforms. PLCβ hydrolyses PIP_2 to generate the second messengers IP_3 and DAG, this mediates Ca^{2+} mobilisation from intracellular stores which is important for integrin activation *via* inside-out signalling.

1.3.3. ADP

In the early 1960's the low molecular weight molecule ADP was identified as a factor released from RBC that could influence the adhesiveness of human platelets (Gaarder et al. 1961) and induce platelet aggregation (Born 1962a). Savage and colleagues described ADP as a low, weak aggregating agonist that, as such, only induces platelet shape change and reversible aggregation (Savage et al. 2001). Apart from ADP found in RBC, platelets store ADP in their dense granules where it is released upon platelet stimulation (Gachet 2001a). ADP then binds in an autocrine/paracrine fashion to ADP receptors on the surface of the originating platelet as well as on those of approaching platelets. Initially, the molecular identity of these receptors remained elusive. However, in 2001 the complete identification of the platelet purinergic 2 (P2) range was reported (Gachet 2001b; Hollopeter et al. 2001), eight years after the first reported cloning of a P2 receptor (Webb et al. 1993).

Three distinct purinergic receptors have been identified on platelets, two GPCR's, $P2Y_1$ and $P2Y_{12}$ and a ligand gated ion channel $P2X_1$, which preferentially binds ATP (Khakh 2001). The $P2Y_1$ receptor is widely distributed in many tissues including the heart (Kumar et al. 2010), blood vessels (Kauffenstein et al. 2010), smooth muscle cells (Govindan et al. 2010) and neural tissues (Heinrich et al. 2008). The coupling of $P2Y_1$ to $G\alpha_q$ triggers Ca^{2+} mobilisation from intracellular stores leading to shape change and weak transient aggregation. Despite this, $P2Y_1$ remains

crucial to the initiation of ADP mediated activation and in the maintenance of collagen induced aggregation of platelets. The $P2Y_{12}$ receptor, which is coupled to G_{i2}, was the last to be cloned (Hollopeter et al. 2001) and has very limited tissue distribution although not exclusive to platelets. $P2Y_{12}$ plays a central role in the amplification of platelet responses stimulated by all platelet agonists including collagen, thrombin, TXA_2, immune complexes, 5-HT and adrenaline (Hechler et al. 2005; Conley & Delaney 2003; Gachet 2006) and is important in ongoing thrombus formation under high shear conditions (Moake et al. 1988; Remijn et al. 2002; Turner et al. 2001). (For a schematic representation of $P2Y_1$ and $P2Y_{12}$ activation by ADP, see Figure 1.4.)

The third P2 receptor found on platelets is $P2X_1$, a ligand-gated cation channel which permits the fast entry of Ca^{2+} induced by ATP. $P2X_1$ receptors are rapidly desensitised impeding the study of their function *in vitro*. Although unable to stimulate platelet aggregation alone, $P2X_1$ has been shown to participate in collagen and shear induced aggregation (Cattaneo et al. 2002; Oury et al. 2001).

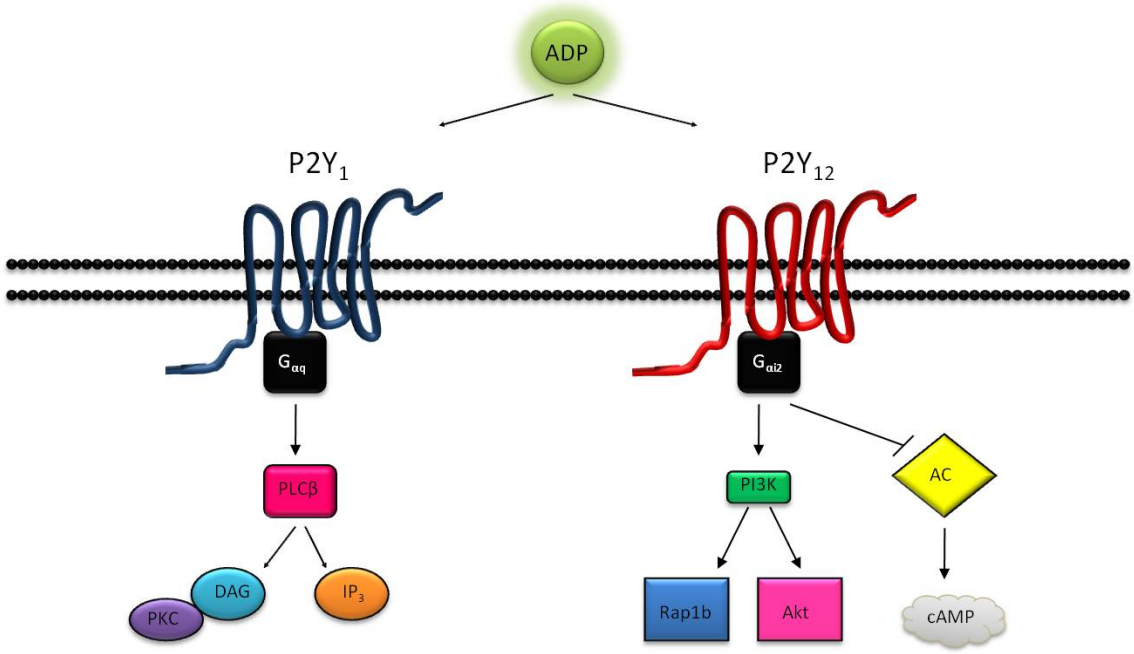


Figure 1.4. Schematic representation of $P2Y_1$ and $P2Y_{12}$ activation by ADP

P2Y receptors are a family of purinergic G protein-coupled receptors and platelets express isoforms 1 and 12. Binding of ADP to $P2Y_1$ leads to mobilisation of intracellular calcium ions *via* activation of PLCβ, causing PIP_2 phosphorylation by PI3Kγ isoforms. PLCβ hydrolyses PIP_2 to generate the second messengers IP_3 and DAG mediating platelet shape change. $P2Y_{12}$ can also act on adenylate cyclase (AC). Active AC dephosphorylates ATP to form cyclic AMP. cAMP directly regulates ion channels in the DTS. Elevated cAMP maintains calcium sequestered in granules or within the DTS. P2Y12 can also regulate downstream signalling molecules such as Rab1b and Akt. Both receptors also functions as a receptor for extracellular ATP.

1.3.4. Thromboxane A_2 (TXA_2)

Thromboxane (TXA_2) is a short lived lipid mediator synthesised by activated platelets. It is released as a local signal, which amplifies activation and recruits other platelets to the site of clot formation (De Groot et al. 1999). TXA_2 is derived from arachidonic acid liberated from membrane phospholipids by phospholipase A2 (PLA_2) as a result of increased cytosolic Ca^{2+} concentration following platelet activation. It is then rapidly metabolised by two distinctly different enzymatic pathways, the first leads to the production of prostaglandin H_2 (PGH_2) by COX, which is subsequently metabolised by thromboxane synthase (TXS) into TXA_2. TXA_2 is able to diffuse across the platelet plasma membrane where it can bind to and activate other platelets. TXA_2 is unstable and is fairly rapidly converted to the more stable lipid, thromboxane B_2 (TXB_2). The other is the production of hydroxy-eicosatetranoic acid (HETE) by lipoxygenase, which is then converted to leukotrienes whose effects on platelets are not yet understood (Lagarde et al. 2010). There are two isoforms of the thromboxane receptors (TP receptors) located on platelets, TPα and TPβ. They differ only in the composition of their C-terminus (Hirata et al. 1996). The association between TXA_2 to these receptors initiates signalling through the $G\alpha_q$ pathway (Shenker et al. 1991) leading to the activation of PLC isoforms (Brass et al. 1987) which are important for Ca^{2+} mobilisation from intracellular stores and the upregulation of integrin activity (Hirata et al. 1991). TP receptors can also couple to the $G\alpha_{12/13}$ pathway which mediates platelet shape

change through the activation of Rho kinase and myosin light chain phosphorylation (Klages et al. 1999).

1.3.5. Other soluble agonist

Adrenaline and 5-HT are weak platelet agonists stored in dense granules and released upon platelet activation. Adrenaline has long been identified as a weak platelet agonist (Born et al. 1967) unable to stimulate platelet aggregation alone, but capable of potentiating the responses of other platelet agonists. Adrenaline mediates its effects on platelets through stimulation the α_2-adrenergic receptor, a GPCR coupled to G_z, a member of the G_i family of G proteins. 5-HT, which is stored in platelet dense granules, is largely derived from the environment surrounding the platelet by two distinct transport systems, both of which are energy dependent. The first is by a plasma membrane 5-HT transporter (SERT) which binds extracellular 5-HT and transports it across the platelet plasma membrane into the cytosol. The second transporter, referred to as vesicular monoamine transporter (VMAT), transports 5-HT across the vesicular membrane back into dense granules (Rudnick et al. 1997) where it is stored.

Upon dense granule secretion, 5-HT is released enabling it to activate surface 5-HT_2A receptors, which are coupled to G_q G-proteins, stimulating signalling pathways. However, 5-HT is incapable of stimulating platelet aggregation in isolation but can synergise with low concentrations of other agonists to amplify their response. In addition, full aggregation can be achieved by combining adrenaline and 5-HT, as this leads to stimulation of both G_i and G_q signalling pathways, which is thought to be a prerequisite for platelet aggregation (Jin & Kunapuli 1998).

In addition to its role in potentiating platelet activation, 5-HT stimulates receptors on endothelial cells, which in turn, signal to the underlying smooth muscle to contract resulting in vasoconstriction, a further physiological mechanism to limit blood loss in the event of vascular injury.

1.4. Negative regulatory signalling pathways

1.4.1. Nitric Oxide

Negative regulation of platelets is essential in preventing uncontrolled haemostasis/thrombosis. Nitric oxide (NO) and prostacyclin (PGI_2) are key negative regulators of platelet function. NO is synthesised from L-arginine by a family of enzymes called NO synthases (NOS). There are three isoforms of NOS which are named after the tissues in which they were first identified. Endothelial (eNOS or NOSIII) and neuronal (nNOS or NOSI) are generally regulated by Ca^{2+}/calmodulin (CaM) and phosphorylation, and inducible NOS (iNOS or NOSII), which as the name suggests, is not expressed constitutively in most tissues but induced upon stimulation (Bruckdorfer 2005).

NO produced by endothelial cells and released into the circulation plays an important role in the negative regulation of platelets. Vascular eNOS generates and releases NO that can diffuse across the platelet membrane passively where it is believed to bind and activate soluble guanylate cyclase (sGS) leading to the generation of cyclic guanosine monophosphate (cGMP) and downstream of this protein kinase G (PKG) (Radomski et al. 1987) leading to the inhibition of integrin activation, platelet adhesive responses and aggregation (Oberprieler et al. 2007a; Oberprieler et al. 2007b; Roberts et al. 2009; Roberts et al. 2008).

Other downstream effectors have been reported, such as the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser^{157} by protein kinase C (PKC) which correlates with reduced activation of integrin aIIbβ3 and an inhibition of platelet aggregation (Horstrup et al. 1994). In addition, phosphorylation of VASP, which occurs downstream of cGMP, also reduces its ability to interact with actin, and therefore negatively regulates actin nucleation and bundling properties (Harbeck et al. 2000). At least one isoform of NOS has been demonstrated in platelets (Radomski et al. 1990) and the majority of reports suggest this to be eNOS (Randriamboavonjy & Fleming 2005), although there is also evidence that iNOS may also be expressed (Chen & Mehta 1996). NO produced by activated platelets inhibits platelet recruitment to aggregates (Freedman et al. 1997) and mice transfused with $eNOS^{-/-}$ platelets display decreased bleeding times when compared to wild-type mice (Freedman et al. 1999) thus highlighting the importance of platelet-derived NO in the regulation of haemostasis. Interestingly, recent evidence suggests that NO may in fact stimulate platelets (Li et al. 2003a), and exert a biphasic response whereby it produces an initial transient stimulatory response, promoting platelet aggregation, followed by an inhibitory response limiting the size of thrombi. This discovery however remains controversial as one research group has challenged whether eNOS is detectable in platelets (Gambaryan et al. 2008). The detailed study by Gambaryan and colleagues attempted to clarify some of the issues relating to platelet NOS. They suggested that neither iNOS nor eNOS are present in either mouse or human platelets, and secondly that cGMP can be synthesised in platelets

independently of NO (Gambaryan et al. 2008). Similarly, Tymvios and colleagues demonstrated a lack of eNOS in human and mouse platelets and found no functional consequence of eNOS ablation (Tymvios et al. 2009). The effects of eNOS on platelet function *in vivo* were also attributed to the vascular endothelium using a mouse model of platelet thromboembolism (Tymvios et al. 2008).

1.4.2. Prostacyclin (Prostaglandin I_2)

Prostacyclin (PGI_2) is a derivative of the unsaturated fatty acid arachidonic acid, which was discovered in 1976 as a substance inhibiting platelet secretion, platelet aggregation and vasoconstriction (Moncada et al. 1976; Gryglewski et al. 1976). The biosynthesis of prostacyclin is part of the arachidonic acid metabolic pathway (similar to that for the generation of TXA_2) and is secreted from the endothelium into the circulating blood (Moncada et al. 1977). After the release from the vessel wall, PGI_2 mediates its inhibitory effects *via* the high-affinity PGI_2 receptors (IP) on the platelet surface thus causing an increase AC activity and cyclic adenosine monophosphate (cAMP) accumulation (Best et al. 1977). The accumulation of cAMP activates protein kinase A (PKA) which causes the phosphorylation and activation of a number of signalling molecules such a myosin light chain kinase (MLCK) (Hathaway & Adelstein 1979), the platelet inositol 1.4.5 triphosphate receptor (IP_3R) (Cavallini et al. 1996) and VASP (Aszódi et al. 1999). These signalling molecules are able to regulate actin polymerisation and maintain platelets in a resting state (Prehoda et al. 1999).

1.5. Platelet Calcium

1.5.1. Introduction

Ca^{2+} homeostasis is tightly regulated in platelets to maintain low resting $[Ca^{2+}]_i$ involving energy expenditure by transporting ions out of the cytosol into the extracellular milieu against a strong chemical gradient.

Platelet Ca^{2+} signalling has been described as being controlled in space, time and amplitude (Berridge et al. 2003) and defines the importance of the link between the Ca^{2+} signal and functionality. Ca^{2+} is also an important secondary messenger in virtually all cells and regulates a range of fundamental cellular processes.

In a resting cell, the concentration of cytoplasmic Ca^{2+} is less than 0.1 µM which is 10,000 fold lower than outside the cell. The membrane potential is negative inside the cell which allows Ca^{2+} entry through open Ca^{2+} channels (across the plasma membrane) as a result of the large electrochemical gradient.

In platelets, the elevation in intracellular calcium concentration $[Ca^{2+}]_i$ contributes to various steps of cellular activation such as the reorganisation of the actin cytoskeleton necessary for shape change (Hathaway & Adelstein 1979) and degranulation or inside-out activation of the integrin αIIbβ3 which is central for platelet aggregation (Shattil & Brass 1987) For a diagrammatical representation of Ca^{2+} regulation see Figure 1.5. The two major sources that drive the elevation of $[Ca^{2+}]_i$ are the release of compartmentalised Ca^{2+} and the entry of extracellular Ca^{2+}

through the plasma membrane. The mechanisms of Ca^{2+} release from intracellular stores have been well established for many years, however the mechanistic processes underlying Ca^{2+} entry remained largely unknown until recently.

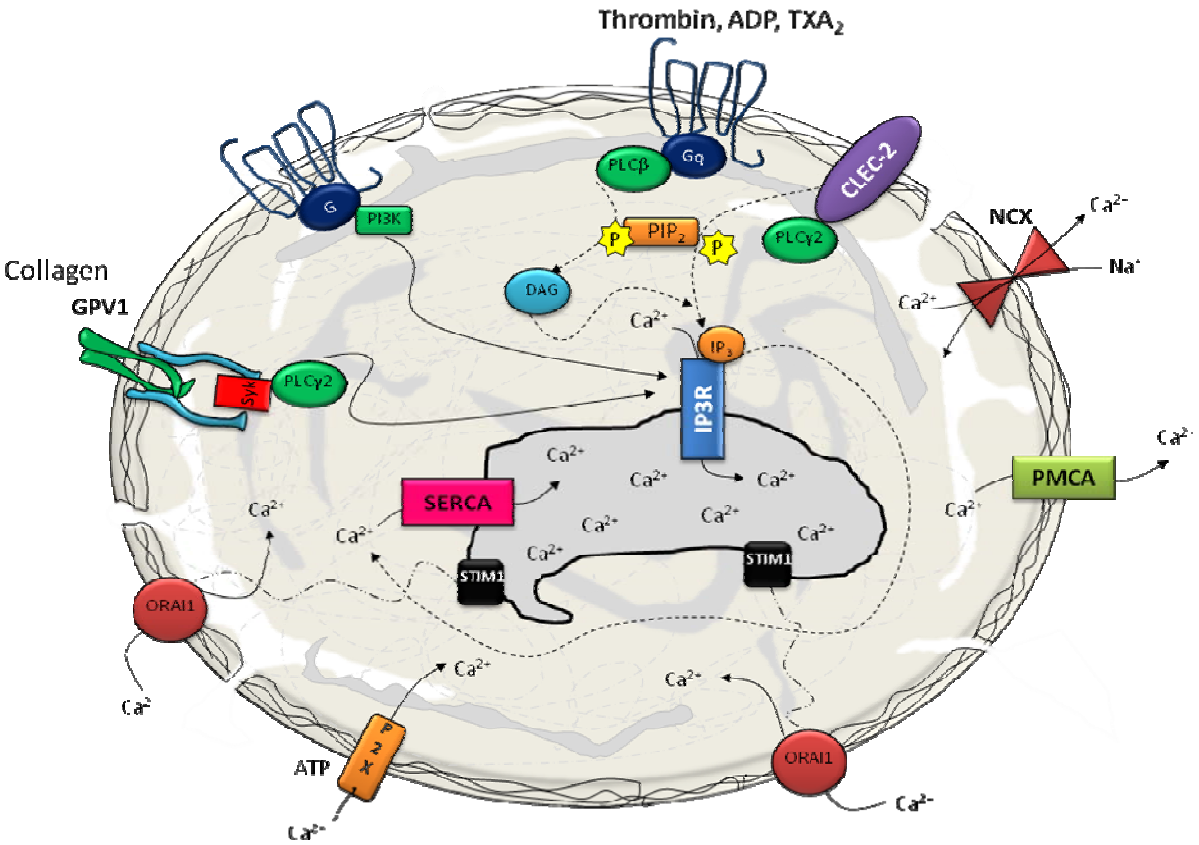


Figure 1.5. Mechanisms involving platelet Ca^{2+} homeostasis

Various transport systems that are responsible for the regulation of cytosolic calcium levels. Intracellular stores are able to release calcium and increasing cytosolic levels. This is achieved *via* the inositol 1,4,5,trisphosphate receptor (IP3R), which in turn leads to a calcium influx through store operated calcium entry through membrane channels such as Orai1 upon stimulation with calcium sensitive molecules in the ER membrane such as stromal interaction molecule 1 (STIM1) *via* protein-protein interaction. The extrusion of intracellular calcium through the plasma membrane is tightly controlled by the plasma membrane calcium ATPase (PMCA) and the Na+/Ca2+ exchanger (NCX). Further sequestration into intracellular stores is achieved *via* the re-uptake by the sarco-endoplasmic reticulum calcium ATPase (SERCA). Calcium mobilisation is also initiated *via* outside in signalling through integrins and glycoproteins located on the plasma membrane such as GPVI, and also through G-protein coupled receptors such as those for thrombin, ADP and thromboxane. Other calcium-dependant receptors such as CLEC-2 are also involved in platelet calcium mobilisation though its association with PLCγ2.

1.5.2. Calcium store release

As mentioned previously, a rise in $[Ca^{2+}]_i$ is a major component of the signalling mechanisms regulating platelet function. Agonist induced stimulation of many platelet receptors leads to the activation of PLC isoforms, namely β, γ and δ, which hydrolyse PIP_2 converting this to IP_3 and DAG. This induces the release of Ca^{2+} from stores. IP_3 generated by PLC, releases Ca^{2+} from the intracellular stores by direct action on the IP_3R in the endoplasmic reticulum (ER), which themselves are Ca^{2+} permeable ion channels. The regulation of these receptors occurs through receptor phosphorylation, most likely through the actions of various protein kinases (Ferris et al. 1991). However, a number of studies have reported inhibition of receptor function after phosphorylation (Komalavilas & Lincoln 1994; Supattapone et al. 1988). IP_3R have also been identified in the plasma membrane and a study performed by El-Daher and colleagues reported that this receptor may be involved in cation entry (El-Daher et al. 2000).

1.5.3. Calcium store entry

Store operated Ca^{2+} entry (SOCE), a mechanism regulated by the filling state of the intracellular Ca^{2+} stores, is a major mechanism for Ca^{2+} influx in non excitable cells (Rosado et al. 2000) and the events that initiate SOCE, i.e. a decrease in the concentration of free Ca^{2+} in the stores have long been characterised (Putney et al. 1986). In the last five years, research concerning the nature of the channels that conduct SOCE has been a matter of debate, for example, the co-expression of the release-activated Ca^{2+} channel protein 1 (Orai1) with the intracellular Ca^{2+} sensor, stromal interaction molecule 1 (STIM1) has been reported to enhance SOCE (Mercer et al. 2006). Physiological agonists such as ADP and thrombin stimulate both the release of Ca^{2+} from intracellular stores and the entry of Ca^{2+} across the plasma membrane. The occupation of surface receptors results in the activation of PLC and the formation of the Ca^{2+} releasing messenger, IP_3, which acts on receptors on the ER. Release of this finite Ca^{2+} store is insufficient for full platelet activation, which in addition, requires Ca^{2+} entry from the external medium. One of the major mechanisms generating Ca^{2+} entry in platelets is the depletion of Ca^{2+} stores.

The mechanism underlying the activation of SOCE is, however, poorly understood. Two hypotheses have been put forward to explain how depletion of Ca^{2+} stores might be signalled to the PM to activate Ca^{2+} entry. One proposal was a direct interaction between proteins in the ER and PM (conformational coupling) and

another proposed a role for a potential diffusible messenger (Berridge 1995; Heemskerk et al. 1994; Parekh & Penner 1997; Sage 1997).

1.5.4. Calcium signalling and disease

A sustained increase of cell Ca^{2+} to micromolar concentrations can impact dramatically to the signalling function of Ca^{2+}. When injurious conditions increase the permeability of the plasma membrane allowing abnormal Ca^{2+} influx, mitochondria try to sustain with the cytosolic Ca^{2+} overload and may serve to prevent cell death should the noxious agent be removed rapidly. In some circumstances, however, a cell may undergo apoptosis due to the activation of Ca^{2+}-dependent hydrolytic enzymes such as proteases. More subtle forms of pathology linked to Ca^{2+} signalling have also been recognised, e.g., the degeneration of the retina caused by gene defects of neuronal Ca^{2+}. Calpain has also been involved in human pathology. The disruption of the gene for muscle isoform p94 (calpain III) causes limb limb gurdle muscular dystrophy type 2A (Richard et al.1995), whereas genetic variation in the gene for another isoform lacking the C-terminal Ca^{2+}-binding domain (calpian 10) favours the onset of type 2 diabetes (Horikawa et al. 2000).

Muscle disturbances resulting in prolonged Ca^{2+} overload have been linked to SERCA pump and ryanodine receptor defects. Malignant hyperthermia is a genetic disease in which inhalation anaesthetics induce skeletal muscle rigidity and extreme hyperthermia (among other symptoms). A leak in the Ca^{2+}- releasing ryanodine receptor of the sarcoplasmic reticulum explains these symptoms. Defects in the genes for the four isoforms of plasma membrane calcium ATPases (PMCA's) have

also been described. PMCA2 and 3 are mostly restricted to neuronal cells, e.g., PMCA2 is very abundant in the outer hair cells of the organ of Corti. Work on knock-out mice and in mice with a phenotype characterised by hearing defects that could be models for hereditary hearing loss in human (Takahashi et al.1999) has revealed essential roles PMCA2.

1.6. Calcium Receptors

1.6.1. Membrane calcium ATPases

Among the structures involved in Ca^{2+} signalling are the Ca^{2+} ATPases which are able to deplete free Ca^{2+} ions from the cytosol. Three families of such proteins are defined depending on the membranes they are inserted in. First, are the plasma membrane Ca^{2+} ATPases (PMCA's) which are responsible for transporting Ca^{2+} ions outside the cell (Carafoli & Brini 2000; Strehler & Zacharias 2001). Second, the sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA's) which are inserted in the ER plasma membrane and are responsible for transporting Ca^{2+} ions into the intracellular ER Ca^{2+} reservoir (Wuytack et al. 2002; Strehler & Treiman 2004; Lipskaia & Lompré 2004) and finally the secretory pathway Ca^{2+} ATPases (SPCA's) which have only emerged in recent years and are found (for example), within the golgi apparatus around the nucleus of mammalian spermatozoa (Harper et al. 2005).

1.6.2. Membrane calcium ATPases: Structure

Both PMCA's and SERCA's are members of the P-type ATPases that transport Ca^{2+} ions against a deep concentration gradient at the expense of ATP.

They share similar structure symmetry and are oriented in the membranes with extramembranous cytosolic loops. They consist of a single polypeptide chain folded into four main domains, a transmembrane domain (M) composed of 10 transmembrane (TM) helices, and three cytosolic domains (N, P and A). The actuator (A) and phosphorylation (P) domains are connected to the M domain whereas the third nucleotide-binding domain (N) is connected to the P domain. The major difference that discriminates PMCA from SERCA structurally is the addition of a C-terminal tail in PMCA. This tail contains a calmodulin (CaM) binding site and acts as an auto-inhibitory segment which is thought to prevent catalytic turnover, keeping PMCA in an inactive state. More recently, the C-terminal end of PMCA has been shown to contain a PDZ binding domain, containing proteins as well as a specific site for caspase-3, one of the main proteases of the apoptotic cascade (Zabe & Dean 2001; Pászty et al. 2007). A representation of the structure of PMCA is shown in Figure.1.6.

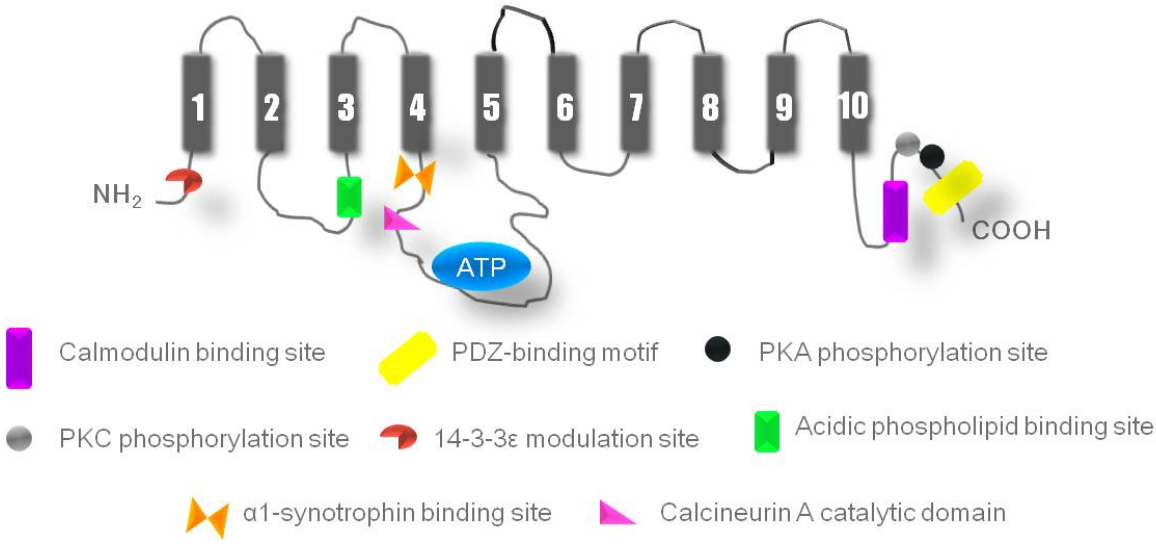


Figure 1.6. Schematic representation of the Plasma Membrane Ca^{2+} ATPase (including regulation sites)

PMCA is organised with 10 transmembrane (TM) domains with approx. 80% of the pump mass protruding into the cytosol with an N-terminal sequence of approximately 90 residues, two main central domains and a long C-terminal tail downstream of the 10th TM domain. The first central domain protrudes between TM domains 2 and 3; it contains a basic stretch of amino acids which is the main site of interaction with activatory acidic phospholipids. It also contains a site for the auto-inhibitory C-terminal calmodulin-binding domain. The second (and largest) cytosolic unit connects TM domains 4 and 5, and contains the active site for ATP It also contains binding sites for α1-synotrophin and calcineurin. Towards the N-terminal end contains a proposed inhibitory site for the 14-3-3 (sigma) protein identified on PMCA (isoforms 4). The cytosolic C-terminal tail contains the most important regulatory sites of the pump, including the calmodulin-binding domain and consensus sites for protein kinases A and C and a newly identified PDZ biding domain. The calmodulin-binding domain, owing to its basic character, also binds acidic phospholipids and thus could also mediate the response of the pump to them.

1.6.3. Membrane calcium ATPases: Conformational states

Both PMCA and SERCA can exist in two conformational states, E1 and E2. E1 binds to ATP and Ca^{2+} in the cytosol to form a high energy aspartylphosphate intermediate. The protein then undergoes a conformational change into the E2 state while translocating Ca^{2+} to the extracellular surface. Due to their low affinity for the E2 state, Ca^{2+} is released into the exoplasm and both revert back into the E1 state as a result of hydrolysation of the acetylphosphate. A report published by Salvador and colleagues suggested that PMCA could have a stoichiometry of 1:1 meaning one Ca^{2+} molecule is translocated for the hydrolysis of each ATP. This contrasts with SERCA, which has a stoichiometry of 2 Ca^{2+} molecules per ATP (Salvador et al. 1998).Thus PMCA has a Hill coefficient of 1 for Ca^{2+} while SERCA has 2 (Grover & Samson 1986).

1.6.4. PMCA in the cardiovascular system

PMCA's are emerging as important regulators of the cardiovascular system (Cartwright et al. 2005). For example, studies using transgenic mice showed that isoform 4b regulates β-adrenergic cardiac contractile responses *via* the interaction and modulation of nNOS activity in cardiomyocytes (Oceandy, Cartwright et al. 2007). Each isoform is said to be expressed in a tissue specific manner (Hammes et al. 1994). Recently, transgenic approaches have led to the elucidation of roles which are specific to PMCA4 in the heart and vasculature. For example, PMCA4 has large intracellular loops and a PDZ-binding domain which allow it to interact with intracellular signalling molecules such as nNOS (Schuh et al. 2001), syntrophin (Williams et al. 2006), calcineurin (Buch et al. 2005; Holton et al. 2007) and Ras association domain-containing protein 1 (RASSF1) (Armesilla et al. 2004). These interactions regulate a number of processes in the cardiovascular system. PMCA4 has also been shown to regulate paracrine secretion (Toescu & Petersen 1995), sperm motility (Schuh et al. 2004), apoptosis (Orrenius et al. 2003) and cell differentiation (Hammes et al. 1994). The fact that PMCA4 acts *via* signalling molecules specific to a particular cell type or a specific intracellular location, such as caveolae (Pang et al. 2005) explains its diverse functions.

1.6.5. PMCA in platelets

There are four genes that encode for PMCA (PMCA1-4) (human genome database nomenclature (ATP2B1-ATP2B4)) and additional isoforms generated *via* alternative RNA splicing of the primary gene transcripts gives rise to over 30 possible isoforms (Brandt & Neve 1992; Strehler & Zacharias 2001). PMCA1 and 4 are expressed ubiquitously; whereas PMCA 2 and 3 are mainly found in excitable cell types i.e. the brain, heart and kidney. Platelets express PMCA1 and PMCA4 (Martin et al. 2000). Very few isomer specific functional studies have been conducted to date; however, PMCA's generally have been shown to form the main mechanism for Ca^{2+} removal across the platelet plasma membrane (Rosado & Sage 2000).

PMCA's are reportedly inactive during platelet activation (to promote a rapid increase in $[Ca^{2+}]_i$) and become active during the disaggregation phase (Wan et al. 2003). Furthermore, PMCA4 interacts with the cytoskeleton and is translocated to filopodia during activation suggesting roles specific to this locality (Zabe & Dean 2001). The physiological relevance of this interaction has been demonstrated by observing impaired clot retraction when association of PMCA4 with β-actin is blocked (Dean & Whiteheart 2004). One study by Zabe and colleagues demonstrated that the inhibition of PMCA4-β-actin interaction does not affect platelet aggregation. However, this observation may be due to functionally relevant interactions occurring later in the activation process (Zabe & Dean 2001). Over expression of constitutively active PMCA4 in platelets has also been shown to

enhance platelet aggregation and this was hypothesised to occur due to the localised removal from Ca^{2+} from unidentified signalling molecules (Schuh et al. 2001).

PMCA's therefore play an important role in platelet Ca^{2+} transport and there is ever more evidence of location-specific and time-specific functional roles of PMCA4 that could be useful in identifying a potential therapeutic target in the treatment of thrombotic associated cardiovascular diseases. The role of PMCA in regulating platelet Ca^{2+} homeostasis and function however, remains incompletely understood.

1.6.6. SERCA in platelets

The SERCA family is issued from three genes named SERCA 1-3 (human genome database nomenclature (ATP2A1-ATP2A3)), that correspond to 3'-end alternatively spliced RNA's and the corresponding protein isoforms (Bobe et al. 1994; Wuytack et al. 1994). Early studies using molecular techniques such as cloning, reverse transcription-polymerase chain reaction (RT-PCR) and immunoblotting identified that human platelets express the SRECA2a isoforms (Enouf et al. 1992). Following this, two other isoforms were identified in platelets, each possessing molecular weights of 100 and 97kD. These were SERCA2b and SERCA3a (Papp et al. 1991; Papp et al. 1992; Papp et al. 1993). Few studies have addressed the physiological relevance of this pump in the removal of Ca^{2+} in either resting or activated platelets, however, some studies have suggested that following platelet stimulation, the activation of SERCA precedes that of the PMCA pump and that during the resting state of platelets, the extrusion of Ca^{2+} through PMCA is the principle mechanism of Ca^{2+}regulation (Rosado & Sage 2000; Redondo et al. 2005). SERCA's are responsible for the sequestration of Ca^{2+}into the ER reservoir and only a minor fraction (about 1%) of the total platelet Ca^{2+} is retained within the ER itself (Brass 1984; Cavallini & Alexandre 1994). When the concentration of intracellular Ca^{2+} increases, this initiates the Ca^{2+}-release mediator IP_3 to release Ca^{2+} from the ER stores, and it is this depletion of Ca^{2+} into the cytosol, that activates the opening of Ca^{2+} influx channels in the plasma membrane (Putney 1990; Meldolesi et al. 1991).

1.6.7. Sodium-Calcium Exchanger (NCX) in platelets

The process of Na^+/Ca^{2+} exchanger (NCX) was first described in the late 1960's through measurements of Na^+-dependent Ca^{2+} fluxes in various tissues (Blaustein & Lederer 1999). The NCX is a protein belonging to a secondary group of transporters, many of which utilise the energy stored in the transmembrane (TM) Na^+ gradient for uphill transport of other substrates, in this case Ca^{2+}. NCX's are generally thought to operate *via* the so-called alternating access model in which a single set of substrate or ion binding sites change their accessibility from the extracellular environment to the cytosol and *vice versa*. Unlike ion channels, each ion translocation event of the NCX is thought to be accompanied by a major conformational change, and these proteins are therefore thought to exist in at least two, and possibly more, distinct conformational states (Altimimi & Schnetkamp 2007).

Another important physiological consequence of the alternating access model is that NCX proteins are expected to be bidirectional. They can mediate both Ca^{2+} extrusion as well as Ca^{2+} import, dependent on the transmembrane Na^+ gradient and to a lesser degree on membrane potential (Schnetkamp 1989). Specifically, events that remove or significantly lower extracellular Na^+ or increase intracellular Na^+ are expected to shift the transport mode from Ca^{2+} extrusion (forward exchange) to Ca^{2+} influx (reverse exchange) which could rapidly lead to Ca^{2+} overload in cells.

The NCX expresses a low affinity but high capacity for Ca^{2+}, transporting up to five thousand Ca^{2+} ions per second (Carafoli et al. 2001).

Therefore, it requires large concentrations of Ca^{2+} to be effective, but is useful for ridding the cell of large amounts of Ca^{2+} in a short time. This suggests that the NCX also plays an important role in regaining the cell's normal Ca^{2+} concentrations after an excitotoxic insult (Kiedrowski et al. 1994). This contrasts to PMCA, which has a much higher affinity and lower capacity for Ca^{2+} and is capable of effectively binding to Ca^{2+} even when its concentrations are quite low. The activities of NCX and PMCA therefore complement each other.

1.7. PMCA inhibitors

1.7.1. Carboxyeosin (CE)

Carboxyeosin (CE) is a derivative of the fluorescein isothiocyanates (FITC) commonly used for covalently labelling proteins and within cellular biology to label and track cells in fluorescence microscopy (for example, flow cytometry) (Vanderkooi et al. 1987). The use of fluorescein derivatives to inhibit plasma membrane Ca^{2+} pumps was first described by Gatto and Millanic in 1993. They demonstrated that CE irreversibly inhibits PMCA's in red blood cells (Gatto & Milanick 1993) and later went onto demonstrate that Eosin (which is the active constituent of CE) inhibited PMCA's in cardiac myocytes without affecting the NCX (Gatto et al. 1995). A number of studies have shown CE to have significant PMCA-inhibitory effects; for example, Moccia and colleagues demonstrated that by inhibiting the PMCA pump with CE, and NCX with benzamil and by removing extracellular Na^{+}, the decay phase of an agonist-evoked Ca^{2+} transient in rat microvascular endothelial cells and the removal of Ca^{2+} is achieved by both the PMCA pump and NCX (Moccia et al. 2002). Recently, CE has been used to inhibit PMCA in other cell types. For example, treatment of live sea urchin sperm with CE resulted in elevations of intracellular Ca^{2+} and loss of flagellar motility (Liang et al. 2004) and within mice, the application of CE reduces sperm motility due to inhibition of PMCA4 (Schuh et al. 2004).

1.7.2. Caloxins

Caloxins are specific extracellular peptide inhibitors of PMCA, and were first described by Chaudhary and colleagues in 2001 who showed that through screening a random peptide phage display library, they were able to select the first extracellular peptide to inhibit PMCA. They identified that Caloxin-2A1 binds to the second extracellular domain sequence of the PMCA pump. This domain links transmembrane domains 3 and 4 and mutagenesis of key residues in domain 4 has been shown to be inhibitory (Guerini et al. 1996). They also investigated the effects of Caloxin-2A1 on endothelium-dependent relaxation of rat aorta where they observed Caloxin-2A1 produced relaxation by activating eNOS (Chaudhary et al. 2001). Caloxin 2A1 was also the first peptide to be identified that inhibited PMCA by binding to the synthetic sequence of exdom domain 2. Since this development, groups have gone onto look at PMCA inhibition using Caloxins in mesenchymal stem cells (Kawano et al. 2003), aortic endothelium, smooth muscle cells (Pande et al. 2006) and in primary cultures of cerebellar granule cells (Vale-González et al. 2006).

1.8. SERCA and NCX inhibitors

1.8.1. Thapsigargin and 2, 5-di-(tert-butyl)-1,4-benzohydroquinone (BHQ)

Thapsigargin and BHQ are widely used as they are membrane permeable and can block ER Ca^{2+} pumps in intact cells (Kass et al. 1989; Thastrup et al. 1990; Moore et al. 1987). Both have been shown to exert their inhibitory action by stabilisation of the E2 conformational state of the ATPase and this is followed by an influx of $Ca^{2+}$from the extracellular medium (Jackson et al. 1988; Thastrup et al. 1990; Wictome, et al. 1992a; Wictome, et al. 1992b). Whereas BHQ and Thapsigargin both act to elevate intracellular Ca^{2+} levels, Thapsigargin has been shown to induce rapid Ca^{2+} store depletion in platelets. In addition, both compounds have differing functional effects. Thapsigargin has been shown to induce aggregation through tyrosine phosphorylation of the PMCA (Rosado & Sage 2000) whereas BHQ does not (Brune & Ullrich 1991) This could be due to the presence of two platelets Ca^{2+} stores with high and low Ca^{2+} leakage rates being previously described (Cavallini et al. 1995; Doni et al. 1994). It has been demonstrated that different SERCA isoforms in platelets are differentially sensitive to either BHQ or Thapsigargin, one that is sensitive to high thapsigargin concentrations and another by low concentrations of thapsigargin but is not affected by BHQ (Cavallini et al. 1995). Both of these compounds are therefore useful in studies examining the impacts of increasing cytosolic Ca^{2+} concentrations within various cell types.

1.8.2. Bepridil (1-N-benzylanilino-2-pyrodinino-3-isobutoxypropane)

Bepridil is a class IV NCX antagonist and used in the treatment of angina pectoris and supraventricular and ventricular fibrillation (Sugi 2006). It decreases Ca^{2+} influx through potential-dependent and receptor-operated sarcolemmic channels and acts intracellularly as a CaM antagonist and Ca^{2+} sensitiser (A. Gill et al. 1992).

In cardiac muscle, it enhances the sensitivity of troponin C to Ca^{2+}, stimulates myofibrillar ATP activity, removes CaM's inhibitory effect on sarcoplasmic reticulum Ca^{2+} release, and inhibits NCX actions that tend to offset the effects of Ca^{2+} influx blockade on cardiac contractile force (Gill et al. 1992; Murakami et al. 2010; Nawada et al. 1995; Watanabe et al. 2006).

The use of Bepridil in platelet studies has been described by Shiraga and colleagues. They reported that by using Bepridil, which is a relatively specific inhibitor of the NCX, inhibition of platelet aggregation induced by various physiological agonists could be achieved (Shiraga et al. 1996).

1.9. PMCA4$^{-/-}$ Mice

Transgenic animals are used as experimental models in biomedical research. As PMCA is thought to play an important role in platelet Ca^{2+} transport, the use of genetically modified mice that lack the PMCA4 gene could be a useful tool in determining isomer specific roles. The first studies were carried out by Schuh and colleagues who showed that homozygous male mice with a targeted gene deletion of PMCA4, which is highly enriched in the sperm tail, are infertile due to severely impaired sperm motility. Embryonic stem cells underwent homologous recombination and were injected into C57Bl/6 blastocysts. These were transferred into pseudo-pregnant foster mice to generate chimeras and later mated with C57Bl/6 females to test for germ line transmission of the targeted PMCA4 allele. PMCA4-deficient mice were finally obtained by appropriate inbreeding of heterozygous offspring and subsequent genotyping (Schuh et al. 2004).

1.9.1 Research objectives

Platelets play a central role in haemostasis and thrombosis and the regulation of Ca^{2+} within platelets is key to their function. PMCA's are currently emerging as having an important role within the cardiovascular system, however their role in platelets remains to be defined. Therefore, the main objectives of this study were to investigate the role of PMCA in platelet function and Ca^{2+} homeostasis. This was achieved using a pharmacological inhibitor of PMCA, carboxyeosin. Following this, isomer specific roles were investigated using PMCA4 knock-out mice and the molecular signalling pathways of PMCA were also explored. Lastly, the consequence of inhibition in comparison to other Ca^{2+} regulators in platelets, SERCA and the NCX was also investigated.

CHAPTER TWO

2.0. Materials & Methods

2.1. Materials

Table 2.1. List of compounds and purchasing companies

Compound	Purchased From
• [^{3}H]-5-HT	Perkin Elmer (Cambridgeshire, UK)
• 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxal in-1-one (ODQ)	Tocris Bioscience (Bristol, UK)
• 1x RIPA solution • Acetic acid solution • Acid Citrate-Dextrose Solution (ACD) • Bovine Serum Albumin Powder • Citric Acid • D-Glucose • EDTA • Ethanol solution • Ethidium Bromide Solution • Fibrinogen (Bovine) • 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid (HEPES) • Imipramine • Isopropanol solution • KCl • Methanol solution • $MgCl_2$ • Milk powder • $Na_2H.PO_4\ 12H_2O$ • NaCl • $NaHCO_3$ • NaOH • PGI_2 • SDS • Sodium Nitroprusside • Thrombin (Bovine) • Tris • Trisodium Citrate • Triton-X 100 • Tween-20 • Urethane	Sigma (Poole, UK)

• 2,5-di-(tert-butyl)-1,4-benzohydroquinone	Calbiochem (Darmstadt, Germany)
• AbGene Reddy-Mix • Enhanced chemiluminescence detection system (ECL™) • Tris (2-Carboxyethyl) phosphine Hydrochloride (TECP.HCl)	Thermo Scientific (Northumberland, UK)
• Agarose powder • Hyperladder II	Bioline (London, UK)
• Bio-Rad protein assay kit • Polyvinylidene difluoride (PVDF) membrane	Bio-Rad (Hertfordshire, UK)
• CEDA-SE 95-(and-6)-Carbon diacetate succinimidyl ester • Proteinase K • PMCA Antibody • PMCA (Neomycin) Antibody	Invitrogen (Paisley, UK)
• Collagen (Collagen fibres predominantly type I from equine tendons)	Nycomed (Munich, Germany)
• Ethly Chloride Spray (Local anaesthetic) - animal use only	Miller Medical Supplies (Newport, UK)
• Horseradish peroxidase-conjugated (HRP) • Secondary antibodies (anti-rabbit IgG, anti-mouse IgG)	Dako (Glostrup, Denmark)
• Hyperfilm ECL • ^{111}Indium Oxine • Molecular Weight Rainbow Marker (full range)	GE Healthcare (Buckinghamshire, UK)
• Phosp - VASP 239 and Total VASP antibody	Cell Signalling (Hertfordshire, UK
• PMCA4 Antibody	SWANT (Bellinzona, Switzerland)
• Protease Inhibitor cocktail I and II	Roche (Hertfordshire, UK)
• Quick-Load 100bp DNA ladder • p-Nitrophenol Phosphate	Biolabs (Hertfordshire, UK)
• Scintillation Fluid	National Diagnostics (Hessle, UK)

Table 2.2. List of equipment and animals used detailing purchasing companies

Equipment	**Purchased From**
• Single point extended area ratio (SPEAR) detector	eV Products (Saxonburg, PA)
• Sysmex F-820 Haematology Analyser	Sysmex (Milton Keynes, UK)
• Spectrum Techniques Software	Mumed Systems (London, UK)
• Chronolog Multi Channel Aggregometer	Chronolog chorporation (Havertown, PA, USA)
• Luminescence Spectrometer LS50B	Perkin Elmer (Cambridgeshire, UK)
• Quartz SUPRASIL Macro/Semi-micro cell cuvette	Perkin Elmer Instruments (Cambridgeshire, UK)
• Liquid Scintillation Counter 1414	WALLUC Instruments (Minnesota, USA)
• Semi-dry transfer blotter • Bio-Rad 18-630 microplate reader	Bio-Rad (Hertfordshire, UK)
Animals	**Purchased From**
PMCA4 C57/SV-129 mice (Male and Female) Approximately 12 weeks old (20-30 grams)	Initial gift from Manchester University, breed at Imperial College London under a heterozygous breeding program

Table 2.3. Buffer compositions

Modified Tyrodes HEPES Buffer (mTHB)	
Compound	Final concentration
D-Glucose	10 mM
HEPES	20 mM
KCl	2.9 mM
$MgCl_2$	10 mM
$Na_2H.PO_4$ $12H_2O$	0.34 mM
NaCl	134 mM
$NaHCO_3$	12 mM
Dissolved in dH_20 and pH to 7.4 - kept for one week only at 4 °C	
Ca^{2+} and Mg^{2+} Free Modified Tyrodes HEPES Buffer (CMFB)	
Compound	Final concentration
D-Glucose	10 mM
KCl	2.9 mM
$Na_2H.PO_4$ $12H_2O$	0.34 mM
NaCl	134 mM
$NaHCO_3$	12 mM
Dissolved in dH_2O – kept for one week only at 4 °C	

Table 2.4. Sequences of the oligonucleotide pair sequences used for genotyping mouse tail tips using PCR

Primer Name	**Sequence**
PMCA Forward	5’-GGTCTATCTGGGAACCCTGC-3’
PMCA Reverse	3’-GAGAATGGCTTACTAGCTCT-5’
PMCA Knock Out (Neomycin) Forward	5’-GAACAAGATGGATTGCACGC-3’
PMCA Knock Out (Neomycin) Reverse	3’-ACAACGTCGAGCACAGCTGC-5’

2.2. EXPERIMENTAL METHODS

2.2.1 Cell preparation techniques

2.2.1.1. Preparation of human washed platelets

Human blood was drawn from consenting, healthy drug-free volunteers at least 1 hour prior to experiments as described previously by (Yanaga et al. 1995). 50 ml of blood was drawn from either a cephalic, basillic or medial vein using a 60 ml pull back syringe connected to a 21 gauge butterfly cannula into 1:9 volume 3.8% (w/v) trisodium citrate.

Platelet rich plasma (PRP) was separated from erythrocytes and leukocytes by centrifugation at 100 *g* for 20 minutes at room temperature. Platelets were then isolated from the plasma by centrifugation at 1400 *g* for 10 minutes in the presence of 125 µg/ml PGI_2 to reduce the risk of activation.

Platelet poor plasma (PPP) was discarded and the pellet was recovered and re-suspended in physiological modified Tyrodes-HEPES buffer (mTHB). A repeated wash was performed in mTHB containing PGI_2 (125 µg/ml). Platelet numbers were determined using a Sysmex F-820 Haematology Analyser (Milton Keynes, UK) and adjusted to a density of 250 x 10^3 cells/ml unless stated otherwise. Platelets were then left to rest at 27 °C for 30 minutes. This study was approved by the Brompton, Harefield and National Heart and Lung Institute Research Ethics Committee (REC

approval number 07/H0708/72) and informed consent was obtained from all participants.

2.2.1.2. Mouse platelet preparation

Blood from either male or female PMCA4 C57/SV-129 mice, approximately 12 weeks old (20-30g) was obtained on the day of the experiment (under terminal anaesthesia Urethane 25% (w/v)) *via* cardiac puncture and followed by termination using a method approved in Schedule 1 of the Animals (Scientific Procedures) Act 1986. Blood was drawn into a syringe containing 150 µl ACD. PRP was obtained by centrifugation at 300 *g* for 3 minutes at room temperature and transferred into new microcentrifuge tubes. 400 µl of CMFB and ACD solution (3:1v/v) containing PGI_2 (1 mg/ml) was then added to the remaining red blood cells (RBC) and the previous centrifugation step repeated. The diluted PRP was removed and added to the previous PRP. The removal of contaminating RBC was achieved by a further centrifugation step at 200 *g* for 2 minutes. PRP was transferred to fresh tubes and platelets isolated by centrifugation at 1500 *g* for 7 minutes. Platelets were finally re-suspended in 200 µl CMFB prior to platelet numbers being determined using a Sysmex F-820 Haematology Analyser. Platelets were adjusted to 4×10^6 cells/ml unless otherwise stated and left to rest for 30 minutes at 27 °C to allow recovery prior to experiments.

2.2.2. Genotyping

2.2.2.1. DNA extraction from mouse tail

Male or female mice were restrained using an appropriate mouse restraining tube and tails sprayed with Ethyl Chloride local anaesthetic spray. Up to 3 mm of mouse tail tip was removed using a sterile scalpel blade, and placed in a sterile microcentrifuge tube. Tail tips were left overnight in 700 µl digestion buffer (50 mM Tris, 100 mM EDTA, 0.5% SDS) along with 35 µl proteinase K (10 mg/ml) at 56 °C). Tail samples were then centrifuged at 13000 *g* for 10 minutes at 4 °C and supernatants transferred into new sterile microcentrifuge tubes containing 700 µl isopropanol. Tubes were gently inverted to mix and left for 30 minutes on ice. DNA was pelleted by centrifugation at 13,000 *g* for 5 minutes and the supernatant discarded. The remaining pellet was washed in 70% ethanol, the supernatant removed and the pellet left to air dry for 20 minutes on ice. Pellets were re-suspended in 50-500 µl Tris-EDTA buffer (TE, 10 mM Tris pH 7.5, 1 mM EDTA pH 8.0) and incubated at 65 °C for 1 hour with occasional agitation to gently dissolve the pellet.

2.2.2.2. Polymerase chain reaction (PCR)

PCR (Mullis & Faloona 1987) was used to amplify DNA from digested mouse tail tip samples (Section 2.2.2.1.). A reaction mix containing the following was prepared for a 25 µl reaction (brackets refer to stock concentrations):

- 12 µl AbGene Reddy-Mix (350 µM each dNTP, 2.25 mM $MgCl_2$, *Taq*, DNA polymerase 1.25 U)
- 1 µl each primer per sample (10 µM)
- 1 µl DNA (1.5 ng/ml)
- and made up to 25 µl using ddH_20

DNA samples were denatured at 94 °C for 23 minutes, prior to 40 cycles of denaturing at 94 °C for 10 seconds, annealing at 56 °C for 30 seconds and extension at 68 °C for 1 minute. The samples then underwent a final extension step at 68 °C for 7 minutes and were held at 4 °C. DNA was visualised on a 1% agarose gel (w/v) in Tris, acetic acid and EDTA buffer (TAE, Tris-base 96.8 g, acetic acid 22.84 ml, EDTA) (40 ml (of 0.5 M) in 1L) containing 10 µl Ethidium bromide (10 mg/ml) by short wave UV light using a UV transilluminator. DNA bands at 344bp represented PMCA (wild-type, +/+), whereas bands at 245bp represented Neomycin resistant PMCA (knock out, -/-). Bands at both positions represented PMCA (heterozygous, +/-) mice.

2.2.3. Molecular techniques

2.2.3.1. Protein Quantification

Protein concentration was measured using a Bio-Rad RC-DC (reducing agent and detergent compatible protein assay which was adapted from (Bradford 1976). Lysates of an unknown concentration (5 µl) or BSA standards (5 µl of 2, 1.5, 1, 0.5, 0.25, 0.125 and 0.0625 mg/ml) were added in triplicate to a 96 well microtiter plate. Ready-made developing reagents were added to each well. The plate was then left in the dark at room temperature for 15 minutes to allow the colour to develop after which, the absorbance of each sample was measured using a Bio-Rad 18-630 microplate reader at 595 nm.

2.2.3.2. Protein isolation and western blotting

Tissue samples were homogenised (placed into liquid nitrogen and crushed using a pestle and mortar) and incubated with a detergent lysis buffer (platelets, were directly treated with the detergent buffer), (1.65 ml 1x RIPA buffer (containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0), 350 µl 1x protease inhibitor cocktail, 2 µl phosphatase inhibitor cocktail I, 2 µl phosphatase inhibitor cocktail II and 2 µl Tris (2-Carboxyethyl) phosphine Hydrochloride (TECP.HCl)) and left for 30 minutes on ice. Cell lysates were then centrifuged at 13,000 *g* to pellet debris and cytoskeletal components and supernatants were removed into fresh microcentrifuge tubes and placed on ice. Proteins were later quantified using a Bio-Rad protein assay kit (Section 2.2.3.1.), electrophoresed on 8-12% SDS-polyacrylamide gels and electrophoretically transferred for 1 hour at 120 volts in a semi-dry blotter (Bio-Rad, UK) onto polyvinylidene difluoride (PVDF) membranes for subsequent probing. Blots were incubated overnight at 4 °C with either 5% (v/v) BSA or milk (depending on antibody used) in 1xPBST (phosphate buffered saline tween-20) to block residual protein binding sites. Membranes were then incubated with primary monoclonal antibodies diluted 1:1000 in 1xPBST for 1 hour at room temperature and washed six times for 5 minutes each with 1xPBST. To detect the primary antibody, blots were incubated with horseradish peroxidise-conjugated anti-mouse, rabbit or goat IgG (depending on primary antibody) diluted 1:10000 in 1xPBST for 1 hour at room

temperature. These were further washed six times for 5 minutes with 1xPBST and exposed to enhanced chemiluminescence reagents between 30 seconds to 4 minutes. Blots were then exposed to pre-flashed photographic film.

2.2.4. Functional assays

2.2.4.1. Aggregation using washed platelets

Washed platelets (Section 2.2.1.1.), (450 µl) were made up to a density of 250 x 10^3 cell/ml and pre-warmed to 37 °C for 90 seconds prior to stimulation with an agonist (50 µl) in a siliconised glass cuvette in an optical platelet aggregometer, under stirring conditions (~1200rpm). Platelet aggregation was measured for up to 3 minutes as described by Born 1962, using light transmission through a sample. Changes in optical density corresponding to shape change and aggregation following agonist administration were recorded using a chart recorder (Chrono-log Corporation, Havertown, PA, USA) (Born 1962b). Platelet agonists were administered in volumes of 50 µl per sample.

2.2.4.2. Adhesion using washed platelets

Platelet adhesion was measured using a modified protocol from Bellavite and colleagues (Bellavite et al. 1994) in which platelet alkaline phosphatase is used as an indicator of the proportion of platelets adhering to an adhesive substrate coated onto the wells of a 96 well plate. Multiple wells of a sterile 96 well plate were coated with 50 µl of 50 µg/ml fibrinogen or collagen (in mTHB) or mTHB control and were incubated overnight at 4 °C. Platelets were isolated (Section 2.2.1.1.), counted on the day of experiment and made up to 1 x 10^8 cells/ml and left to rest until later use.

Excess fibrinogen or collagen, incubated the night before, was removed by washing the wells twice with 200 µl mTHB and non specific adhesion was blocked with 50 µl (0.5% w/v) BSA for 1 hour at 37 °C. The wells were then washed twice with 200 µl mTHB and 50 µl platelets with/out any additional drugs were added and allowed to bind for 1 hour at 37 °C. Non-adherent platelets were removed by washing each well twice with 200 µl mTHB. Wells containing adherent platelets were incubated with 150 µl per well of acid phosphatase substrate solution (0.1 M citrate buffer, pH 5.4, containing 5 mM *p*-nitrophenol phosphate and 0.1% Triton X-100) and after 1 hour of incubation at room temperature, the reaction was stopped and the colour developed by the addition of 100 µl NaOH (2 M). The plate was immediately read on a Bio-Rad 18-630 microplate reader in the λ1-λ2 mode at wavelengths of 405-600 nm. The percentage of adherent platelets was calculated from a standard curve

obtained with known numbers of platelets normalised as a percentage of control. All experiments were performed in triplicate.

2.2.4.3. Clot Retraction

The measurement of clot retraction was adapted from Landesberg and colleagues 2005, (Landesberg et al. 2005). Human blood was drawn into 3.8% (w/v) trisodium citrate and platelet rich plasma obtained by centrifugation at 100 *g* for 20 minutes. Platelet rich plasma was counted and adjusted to 250 x 10^3 cells/ml by the addition of platelet poor plasma (obtained by centrifugation of PRP at 1400 *g* for 10 minutes) and made up to 1 ml with/out the additional drugs into sterile test tubes. 5 µl of the donor's erythrocytes were added to enable visualisation. Thrombin (2.5 U/ml) was then added and a sealed glass Pasteur pipette was fixed in place to promote the generation of fibrin. Tubes were left at room temperature for 2 hours. Photographs of the clots formed were taken and the clot weighed and measured.

2.2.4.4. $[Ca^{2+}]_i$ measurements using washed human platelets

Measurements of Ca^{2+} were adapted from Rosado and colleagues 2000, (Rosado & Sage 2000). Platelets were isolated (Section 2.2.1.1.) and adjusted to 250 x 10^3 cells/ml and loaded with Fura-2 by incubation with Fura-2-acetoxymethyl ester (Fura-2AM, 5 μM) after the first wash stage. Fura-2 was left to penetrate cells for 40 minutes (in the dark) at 37 °C before continuing with the second wash. Pre-warmed platelets were stimulated with a chosen agonist (50 μl) in a Quartz SUPRASIL Macro/Semi-micro cell cuvette in a Perkin Elmer Luminescence Spectrometer LS50B with constant stirring at 1200 rpm at 37 °C. Excitation wavelengths were adjusted to 340 and 380 nm and emission at 509 nm. Changes in $[Ca^{2+}]_i$ were measured using the Fura-2 340/380 fluorescence ratio (which is directly correlated to $[Ca^{2+}]_i$) as it automatically cancelled out confounding variables, such as variable dye concentration and cell thickness. Data was stored on time drive files using FL-WinLab software (Perkin Elmer).

2.2.4.5. Dense granule secretion

5-HT release from platelets was determined as a measure of dense granule secretion modified from Poole and colleagues (Poole et al. 1997). Human PRP was obtained as previously described in section 2.2.1.1. Platelets were then loaded with 1 μCi/ml [^{3}H] 5-HT and left to rest for 1 hour at 37 °C. Platelets were then isolated from PRP as described previously (Section 2.2.1.1.) and adjusted to a density of 250 x 10^3 cells/ml with the addition of 1 μM imipramine to inhibit any reuptake to 5-HT into storage granules. After platelets were rested for 30 minutes, platelet aggregometry was performed in either resting or stimulated platelets at 37 °C under stirring conditions as described in section 2.2.4.1. Stimulations were terminated after 90 seconds with an equal volume of ice-cold 6% glutaraldhyde (v/v) and the samples immediately transferred to fresh microcentrifuge tubes on ice. Samples were centrifuged at 16, 000 *g* for 5 minutes to remove the platelets. Levels of [^{3}H] 5-HT released into the supernatant was then measured by transferring 800 μl of the supernatant from each sample to scintillation vials containing 4 ml scintillation fluid and subjecting the samples to scintillation spectrometry using a WALLUC® scintillation analyser. [^{3}H] 5-HT release was expressed as a percentage of total platelet content minus release in the presence of vehicle alone.

2.2.5. In vivo measurements of thromboembolism

2.2.5.1. ^{111}Indium Oxine labelling of platelets

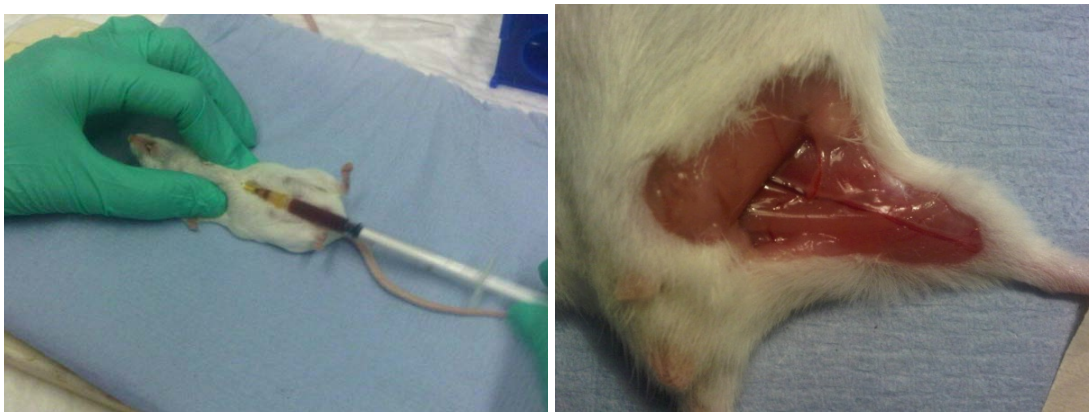
Mouse platelets were collected as described previously (Section 2.2.1.2.).

The surface of the platelet pellet was then washed carefully three times with 200 µl CMFB. Platelets were gently re-suspended in 1.5 ml of CMFB and incubated for 10 minutes at room temperature wit 1.8 MBq ^{111}Indium Oxine (^{111}In). During this step, the radioactivity of the solution was measured by a γ-radiation spectrometer. A further centrifugation step (670 *g* for 7 minutes) was performed and the supernatant removed and the final radiolabelled platelet pellet re-suspended in CMFB (200 µl per mouse used). The radioactivity of platelets was again measured to calculate the percentage of labelling.

2.2.5.2. Radiolabelled platelet monitoring

^{111}In labelled platelets were injected intravenously through the femoral vein after minor surgery to expose the vein (Fig. 2.2B). Circulating platelets were monitored in the pulmonary circulation using a using a single point extended area ratio (SPEAR) detector (eV Products, Saxonburg, PA) probe located over the thorax (Fig. 2.2C). Counts were continuously recorded using a UCS-20 spectrometer connected to a laptop (Fig. 2.2D). Specialised software supplied by Mumed Systems (London, UK) was used to measure changes in counts over time.

(A) (B)



(C) (D)

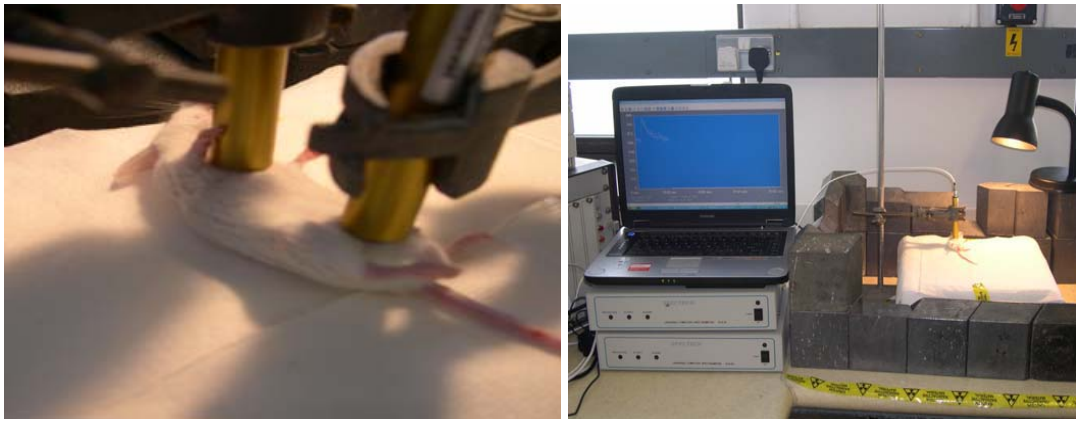


Figure 2.2. Measurement of thromboembolic platelet responses in the mouse
Figure A shows platelet collection *via* cardiac puncture from anaesthetised donor mice. (B) The femoral vein exposed prior to injection of ^{111}Indium Oxine radiolabelled washed platelets. (C) Placement of probes over the thoracic region and abdomen and (D) Equipment used to monitor radiolabelled platelets.

2.2.5.3. Administration of compounds

^{111}In labelled platelets were allowed to equilibrate within the circulation for 30 minutes prior to injection with experimental compounds or relevant control. Platelet aggregation was induced by intravenous injection (i.v) of collagen (50 µg/kg). The concentration of collagen was selected based on published data from our group (Tymvios et al. 2008). All compounds were administered intravenously in a 50µl volume, through the femoral vein and extra care was taken so as to ensure the vein did not collapse. A characterised "whitening" of the vein was apparent when the agent had been introduced successfully. The full time course of the platelet aggregation response was measured and recorded as changes in radioactive counts representing platelet aggregation and disaggregation in the pulmonary vasculature (Tymvios et al. 2008).

Data was presented as percentage changes from stable baseline values recorded prior to administration of platelet agonists.

2.3. STATISTICS

Data was analysed and all bar graphs were drawn using the GraphPad Prism software package (GraphPad, U.S.A). All results were given as the mean ± the standard error of mean (SEM). A one way ANOVA (Kruskal-Wallis) was used to determine significance followed by a multiple comparison test (Dunn's correction) to identify significance between groups.

Where indicated, paired t-Tests were performed on the results using Microsoft Excel after confirming normality. P values <0.05 were considered as significant.

Details of statistical analysis for each data set are provided in the legends of accompanying figures.

CHAPTER THREE

3.0. The Plasma Membrane Calcium ATPase (PMCA) in platelets

3.1. INTRODUCTION

PMCA's extrude Ca^{2+} from a variety of cell types and have recently been shown to regulate signalling events and function in the cardiovascular system (Oceandy et al. 2007; Oceandy, Cartwright et al. 2007; Choi & Eisner 1999). However, the functional and signalling roles of PMCA in platelets remain undefined.

In this chapter, the effects of a pharmacological PMCA inhibitor, Carboxyeosin (CE) on platelet function were examined using various functional assays and its effects on Ca^{2+} homeostasis determined. The validity of CE as a selective inhibitor of PMCA was also investigated.

CE has been used to inhibit PMCA in a variety of different cell types, including cardiomyocytes (Choi & Eisner 1999), embryonic stem cells (Yanagida et al. 2004) and in sperm (Schuh et al. 2004). It exerts its effects by crossing the plasma membrane where it is cleaved by intracellular esterases to yield eosin. Eosin further interacts with amine groups in proteins and inhibits PMCA independently of the ATPase site (Gatto & Milanick 1993; Gatto et al. 1995).

The role of PMCA was investigated, not only through the use of CE, but also using PMCA4 knock-out mice to determine isomer specific roles. In addition, the signalling pathways mediating the effects of PMCA were investigated.

3.1.1. Aims of the study

- To determine effective concentrations of CE that would impair Ca^{2+} extrusion through PMCA in platelets.
- Validate CE as a selective pharmacological inhibitor of PMCA.
- Ascertain the role of PMCA in regulating Ca^{2+} homeostasis in platelets.
- Examine the consequences of PMCA inhibition by investigating the early and later stages of platelet activation using various functional assays.
- Investigate the functional role of PMCA4 in platelets using PMCA4 knock-out (-/-) mice.
- Explore the molecular signalling mechanisms associated with PMCA in platelets.

3.2. RESULTS

3.2.1. PMCA regulates Ca^{2+} in resting and stimulated platelets

PMCA is thought to be the main route of Ca^{2+} extrusion within platelets (Rosado & Sage 2000), and changes in intracellular calcium concentration $[Ca^{2+}]_i$ are crucial for platelet activation. Furthermore, it has been previously shown in other cell types, for example within ventricular myocytes, that PMCA can be selectively inhibited using the pharmacological inhibitor Carboxyeosin (CE). However, no studies have used CE to inhibit PMCA in platelets. Preliminary studies were therefore required to determine effective concentrations of CE that could inhibit PMCA in platelets to allow future experiments to be conducted using a suitable concentration range. Following this, CE was used to identify whether PMCA could regulate Ca^{2+} homeostasis by observing Ca^{2+} measurements in either resting platelets or upon stimulation.

Washed human platelets were loaded firstly with the Ca^{2+} sensitive loading dye Fura-2 for 40 minutes in the dark (Section 2.2.4.4.) and later with concentrations of CE (10-40 μM) or DMSO control (0.4%) for 30 minutes prior to stimulation. Ca^{2+} responses were recorded in the presence of EGTA (100 μM) and platelet were stimulated using thapsigargin (1 μM) plus ionomycin (50 nM).

Stimulation of platelets with thapsigargin (1 μM) plus ionomycin (50 nM) induced a rapid and transient increase in fluorescence indicating Ca^{2+} release from intracellular stores and an increase in $[Ca^{2+}]_i$.

Once Ca^{2+} had been released from intracellular stores, extrusion of Ca^{2+} from the cytosol, which has been shown to be mediated through PMCA under these conditions (Rosado & Sage 2000), was seen as a decay of the Ca^{2+} transient towards a stable baseline (Fig. 3.1A control blue line).

The addition of CE to platelets resulted in Ca^{2+} extrusion being inhibited concentration dependently compared to the DMSO control, and this was observed as a slower return to baseline following stimulation (Fig. 3.1A). The mean percentage increase in $[Ca^{2+}]_i$ remaining 3 minutes after stimulation was calculated for each concentration of CE and was increased concentration dependently compared to the DMSO control (normalised to 100%) (Fig. 3.1B): (10 μM) 124±2.3%, (20 μM) 128±2.7%, (30 μM) 145±4.9% ($p<0.01$) and (40 μM) 144±4.2% ($p<0.01$).

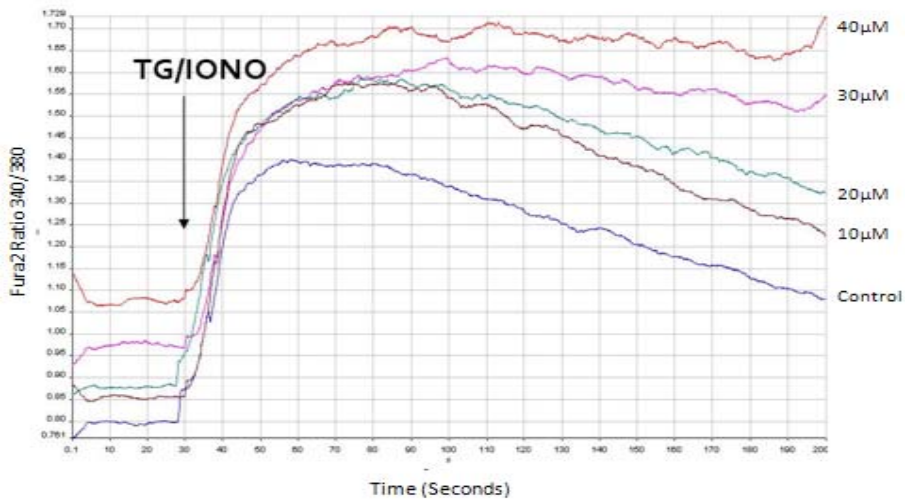
There was no evidence of an effect of CE on Ca^{2+} store loading since Ca^{2+} release (measured as peak minus basal) was unaffected by this treatment (Fig. 3.1C) Calculation were normalised to the DMSO control (100%): (10μM) 94±0.4%, (20μM) 97±0.2%, (30μM) 100±0.05% and (40μM) 95±0.3%.

CE also concentration dependently increased $[Ca^{2+}]_i$ in resting platelets compared to the DMSO control (normalised to 100%): (10 μM) 106±0.7%, (20 μM) 118±1.8%, (30 μM) 130±3.2% ($p<0.05$) and (40 μM) 141±4.3% ($p<0.01$) (Fig.3.1D), indicating a role for PMCA in maintaining basal $[Ca^{2+}]_i$.

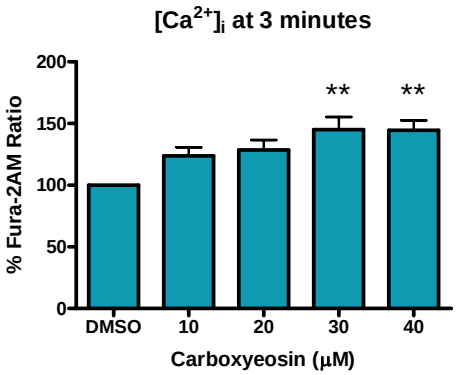
Figure 3.1. The effects of carboxyeosin on thapsigargin plus ionomycin induced Ca^{2+} release

Washed human platelets were loaded with Fura-2 and incubated with CE (10-40 μM). Fura-2 ratio emission at 340/380 nm was measured in response to thapsigargin (1 μM) and ionomycin (50 nM) in the presence of EGTA (100 μM). Graph (A) shows a typical time course of response expressed as a Fura-2 340/380 nm emission ratio against time. Figure (B) shows the % of $[Ca^{2+}]_i$ remaining at 3 minutes post stimulation. Figure (C) shows the % peak $[Ca^{2+}]_i$ minus the basal $[Ca^{2+}]_i$ post stimulation and figure (D) shows the % of $[Ca^{2+}]_i$ in resting platelets pre-stimulation. Data represents mean percentage ±S.E.M relative to DMSO (0.4%). Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values, *P<0.05, **P<0.01, n=9.

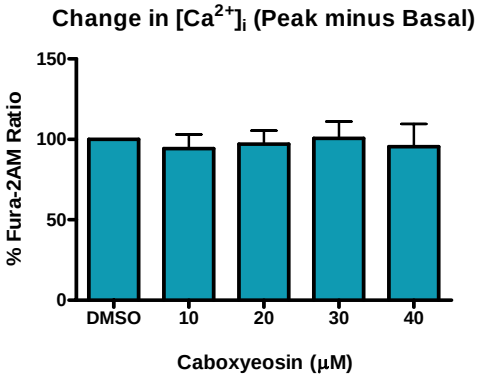
(A)


TG/IONO
40μM
30μM
20μM
10μM
Control
Fura2 Ratio 340/380
Time (Seconds)

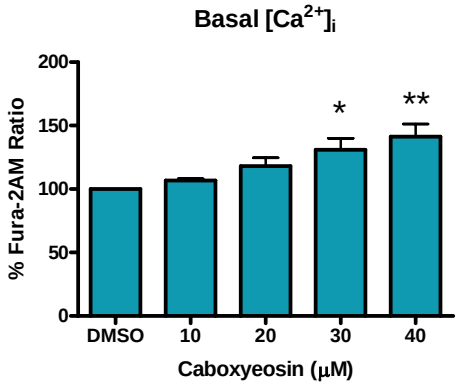
(B)


[Ca2+]i at 3 minutes
% Fura-2AM Ratio
200
150
100
50
0
**
**
DMSO
10
20
30
40
Carboxyeosin (μM)

(C)


Change in [Ca2+]i (Peak minus Basal)
% Fura-2AM Ratio
150
100
50
0
DMSO
10
20
30
40
Caboxyeosin (μM)

(D)

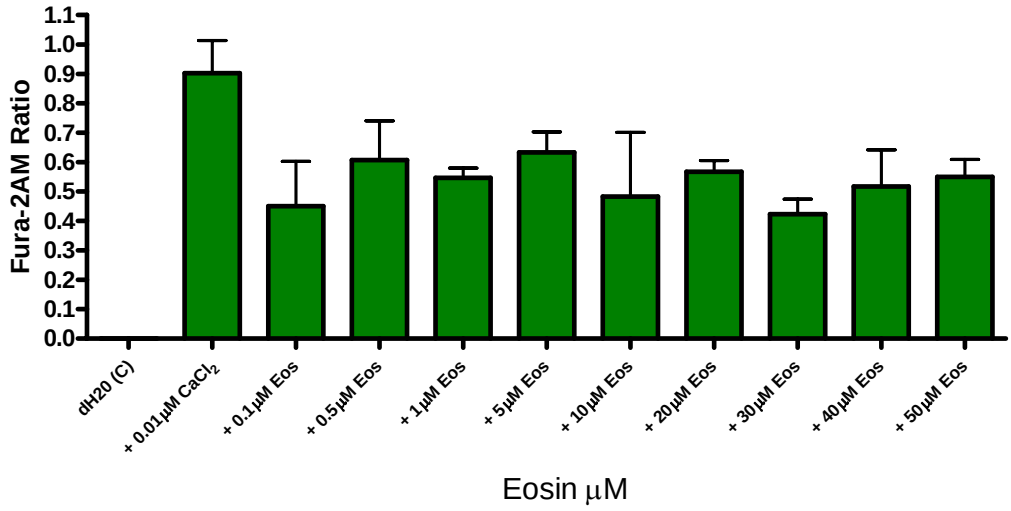

Basal [Ca2+]i
% Fura-2AM Ratio
200
150
100
50
0
*
**
DMSO
10
20
30
40
Caboxyeosin (μM)

3.2.2. Validation of carboxyeosin as a PMCA inhibitor

Although there appeared to be a significant change in the extrusion of platelet Ca^{2+} in the presence of CE, the specificity of CE needed to be verified. As described previously, CE is taken up into cells where it is cleaved to form eosin, which is fluorescently active. Therefore, since measurements to determine Ca^{2+} flux in platelets within previous experiments were conducted in the presence of Fura-2 (see Fig. 3.1.) which possesses a fluorescence emission ratio close to eosin (eosin: 538nm, Fura-2: 510 nm), experiments were conducted to see if eosin alone could concentration dependently increase Fura-2 emission independently of Ca^{2+}. This would eliminate any confounding fluorescence issues that may have affected previous results observed with CE in the presence of Fura-2 in prepared platelet suspensions. A series of experiments was therefore conducted in distilled water (dH_20), using a broad range of eosin concentrations and measured using a LS50B fluorescence spectrometer in the presence of Fura-2 (0.05 μM) and $CaCl_2$ (0.01 μM). Eosin (0.1-50 μM) caused an apparent suppression of 340/380 nm emission that was neither significant nor concentration dependent compared to the control (+0.01 μM $CaCl_2$ 0.9±2.7): (0.1 μM) 0.4±3.0, (0.5 μM) 0.6±1.9, (1 μM) 0.5±2.3, (5 μM) 0.6±1.8, (10 μM) 0.4±2.8, (20 μM) 0.5±2.2, (30 μM) 0.4±3.2, (40 μM) 0.5± 2.5 and (50 μM) 0.5±2.3 (Fig. 3.2A). In previous Ca^{2+} experiments (Fig. 3.1.), excess CE was not removed from platelets prior to analysis, so additional experiments showed that CE alone in dH_20 (0.1 –50μM) did not affect Fura-2

340/380 nm emission in the presence of $CaCl_2$ (+0.01 μM $CaCl_2$ 0.94±1.7): (0.1 μM) 0.99±0.5, (0.5 μM) 1.0±0.8, (1 μM) 1.1±0.8, (5 μM) 1.1±1.7, (10 μM) 1.0±0.7, (20 μM) 1.0±1.0, (30 μM) 1.2±1.8, (40 μM) 0.9± 0.06 and (50 μM) 1.0±0.5 (Fig. 3.2B) In addition, DMSO did not cause any interference with fluorescence when placed in dH_20 (DMSO: 0.5% (0.002±0.01)). These experiments demonstrated that although the observed increases in Fura-2 emission in resting platelets containing eosin may have been suppressed (i.e. underestimated) and quantitative studies could not be performed, the finding of a concentration dependent increase in Fura-2 emission with CE validates that PMCA regulates basal $[Ca^{2+}]_{i.}$

(A)



(B)

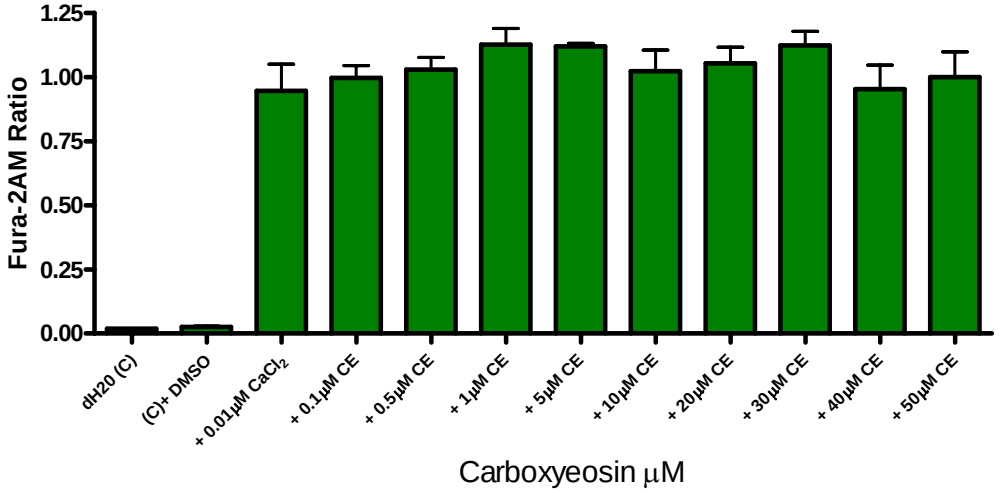


Figure 3.2. The effects of eosin and carboxyeosin on Fura-2 emission spectra
Measurements of Fura-2 340/380nm emission ratio in suspensions of dH_20 containing Fura-2 (0.05 µM) and $CaCl_2$ (0.01 µM) in the presence of either (A) eosin (0.1 – 50 µM) or (B) carboxyeosin (0.1 – 50 µM) were recorded using a LS50B fluorescence spectrometer. Mean stable 340/380 ratio was measured over a 3 minute period. Blank readings were taken in dH_2O (dH_20(C)) and with DMSO (0.5 %) ((C)+DMSO). Data represents mean percentage ±S.E.M relative to control. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values. No significant changes were observed, n=5.

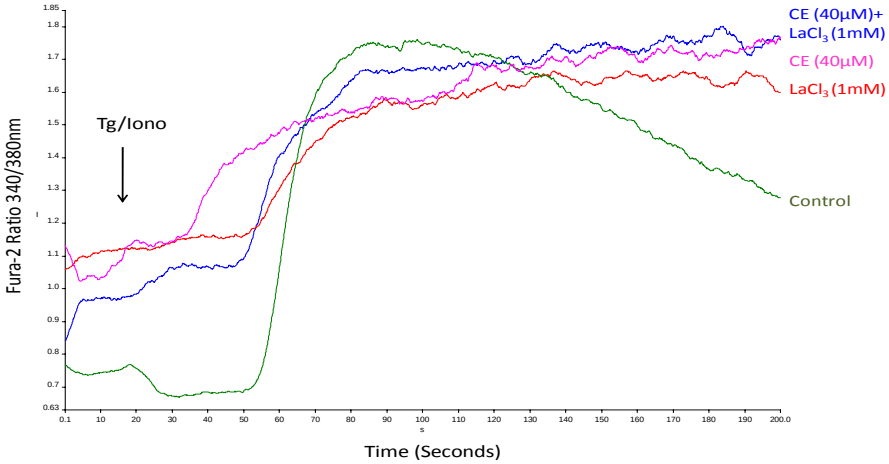
3.2.3. Selectivity of carboxyeosin in platelets

To further investigate the selectivity of CE for PMCA, platelets were incubated with the non-selective cationic channel antagonist Lanthanum Chloride ($LaCl_3$). $LaCl_3$ has been used in several studies to interfere with Ca^{2+} channels in human and animal cells such as platelets and smooth muscle cells (Ng et al. 2009; London et al. 2006; Yu Shi et al. 2009). Washed human platelets were loaded with Fura-2 (5 μM) and later with either CE (40 μM), $LaCl_3$ (1 mM), both compounds, and the relevant DMSO control (0.4%) for 30 minutes. Ca^{2+} responses were recorded in the presence of EGTA (100μM) and platelets stimulated with thapsigargin (1 μM) plus ionomycin (50 nM). Stimulation induced a rapid and transient increase in fluorescence indicating Ca^{2+} release from intracellular stores and an increase in $[Ca^{2+}]_i$ (Fig. 3.3A). Extrusion of Ca^{2+} from the cytosol was seen as a decay of the Ca^{2+} transient towards a stable baseline (Fig. 3.3A DMSO control green line). The addition of CE (40 μM), $LaCl_3$ (1 mM) or both resulted in the inhibition of Ca^{2+} extrusion observed as a sustained elevation of Ca^{2+} at three minutes compared to the DMSO control: (normalised to 100%) CE (40 μM) 180±3.3% ($p<0.001$), $LaCl_3$ (1 mM) 181±3.1% ($p<0.001$) and CE (40 μM)+$LaCl_3$ (1 mM) 193±3.6% ($p<0.001$) (Fig. 3.3B). Similar increases were observed in basal $[Ca^{2+}]_i$ with CE (40 μM) and $LaCl_3$ (1 mM) compared to the DMSO control (100%): CE (40 μM) 155±5.9% ($p<0.001$), $LaCl_3$ (1 mM) 152±5.5% ($p<0.001$) (Fig. 3.3C) confirming a comparable increase in $[Ca^{2+}]_i$ following inhibition of Ca^{2+} extrusion through two entirely

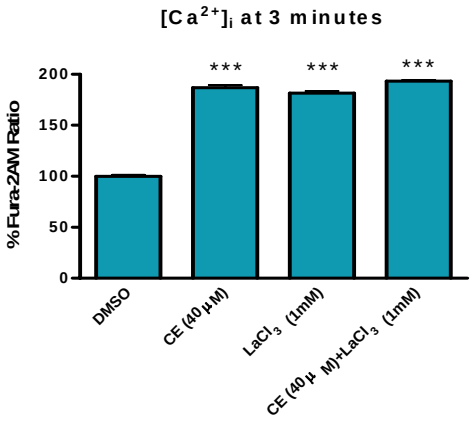
independent mechanisms and in the absence of potentially confounding fluorescence issues. This data also shows that the effect of CE on Ca^{2+} homeostasis did not occur in platelets pre-treated with $LaCl_3$, indicating that the effect of CE on Ca^{2+} homoeostasis was exerted at the plasma membrane rather than at a location independent of PMCA.

Figure 3.3. The effects of Ca^{2+} extrusion blockade on platelet Ca^{2+} homeostasis
Washed human platelets were loaded with Fura-2 and incubated with CE (40 μM), $LaCl_3$ (1 mM) or both compounds. Fura-2 ratio emission was measured at 340/380 nm in response to thapsigargin (1 μM) and ionomycin (50 nM) in the presence of EGTA (100 μM). Graph (A) shows a typical time course of response expressed as 340/380 nm emission ratio against time. Figure (B) shows the % of $[Ca^{2+}]_i$ remaining at 3 minutes post stimulation. Figure (C) shows the % of $[Ca^{2+}]_i$ in resting platelets pre-stimulation. Data represents mean percentage ±S.E.M relative to DMSO (0.4%). Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values, * $P<0.05$ ***$P<0.001$, n=4

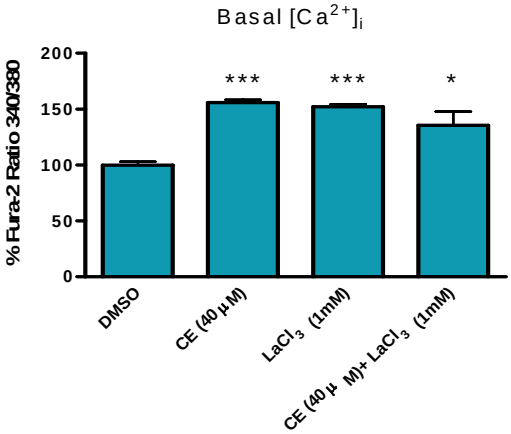
(A)


Fura-2 Ratio 340/380nm
Tg/Iono
CE (40µM)+ LaCl3 (1mM)
CE (40µM)
LaCl3 (1mM)
Control
Time (Seconds)

(B)


[Ca2+]i at 3 minutes
% Fura-2AM Ratio
***
***
***
DMSO
CE (40 µM)
LaCl3 (1mM)
CE (40 µM)+LaCl3 (1mM)

(C)


Basal [Ca2+]i
% Fura-2 Ratio 340/380
***
***
*
DMSO
CE (40 µM)
LaCl3 (1mM)
CE (40 µM)+ LaCl3 (1mM)

3.2.4. PMCA regulates platelet aggregation in isolated human platelets

To investigate whether CE elicits a functional effect on washed platelets, cells were pre-incubated with concentrations of CE previously shown to selectively inhibit Ca^{2+} extrusion through PMCA and aggregation measured upon stimulation by two potent agonists, collagen or thrombin.

Washed human platelets were incubated with CE (10-50 μM) or control DMSO (0.5%) and stimulated with either collagen (5 μg/ml) or thrombin (0.1 U/ml) which induced platelet aggregation shown as a fall in optical density (Fig. 3.4A and B). Maximal aggregation was achieved at around 3 minutes. In the presence of CE (10-50μM) platelets exhibited a concentration dependent inhibition of aggregation following stimulation (Fig. 3.4C and D). The mean percentage decrease in platelet aggregation was calculated for each concentration of CE stimulated with collagen (5 μg/ml) which was significant between 30-50 μM: (10 μM) 80±1.5%, (20 μM) 56±3.2%, (30 μM) 40±4.4% ($p<0.05$), (40 μM) 32±5.1% ($p<0.01$), and (50 μM) 22±5.8% ($p<0.01$) compared to the DMSO control (normalised to 100%) and thrombin (0.1U/ml) which was significant at 40 and 50μM: (10 μM) 94±0.4%, (20 μM) 70±2.3%, (30 μM) 59±3.1%, (40 μM) 37±4.8% ($p<0.01$) and (50 μM) 31±5.3% ($p<0.001$) compared to the DMSO control (normalised to 100%). The effect of CE on isolated human platelets demonstrates the functional consequences of CE mediated inhibition of Ca^{2+} extrusion *via* PMCA.

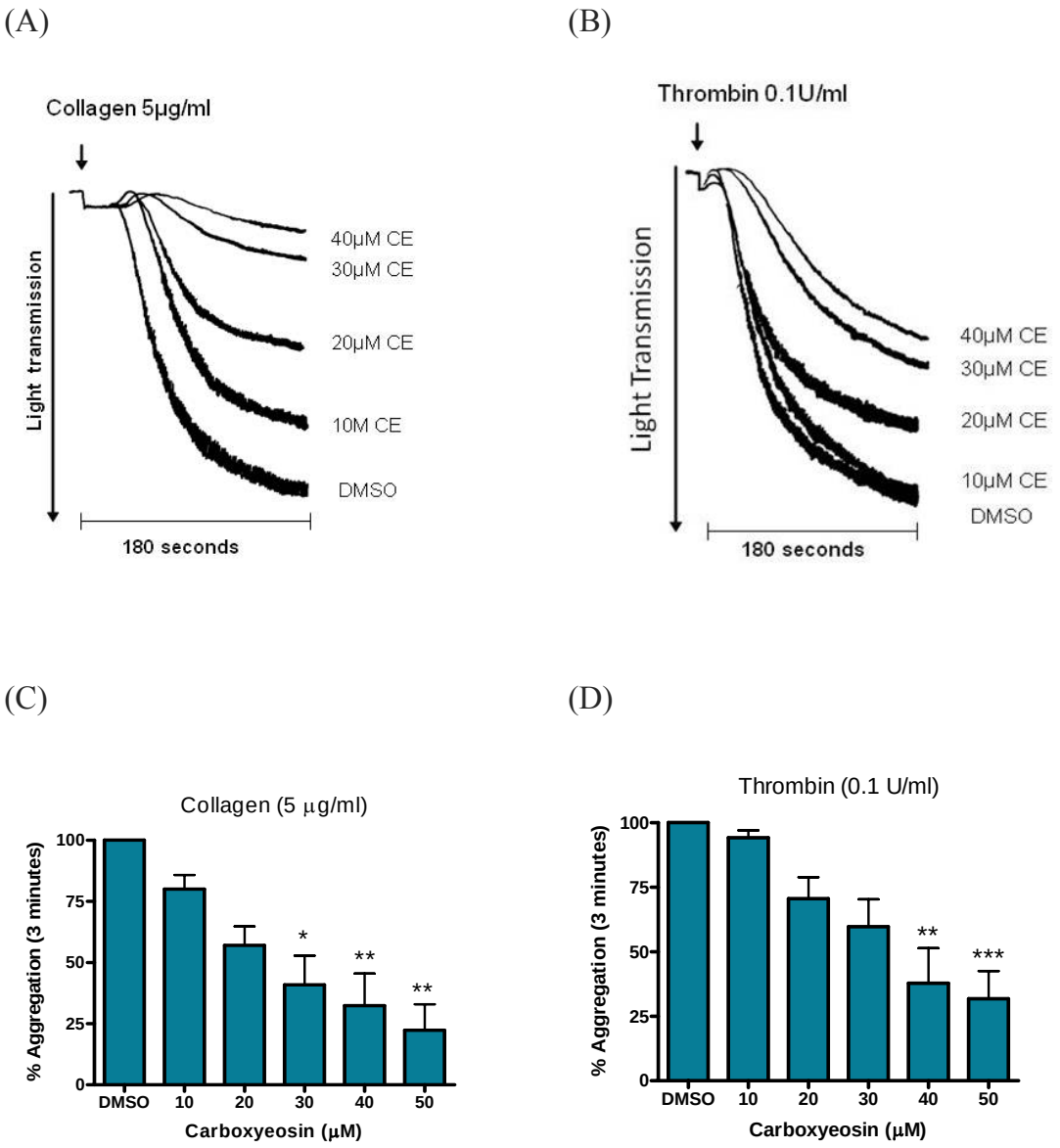


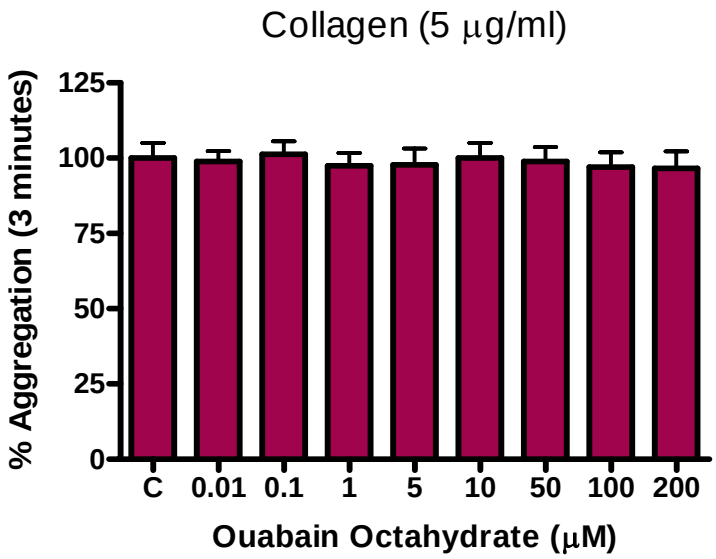
Figure 3.4. The effects of carboxyeosin on collagen and thrombin mediated platelet aggregation

Washed human platelets were pre-incubated with CE (10-50 µM) or DMSO (0.5%) prior to stimulation. Optical aggregometry was used to measure aggregation in response to thrombin (0.1 U/ml) or collagen (5 µg/ml) for 180 seconds with constant stirring (~1200 rpm). Figure (A) and (B) show typical aggregation traces representing inhibition in the presence of CE, (C) and (D) represent mean percentage aggregation ±S.E.M relative to DMSO. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values *P<0.05, **P<0.01, ***P<0.001, n=4.

3.2.5. Validation of carboxyeosin as a selective PMCA inhibitor

Previous reports have suggested that CE has an inhibitory effect at targets other than PMCA, notably the Na^+/K^+-ATPase (Ogan et al. 2007) and also inhibits transients during Ca^{2+} repletion (Zimlichman et al. 1987). Therefore, to investigate whether the inhibition of the Na^+/K^+ ATPase functionally affects platelets, a known inhibitor of this pump (ouabain) was investigated and the effects upon platelet aggregation measured. Washed platelets were pre-incubated with concentrations of ouabain previously shown to inhibit Na^+/K^+-ATPases (1-10 μM) (Ogan et al. 2007) and excess concentrations (200 μM). Platelets were then stimulated with either collagen (5 μg/ml) or thrombin (0.1 U/ml) and aggregation recorded. In the presence of ouabain (0.01-200 μM) the mean percentage of platelet aggregation displayed a non significant effect on both collagen and thrombin mediated aggregation (Fig. 3.5A and B). Therefore, although CE may exert effects *via* Na^+/K^+ ATPases, such actions are unlikely to explain the effects of CE on platelet function reported here, since inhibition of the Na^+/K^+ ATPase has no effect on collagen or thrombin induced platelet aggregation.

(A)



(B)

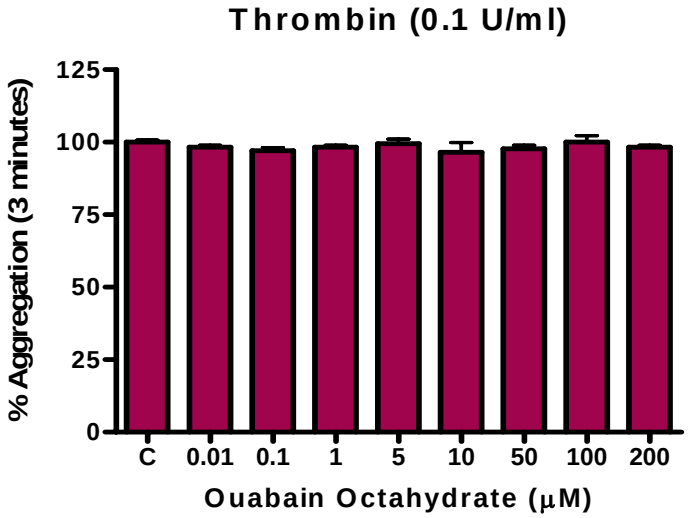


Figure 3.5. The Na^+/K^+ ATPase does not modulate platelet aggregation
Washed human platelets were pre-incubated with ouabain (0.01 – 200 μM) or vehicle control (dH_2O) prior to stimulation. Optical aggregometry was used to measure aggregation in response to (A) thrombin (0.1 U/ml) or (B) collagen 5 μg/ml. Data represents mean percentage aggregation ±S.E.M relative to control responses. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values. No significant changes were observed. P>0.05, n=4.

3.2.6. The effects of carboxyeosin on platelet adhesion

To investigate another functional consequence of PMCA inhibition in platelets, platelet adhesion onto collagen (50µg/ml) and fibrinogen (50µg/ml) coated surfaces was measured spectrophotometrically using the colorimetric platelet phosphatase assay as described by Bellavite and colleagues (Bellavite et al. 1994) (Section 2.2.4.2.) following pre-incubation of platelets with either CE (10-40 µM) or DMSO control (0.4%). Platelet adhesion onto fibrinogen coated surfaces was enhanced in a concentration-dependant manner in the presence of CE and was significant at concentrations of 30 and 40 µM compared to the DMSO control (normalised to 100%): (30 µM) 148±4.4% ($p<0.05$) and (40 µM) 153±1.4% ($p<0.05$) (Fig. 3.6A). However, platelet adhesion to collagen showed a slight reduction (Fig. 3.6B) that was neither concentration dependent nor significant at any concentration tested.

(A) (B)

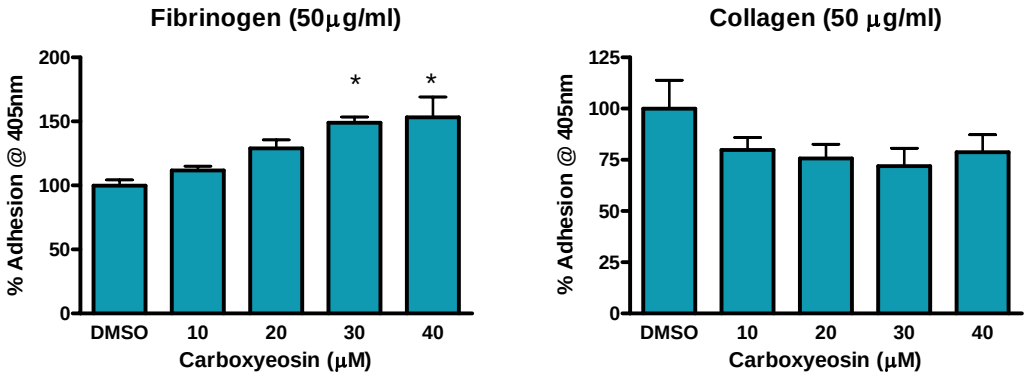


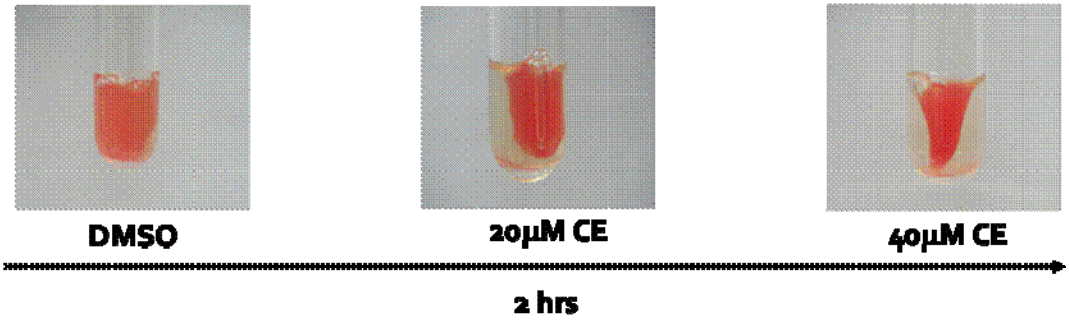
Figure 3.6. Platelet adhesion to fibrinogen and collagen coated surfaces
Platelet adhesion to collagen (50 µg/ml) and fibrinogen (50 µg/ml) coated surfaces was measured using an acid phosphatase static adhesion assay and absorbance's for each well read at 405 nm. Absorbance values for CE treated cells (10-40 µM) or DMSO (0.4%) were normalised to percentage values obtained for non treated cells. Figure (A) Shows the normalised % adhesion of platelets onto fibrinogen coated plates. (B) Shows the normalised % adhesion onto collagen coated plates. Data represents mean ±S.E.M in relative units to control responses. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values, **P<0.05, n=4.

3.2.7. The effects of carboxyeosin on clot retraction

To investigate whether the inhibition of PMCA elicits an effect on the later stages of platelet activation, platelet clot retraction was measured. Human PRP was pre-incubated with CE (10-40 μM) or DMSO control (0.4%) and clot retraction measured as described in section 2.2.4.3. Figure 3.7A displays a representative set of clots which have retracted over 2 hours and shows more retracted clots in the presence of CE (20 and 40 μM) compared to a DMSO control. The % clots weight was calculated based on the weight of serum subtracted from the serum weight of a non-stimulated sample. Data was expressed as the % clot weight of each sample as shown in Fig. 3.7B. Clots formed in the presence of CE (10-40 μM) expressed an enhancement i.e. a smaller clot weight compared to the control (normalised to 100%) which was significant at 30 μM (71±3.1% ($p<0.05$)) and 40 μM (60±4.4% ($p<0.01$)) (Fig.3.7B).

In contrast to platelet aggregation, which is suppressed following PMCA inhibition, clot retraction was enhanced suggesting that PMCA positively regulates platelet aggregation but negatively regulates clot retraction and platelet adhesion.

(A)



(B)

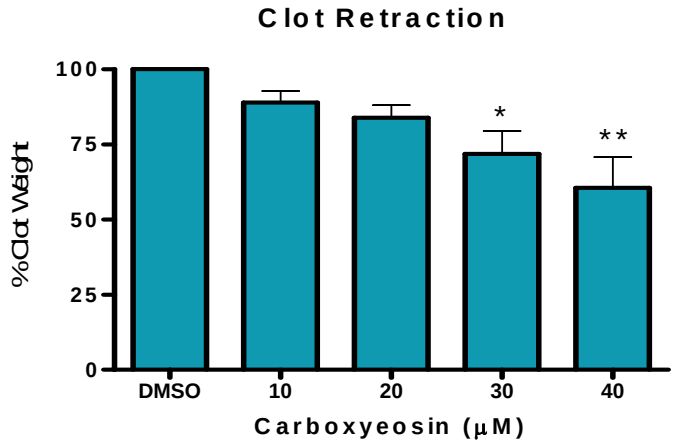


Figure 3.7. Inhibiting PMCA accelerates clot retraction

The effect of PMCA inhibition by CE on clot retraction was measured. Human PRP was incubated with CE (10-40 µM) or DMSO control (0.4%). Figure (A) shows the clots formed and retracted in the presence of DMSO or CE (20 and 40 µM) at 2 hours. (B) shows mean data for the clot weights (normalised to control 100%) ±S.E.M weight in the presence of CE (10-40 µM) or DMSO control (0.4%). Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values, *P<0.05, **P<0.01, n=5.

3.2.8. Carboxyeosin inhibits aggregation of wild-type mouse platelets

The functional observations in human platelets characterised the effects of PMCA *in vitro*. Before PMCA4 knock-out (-/-) mouse studies could be performed, we wanted to establish whether effects recorded in human platelets could be reproduced in wild-type (+/+) mice.

Washed mouse platelets (Section 2.2.1.2) were incubated with CE (10-40 μM) or control DMSO (0.4%) and stimulated with either collagen (5 μg/ml) or thrombin (0.1 U/ml) which induced platelet aggregation shown as a fall in optical density (Fig. 3.8A and B). Maximal aggregation was achieved at around three minutes. In the presence of CE (10-40 μM), platelets exhibited a concentration dependent inhibition of aggregation upon stimulation. The mean percentage decrease in platelet aggregation was calculated for each concentration of CE stimulated firstly with thrombin (0.1 U/ml) (Fig. 3.8C) that was significant between 20 and 40 μM compared to the DMSO control (normalised to 100%): (10 μM) 39±1.3%, (20 μM) 24±1.8% ($p<0.05$), (30 μM) 14±1.8% ($p<0.01$) and (40 μM) 7±1.6% ($p<0.001$) and collagen (5 μg/ml) which was significant at 40 μM only 38±3.8% ($p<0.05$) compared to the DMSO control (Fig. 3.8D). The effect CE has on mouse platelets is similar to that observed in human platelets, enabling progression to functional studies in PMCA4$^{-/-}$ mice.

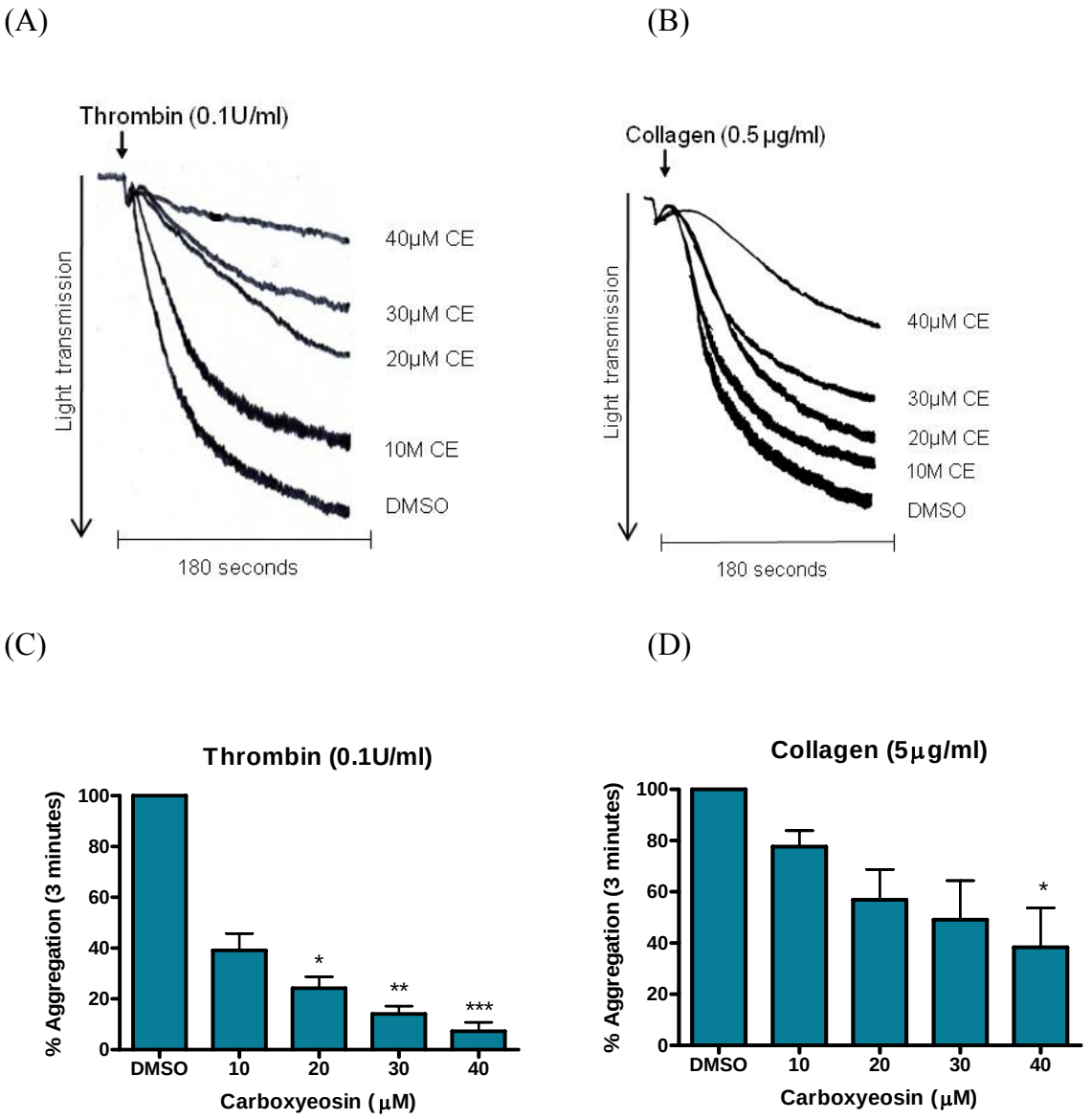


Figure 3.8. The effect of carboxyeosin on collagen and thrombin mediated mouse platelet aggregation

Washed mouse platelets were pre-incubated with CE (10-40 μM) or DMSO (0.4%) prior to stimulation. Optical aggregometry was used to measure aggregation in response to thrombin (0.1 U/ml) or collagen (5 μg/ml) for 180 seconds with constant siring (~1200rpm). Figure (A) and (B) show typical aggregation traces representing inhibition, (C) and (D) represent the mean percentage aggregation ±S.E.M relative to DMSO. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values, *P<0.05, **P<0.01, ***P<0.001, n=4.

3.2.9. Identification of PMCA in Mice

Before identifying isomer specific roles of PMCA in mouse platelets, the expression of PMCA (isoform 4) platelets from (+/+) mice was investigated. Tissue samples were collected and snap-frozen into liquid nitrogen to prevent protein degradation. The aorta, kidney, heart, lung and platelets were homogenised/lysed (Section 2.2.3.2.) and protein quantified (Section 2.2.3.1.). PMCA4 expression was finally identified using western blot methods (Section 2.2.3.2). Although PMCA4 is ubiquitously expressed in all major cell types (Carafoli 1991), experiments showed little expression in most tissue samples according to the kD band location (Fig. 3.9). A protein band was however identified for PMCA4 in the platelet samples. This was located at 145kD (indicated by the arrow).

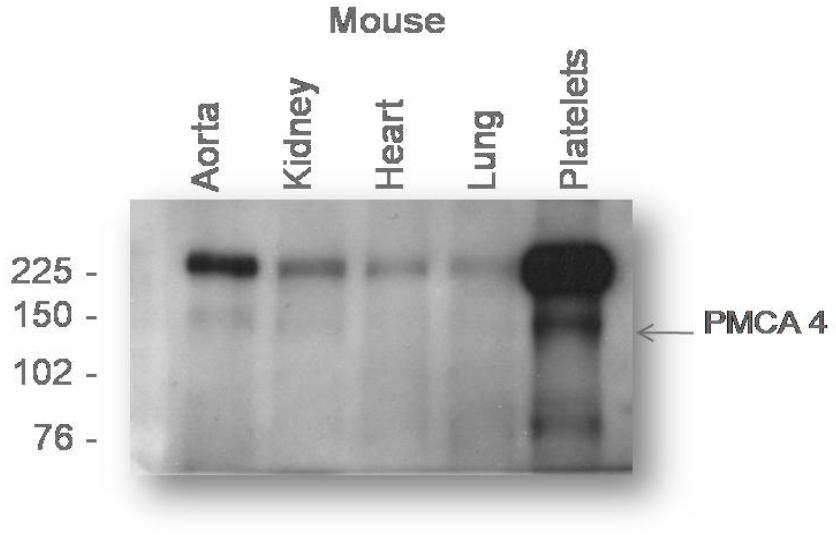


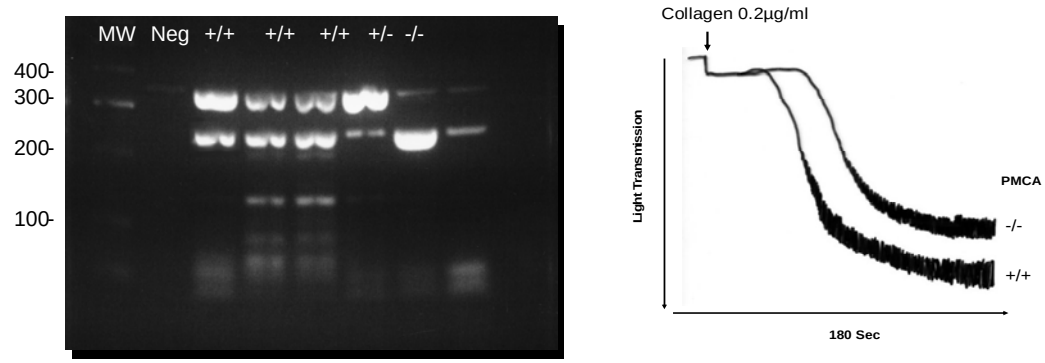
Figure 3.9. PMCA4 expression in the wild-type mouse tissue samples
Protein lysates were separated and blotted using a PMCA 4 antibody (SWANT, Bellinzona, Switzerland) (1:1000), Immuno-blots were developed using an ECL detection system. (PMCA4 is ≈145 kD, as indicated by the arrow).

3.2.9.1. Ablation of mouse platelet PMCA4 inhibits agonist mediated aggregation and adhesion

It has been well documented that there are two PMCA isoforms present on the surface of human platelets PMCA1 and 4 (Martin et al. 2000). The availability of PMCA4 (-/-) mice allowed the determination of isoform specific roles of PMCA4 in regulating platelet function. Tail tips from heterozygous (+/-), (+/+) and (-/-) mice were digested and DNA amplified using PCR to identify zygosity (Section 2.2.2.1.). Bands corresponding to the PMCA insert showed at 344bp and neomycin at 245bp. Mice expressing heterozygous zygosity had bands appearing at 344bp, wild-type at both 344 and 245bp and knock-out expressing only 245bp (Fig. 3.9.1A). Once mice were genotyped, washed mouse platelets from (-/-) and (+/+) mice were stimulated with collagen (0.2 μg/ml) and aggregation recorded using optical aggregometry over a three minute period to establish maximal aggregation. Figure 3.9.1B shows a representative trace of platelets from (+/+) and (-/-) mouse aggregations and mean data calculated over four separate experiments is shown in Figure 3.9.1C. (-/-) mouse platelets exhibited a significant decrease in platelet response when stimulated with collagen (0.2 μg/ml) compared to the (+/+) control (normalised to 100%): (+/+) 100±1.5%, (-/-) 82±7.2% ($p<0.05$). In addition, platelet adhesion to collagen (50 μg/ml) was performed in platelets from (+/+) and (-/-) mice as previously described (Section 2.2.4.2.). Absorbance values for (+/+) mouse platelets were normalised to 100% and absorbance values for (-/-) platelets defined as a percentage of this. Platelet adhesion of (-/-) mice exhibited a small but significant decrease in adhesion

compared to (+/+) controls: (-/-) 72±2.6% ($p<0.05$) (Fig. 3.9.1D) indicating reduced adhesive response to collagen when PMCA4 is ablated.

(A) (B)



(C) (D)

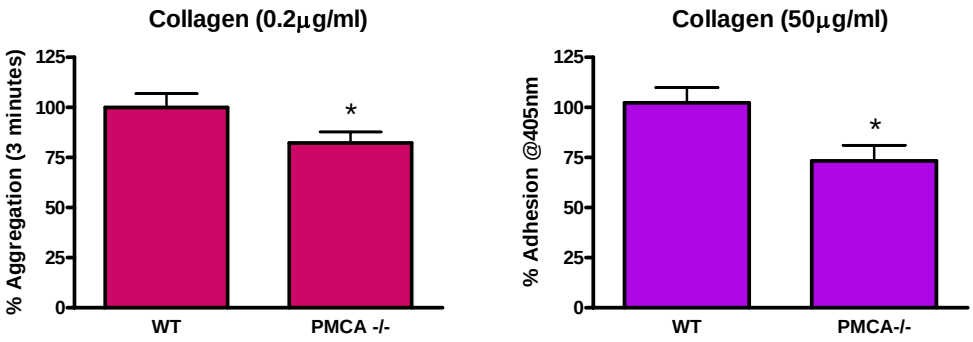


Figure 3.9.1. PMCA4 expression and inhibition of platelet function

(A) PCR blot from tail tips genotyped from (+/-), (+/+) and PMCA4 (-/-) mice. (B) Shows a typical aggregation trace from platelets isolated from PMCA4 (-/-) mice and (+/+) controls stimulated with 0.2µg/ml collagen. (C) Graph represents mean percentage aggregation ±S.E.M relative to (+/+) control. Figure (D) Represents the mean percentage ±S.E.M adhesion to collagen (50 µg/ml) compared to (+/+) control. Aggregations n=4. Adhesion n=8. Where statistical comparisons were made a T-test was performed,* $P<0.05$.

3.2.9.2. PMCA inhibition does not desensitise platelets

Raised basal Ca^{2+} levels and impaired Ca^{2+} extrusion accompanied a decrease in platelet aggregation observed with CE (Fig.3.1 and 3.4). Therefore, to assess the mechanisms underlying these effects and to determine if these effects were as a result of platelet pre-activation, platelet dense granule secretion and light transmission were measured in resting platelet suspensions incubated with CE (10-40 μM). In addition, and for comparison, granule secretion and light transmission were also determined in resting platelets incubated with the SERCA inhibitor thapsigargin (0.025-5 μM) which also leads to increased $[Ca^{2+}]_i$.

Human platelets were loaded with [^{3}H] 5-HT and dense granule secretion measured (Section 2.2.4.5.). Loaded platelets were pre-incubated with concentrations of thapsigargin (0.05-5 μM), CE (10-40 μM) or control DMSO (0.05 or 0.4%). Platelets pre-treated with thapsigargin (0.05-5 μM) displayed an increase in granule secretion that was significant at 5 μM: 32±4.5% ($p<0.01$) compared to the DMSO control (DMSO 3±1.3%) (Fig. 3.9.2A). However, platelets pre-treated with CE (10-40μM) showed no increase in dense granule secretion at any concentration compared to the DMSO control (2.9±1.1%) (Fig. 3.9.2B).

To further investigate platelet pre-activation, light transmission was measured in platelet suspensions in the absence of platelet agonists. Washed human platelets were incubated with either thapsigargin (0.025-1 μM), CE (10-40 μM), or DMSO (0.0025 or 0.4%) and light transmission measured by optical aggregometry.

Maximal aggregation was calculated as the largest fall in optical density from a baseline. A fall in optical density indicated pre-activation of platelets. Platelets were then stimulated with thrombin (0.1 U/ml) to assess desensitisation.

Thapsigargin pre-treatment of platelets caused a concentration dependent increase in light transmission that was significant between 0.25-1μM: (0.25 μM) 20±4.2% ($p<0.5$), (0.5 μM) 41±2.1% ($p<0.01$) and (1 μM) 43±2.9% ($p<0.01$) compared to the DMSO control (normalised to 100%) (Fig. 3.9.2C). In contrast platelets pre-treated with CE (10-30 μM) exhibited no increase in light transmission with the exception of 40μM where there was a small but significant increase compared to the DMSO control (normalised to %) (40 μM) 7.9±1.5% ($p<0.05$) Fig. 3.9.2D. These results suggest that like dense granule secretion, there appeared to be no indication of pre-activation of platelets pre-incubated with CE. When platelets were subsequently stimulated with thrombin, there was a concentration dependent reduction in aggregation in the presence of CE that was significant at 30 and 40 μM compared to the DMSO control (30 μM) 75± 0.02 ($p<0.05$), (40 μM) 58± 1.2 ($p<0.01$). Thapsigargin pre-treatment however, had no effect on subsequent thrombin induced aggregation.

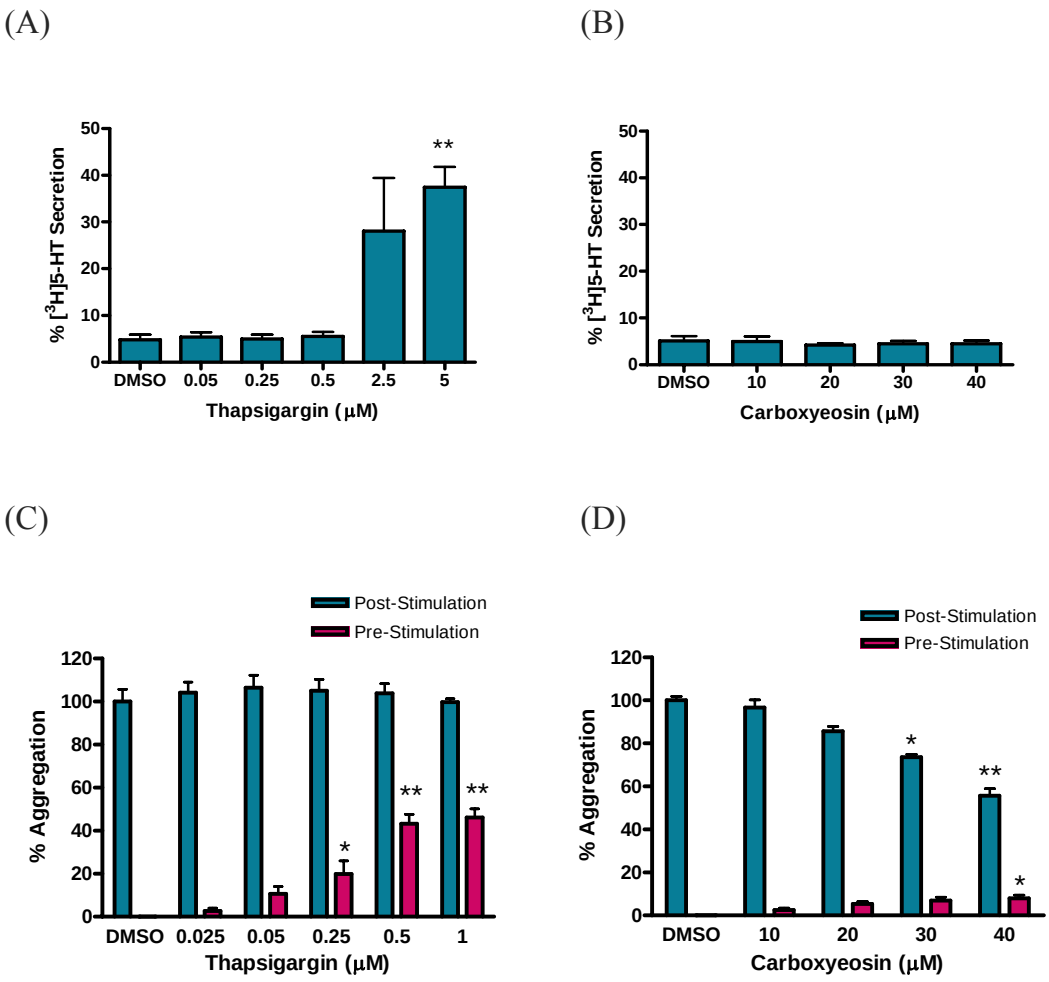


Figure 3.9.2. Increased $[Ca^{2+}]_i$ following PMCA inhibition does not desensitise platelets

Washed platelets were pre-incubated with either (A) thapsigargin (0.05-5μM) or (B) CE (10-40μM) or DMSO control (0.4%) and [^{3}H] 5-HT release measured from un-stimulated platelets. Light transmission of un-stimulated platelet suspensions in the presence of (C) thapsigargin (0.025-1μM) or (D) CE (10-40μM) or DMSO (0.4%) was measured using optical aggregometry and compared to stimulated (thrombin 0.1 U/ml) preparations. Data represents mean percentage ±S.E.M in relative units or percentage aggregation. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values, *P<0.05, **P<0.01, n=4.

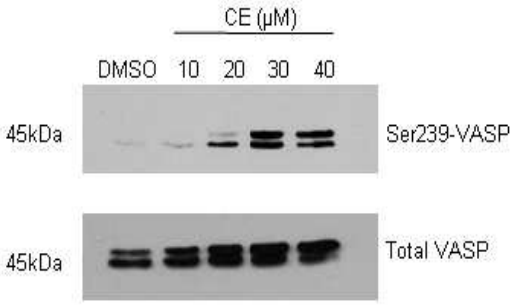
3.2.9.3. Carboxyeosin enhances VASP phosphorylation independently of cGMP

To address other possible mechanisms behind the inhibitory effects observed upon platelet aggregation with CE (Fig. 3.4), possible inhibitory pathways associated with PMCA were investigated. It has been documented that PMCA can regulate inhibitory signalling within cardiomyocytes (Oceandy, Cartwright et al. 2007) and vascular smooth muscle where PMCA associates with nNOS (Schuh et al. 2003). Therefore, it was hypothesised that CE could exert these inhibitory effects in platelets by raising Ca^{2+} locally to allow PMCA to activate inhibitory signalling molecules such as VASP. Aggregations were performed with washed platelets pre-incubated with CE (10-40 μM) and stimulated with collagen (5 μg/ml), platelets were lysed and western blotting performed (Section 2.2.3.2.) to identify Ser^{239}-VASP phosphorylation and demonstrated a concentration dependent increase in Ser^{239}-VASP phosphorylation in platelets incubated with CE that was significant at 30 and 40μM compared to the DMSO control: (DMSO) 0.17±0.2, (30 μM) 0.75±1.2 ($p<0.001$) and (40 μM) 0.89±1.4 ($p<0.001$) (Fig. 3.9.3A). Data was normalised to total VASP levels and arbitrary units were analysed using the ImageJ densitometry software (NIH, USA) (Fig. 3.9.3B) indicating that PMCA may act to prevent VASP phosphorylation.

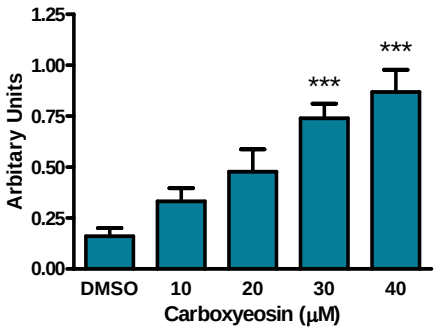
To further investigate these findings, platelet aggregation in the presence of the guanylate cyclise (SGC) inhibitor (ODQ) was investigated to determine the role of SGC in mediating the inhibitory effects of CE. Washed platelets were pre-incubated

with NO donor sodium nitroprusside (SNP) (1 μM), and stimulated with collagen (5 μg/ml) which almost completely inhibited platelet aggregation compared to control (normalised to 100%): (1 μM) 3±0.9% ($p<0.001$) (Fig. 3.9.3C). This inhibition was significantly reversed by ODQ (10 μM): 97.99±1.4% ($p<0.001$) indicating effective inhibition by ODQ. CE (40 μM) induced inhibition of aggregation that was significant compared to the control: (40 μM) 76±1.2% ($p<0.01$). However, the inhibitory effect of CE was not impaired by ODQ (10 μM) 1±0.9% ($p>0.05$) suggesting that the inhibition caused by CE, although mediated through VASP, was independent of cGMP.

(A)



(B)



(C)

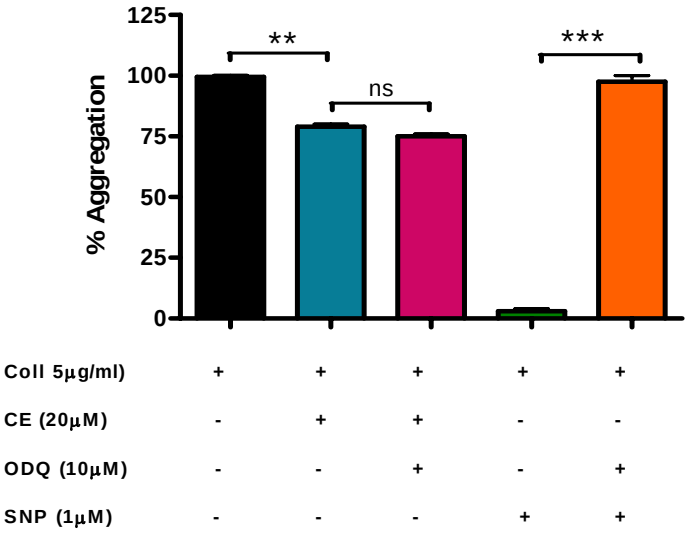


Figure 3.9.3. PMCA enhances VASP phosphorylation independently of cGMP
Platelet lysates from collagen (5 μg/ml) stimulated platelets pre-incubated with CE (10-40 μM) were immunoblotted for phospho-VASP (Ser^{239}) or total VASP (A) and phospho-VASP levels normalised and densitometry performed to calculate arbitrary units (B). Optical aggregometry was used to measure aggregation in response to collagen (5 μg/ml). Figure (C) shows the mean percentage aggregation of platelets pre-incubated with carboxyeosin (CE, 20 μM) or (SNP, 1 μM) in the presence and absence of ODQ (10 μM) relative to a collagen control. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values, **P<0.01 ***P<0.001, n=4.

3.2.9.4. The effects of carboxyeosin on platelet aggregation *in vivo*

CE has been shown to inhibit platelet aggregation *in vivo* when applied to platelets prior to mouse infusion *via* the femoral vein (S. Jones, personal communication). Therefore, to determine whether systemic infusion of CE causes an anti-platelet effect *in vivo*, CE (30 μM) or control DMSO (0.3%) was infused systemically into anaesthetised mice prior to induction of *in vivo* platelet aggregation with collagen (50 μg/kg). Washed platelets were isolated *via* cardiac puncture from anaesthetised C57/SV-129 donor mice and radio-labelled as described in sections 2.2.1.2. and 2.2.5.1.

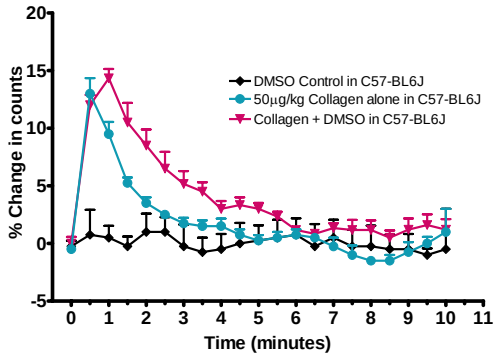
An initial dose of collagen (50 μg/kg) was administered *via* the femoral vein to ensure platelet responsiveness. An increase in counts was observed after administration followed by a return to baseline indicating platelet aggregation and dissolution (Fig. 3.9.4A blue line and B orange line). CE (30 μM) or DMSO (0.3%) was then administered and left to equilibrate for a further 10 minutes (Fig. 3.9.4A black line and B green line). Finally, collagen (5 μg/kg) was administered to determine whether the prior-administration of either CE (30 μM) or DMSO (0.3%) had an effect on platelet aggregation (Fig. 3.9.4A pink line and B grey line).

Mean data from four separate experiments indicate that DMSO (0.3%) had a non significant effect on basal counts compared to CE alone: (CE) 1.45±0.2%, (DMSO) 0.35±0.01% ($p>0.05$) (Fig. 3.9.4C) Platelet aggregation measured in the presence of CE (30 μM) exhibited a slight decrease (% maximum response) however, this was

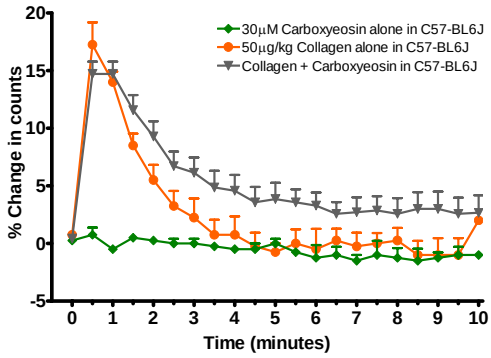
not significant compared to the collagen control: (collagen) 18±3.4%, (CE+collagen) 16±3.1% ($p>0.05$) (Fig. 3.9C). There appeared to be a slight delay in platelet counts returning to base-line in the presence of CE (Fig. 3.9.4B grey line), however mean data representing AUC demonstrated that this was also not significant compared to collagen: (collagen) 39±4.2, (CE+collagen) 52±5.6 ($p>0.05$) (Fig. 3.9.4D). Whilst these experiments were conducted to determine whether CE (30 μM) had any anti-thrombotic effect when infused systemically into an *in vivo* model of platelet aggregation, results indicate that the presence of CE (30 μM) had no effect on the platelet aggregate formation but may indicate a trend towards a delay in aggregate dissolution.

Figure 3.9.4. Measurement of *in vivo* platelet aggregation responses in the mouse
Washed mouse platelets were isolated form donor mice and radiolabelled with ^{111}Indium Oxine. Recipient mice were injected with either collagen (5 μg/kg), DMSO (0.3%) or CE (30 μM) and platelet counts recorded. Traces (A) and (B) represent the mean percentage change in radioactive counts against time. Graphs (C) represents the mean ±SEM maximal percentage increase in radioactive counts and (D) mean ± SEM area under curve (AUC) in counts. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values. No significant observations were found, ($P>0.05$), n=4.

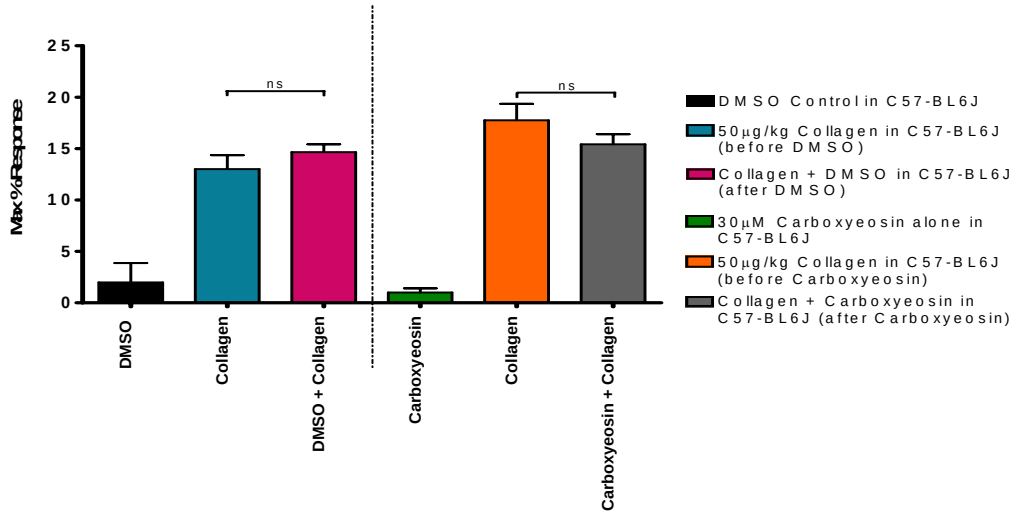
(A)


% Change in counts
Time (minutes)
DMSO Control in C57-BL6J
50µg/kg Collagen alone in C57-BL6J
Collagen + DMSO in C57-BL6J

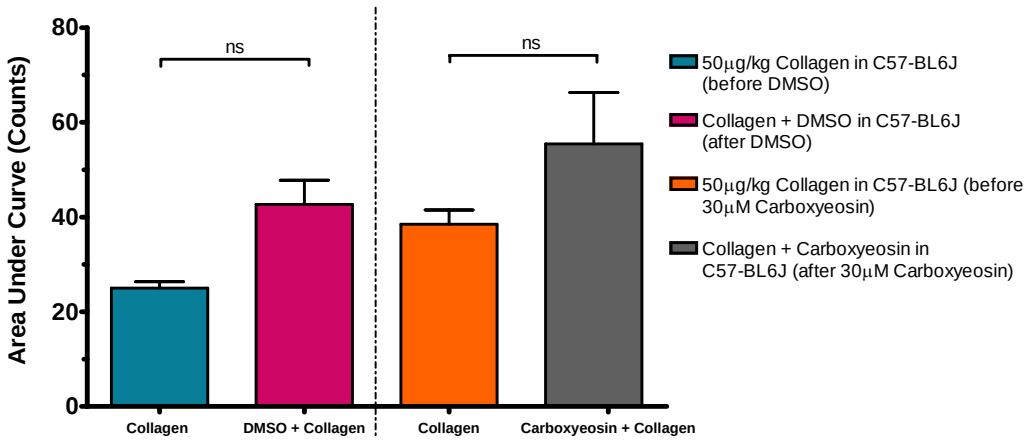
(B)


% Change in counts
Time (minutes)
30µM Carboxyeosin alone in C57-BL6J
50µg/kg Collagen alone in C57-BL6J
Collagen + Carboxyeosin in C57-BL6J

(C)


Max %Response
ns
ns
DMSO
Collagen
DMSO + Collagen
Carboxyeosin
Collagen
Carboxyeosin + Collagen
DMSO Control in C57-BL6J
50µg/kg Collagen in C57-BL6J (before DMSO)
Collagen + DMSO in C57-BL6J (after DMSO)
30µM Carboxyeosin alone in C57-BL6J
50µg/kg Collagen in C57-BL6J (before Carboxyeosin)
Collagen + Carboxyeosin in C57-BL6J (after Carboxyeosin)

(D)


Area Under Curve (Counts)
ns
ns
Collagen
DMSO + Collagen
Collagen
Carboxyeosin + Collagen
50µg/kg Collagen in C57-BL6J (before DMSO)
Collagen + DMSO in C57-BL6J (after DMSO)
50µg/kg Collagen in C57-BL6J (before 30µM Carboxyeosin)
Collagen + Carboxyeosin in C57-BL6J (after 30µM Carboxyeosin)

3.2.9.5. Carboxyeosin selectively inhibits PMCA4 in mice

To determine the selectivity of CE on PMCA4, aggregations were performed in platelets isolated from PMCA4 (+/+) mice and PMCA4 (-/-) mice in the presence of CE. Washed mouse platelets were incubated with CE (40 μM) and stimulated with either collagen (5 μg/ml) or thrombin (0.1 U/ml) (Fig. 3.9.5.A and B) which induced maximal aggregation at around three minutes. Platelets isolated from (-/-) mice aggregated normally upon stimulation despite the presence of CE in comparison to platelets from (+/+) mice, where the response was inhibited, demonstrating that CE selectively exerts its effects *via* PMCA4.

(A) (B)

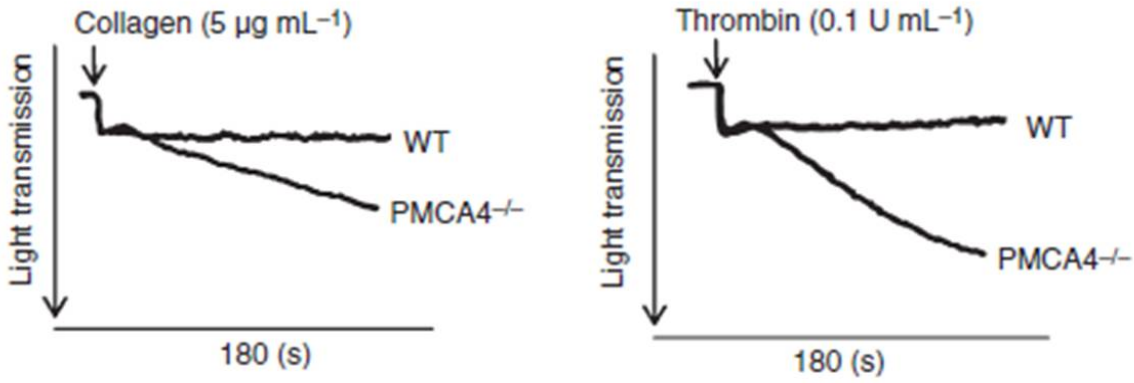


Figure 3.9.5. Carboxyeosin exerts its effects selectively at PMCA4
Washed mouse platelets from PMCA4 +/+ and -/- mice were pre-incubated with CE (40 μM) prior to stimulation. Optical aggregometry was used to measure aggregation in response to thrombin (0.1U/ml) or collagen (5μg/ml) for 180 seconds with constant stirring (~1200rpm). Figure (A) and (B) show typical aggregation traces representing inhibition of aggregation in wild-type but not PMCA4$^{-/-}$ platelets. n=2.

3. 3. Discussion

The aims of the research carried out in this chapter were to determine whether PMCA plays a role in regulating Ca^{2+} homeostasis and function in human and in mouse platelets. Further work was conducted using PMCA4 (-/-) mice to examine the specific roles of PMCA4 and the possible molecular signalling pathways associated with PMCA were also considered. The aims were addressed using a pharmacological inhibitor of PMCA, CE, to inhibit the activity of PMCA at the platelet membrane, using a variety of functional, molecular and *in vivo* techniques.

Ca^{2+} regulation

To identify whether PMCA could influence Ca^{2+} homeostasis, Ca^{2+} measurements were investigated in resting platelets and upon stimulation. Our initial hypothesis was that any inhibition of PMCA would impair Ca^{2+} extrusion and elevate $[Ca^{2+}]_i$ levels, leading to platelet activation by sub-threshold concentrations of platelet agonists.

Preliminary experiments demonstrated an elevation of basal Ca^{2+} levels in the presence of increasing concentrations of CE. Following this elevation, subsequent stimulation with thapsigargin produced a rise in $[Ca^{2+}]_i$ (also demonstrated by Rosado and colleagues (Rosado & Sage 2000)) followed by a concentration dependent inhibition of Ca^{2+} extrusion in the presence of CE. The extrusion of Ca^{2+} under the experimental conditions employed in this thesis has been shown to occur

exclusively through PMCA, with a negligible contribution by other Ca^{2+} removal mechanisms (Rosado & Sage 2000). In addition, an inhibition of Ca^{2+} extrusion was also reported by S. Jones, who observed similar effects when platelets were stimulated with collagen and thrombin (personal communication, data not shown). These initial experiments determined concentrations of CE that could inhibit PMCA and indicates the role of PMCA in the maintenance of low resting Ca^{2+} in platelets as described by Hammes and colleagues in other cell types (Hammes et al. 1994). The data also suggests that PMCA acts to propagate the rise in $[Ca^{2+}]_i$ following stimulation as CE is able to inhibit agonist induced intracellular Ca^{2+} release shown as a reduced peak response. The impaired Ca^{2+} extrusion through PMCA following stimulation with thapsigargin may have been due to differences in Ca^{2+} store release. However, there appeared to be no change in Ca^{2+} release (measured as peak minus basal) indicating normal store loading in the presence of CE and an equal amount of Ca^{2+} release into the cytosol upon stimulation. In conclusion, PMCA is a key modulator of Ca^{2+} homeostasis at rest and during platelet activation. In addition, CE at 10-40 μM, inhibits Ca^{2+} extrusion through PMCA.

Validation of carboxyeosin

CE has been used to determine function in a variety of different cell types for example, the application of CE in mouse embryonic stem cells induces a large increase in $[Ca^{2+}]_i$ (Yanagida et al. 2004). Furthermore, in rat ventricular myocytes, CE had been used to demonstrate the role of PMCA in controlling resting intracellular Ca^{2+} (Choi & Eisner 1999). The question as to whether the elevation of basal Ca^{2+} levels was due to PMCA inhibition by CE and not a consequence of Fura-2 fluorescence interference in prepared platelet suspensions, was addressed by performing experiments to see if eosin (the cleaved form of carboxyeosin) or CE itself could concentration dependently increase Fura-2 emission independently of Ca^{2+}. These results clarified that there was no significant difference between fluorescence readings using a wide range of concentrations of CE or eosin supporting the initial Ca^{2+} experiments where CE elevated Ca^{2+} levels in resting platelets, concluding that PMCA regulates basal $[Ca^{2+}]_i$. Further validation of CE as a selective inhibitor of PMCA was confirmed with the use of $LaCl_3$, where similar increases in basal Ca^{2+} levels also occurred following inhibition of Ca^{2+} extrusion at the plasma membrane confirming that CE exerted its inhibitory effects at the plasma membrane. These experiments do not exclude the possibility that CE could exert its effects at other sites located on the plasma membrane for example at the NCX or NKA, and so, further validation studies were conducted. We found no evidence of a role for the Na^+/K^+ ATPase in modulating platelet aggregation. Therefore, although CE exerts inhibitory effects at Na^+/K^+ ATPases (Ogan et al. 2007), the effects of CE

on platelet aggregation is unlikely to be mediated through inhibition of Na^+/K^+ ATPase since inhibition of this pump at excessive concentrations of ouabain had no effect on platelet aggregation.

Platelet function

Platelet function was assessed using a range of standard platelet functional assays with collagen or thrombin as the two potent agonists chosen. In human washed platelet aggregation assays, CE was found to inhibit platelet aggregation in a concentration dependant manner indicating a positive regulatory role of PMCA during platelet activation. This was surprising, as one would expect platelets to activate as a result of elevated $[Ca^{2+}]_i$. One possible mechanism, by which PMCA inhibition could impair aggregation, could include partial activation and subsequent desensitisation of platelets. This would be in line with elevations of $[Ca^{2+}]_i$ and will be discussed later. The formation of a platelet plug has been described as a spatiotemporal process (Brini 2009), from initial adhesion to clot retraction. Contrary to the inhibitory effect of CE on platelet aggregation, platelet adhesion to fibrinogen was enhanced. Adhesion, which is one of the initial steps in clot formation, occurs when receptor integrins (notably αIIbβIII) express themselves in response to elevated $[Ca^{2+}]_i$. These receptors have a high affinity towards the fibrinogen receptor located on the sub-endothelial matrix of exposed/damaged tissue. The elevated basal Ca^{2+} levels seen in initial experiments suggest that in the early stages of platelet activation PMCA acts to suppress both Ca^{2+} and activation.

This appears to be consistent with the Ca^{2+} dependence of the fibrinogen receptor αIIbβIII. Exposed collagen, which is also present during the early stages of adhesion, appeared to show a decreased trend in platelet adhesion compared with fibrinogen. This could suggest that PMCA negatively regulates adhesion to fibrinogen however, in the case of collagen, which activates the platelet through additional and different pathways, the negative effect on adhesion is cancelled out by a positive effect on collagen induced aggregation i.e. there is an aggregation component to the adhesion assay when collagen is used but with fibrinogen only adhesion of platelets occurs resulting in different functional effects.

Platelets provide an excellent model with which to address the issue of matrix retraction, since platelets develop contractile activity, fibrin clots and retraction is easy to assess. It is also a good functional assay to observe integrin αIIbβ3 activity. The first evidence for a role of αIIbβ3 in retraction has been deduced from studies of a human hereditary hemorrhagic condition termed Glanzmann thrombasthenia, which is characterised by a quantitative or a qualitative defect in αIIbβ3 (Nurden 2005).

Following platelet activation through inside-out signalling, the fibrinogen receptor (integrin αIIbβ3) undergoes a conformational change in which it facilitates fibrinogen binding. Downstream of this, PKCβ activation assists interactions with cytoskeletal actin (Buensuceso et al. 2005) leading to the generation of contractile forces from the platelet actin cytoskeleton. These forces are exerted on the fibrin matrix which induces contractions of the fibrin fibres and creates a ‘pulling in’ effect

in which the clot shrinks and becomes more compact and stable (Osdoit & Rosa 2001). Therefore, the analysis of clot retraction is a commonly used measure of outside-in signalling through integrin αIIbβ3 and any alterations involved in this pathway may be investigated.

Upon investigation, an enhanced clot retraction was observed when PMCA was inhibited using CE. This could be as a result of prolonged elevations of $[Ca^{2+}]_i$ driving Ca^{2+} dependent myosin activity which is essential for the clot retraction process. These findings are consistent with results published by Dean and colleagues who observed that PMCA association with the cytoskeleton during platelet activation results in translocation of PMCA to filopodia and affects later stages of platelet activation measured as clot retraction (Dean & Whiteheart 2004). This suggests that PMCA acts to slow the rate of clot retraction towards the later stages of platelet activation, presumably to ensure effective healing of underlying damaged tissue so that PMCA may possess an important role in blood vessel integrity and disease for example in atherosclerosis. In conclusion, PMCA differentially regulates the various stages of platelet activation. It positively drives platelet aggregation since inhibition with CE leads to reduced aggregation but negatively regulates the earlier and later stages of activation measured as enhanced adhesion to fibrinogen and clot retraction respectively.

PMCA4 studies

To determine whether the inhibitory effects observed previously in human platelets were specific to PMCA4, studies were carried out firstly in mouse platelets to investigate whether the effects observed in human platelets could be reproduced in mice and subsequently determine the isomer specific roles of PMCA4 using PMCA4 (-/-) mice. Experiments were initially conducted in platelets of wild-type (+/+) mice to determine whether the inhibition of PMCA with CE affected mouse platelet aggregation. In (+/+) mice, platelets were incubated with varying concentrations of CE and upon stimulation with either collagen or thrombin exhibited the same inhibitory effects as in isolated human platelets suggesting that PMCA regulates platelet function in both human and mouse platelets.

As male mice are infertile (Schuh et al. 2004), knock-out mice were bred through a heterozygous breeding programme. Once litters were old enough, mice were genotyped to identify the knock-out populations, and once identified, platelets were isolated from (-/-) mice and used to perform functional studies. In addition, PMCA expression was also investigated in mouse platelets and other tissues using western blotting methods. Platelets isolated from (-/-) mice demonstrated an impaired aggregatory response when stimulated with collagen in comparison to wild-type (+/+) mice and also an impairment to platelet adhesion onto fibrinogen was apparent. The effects of PMCA4 ablation on platelet aggregation was mild compared to that observed with CE. This may reflect compensatory changes in PMCA4 $^{-/-}$ mice. Alternatively, their may be a role of PMCA1 that is apparent

following application of CE which does not have isoform specificity. In addition, it is possible that CE exerts non-specific effects at other ATPases that have yet to be identified in PMCA4$^{-/-}$ mice.

Further aggregation studies were carried out in platelets isolated from (-/-) and (+/+) mice incubated with CE and stimulated with thrombin. Preliminary data showed that CE inhibited aggregation in (+/+) mice but not PMCA4 (-/-) mice suggesting that although CE inhibits all isoforms of PMCA, its effects in platelets are at least partly mediated *via* inhibition of PMCA4 and that the PMCA4 isoform is a key modulator of platelet function. The possibility that other isoforms of PMCA (particularly PMCA1), may have important functional roles in platelets cannot be ruled out.

Whilst mouse platelets possess both PMCA 1 and 4, genetically modified ablation of a particular isoform (in this case PMCA4) could present questions as to whether the effects of CE in platelet function are mediated exclusively through this isoform. One way to distinguish between isomer specific roles would be to perform functional and *in vivo* studies using isomer selective antagonists or knock-out mice. In the case of PMCA1, which is crucial for embryonic development (Tempel & Shilling 2007), and is also ubiquitously expressed, the use of platelets from a PMCA1 (-/-) mouse would allow comparison of the role of PMCA1 with PMCA4 by contrasting data from PMCA4 (-/-) mice. Unfortunately, PMCA1 (-/-) strains are embryonically lethal making these experiments impossible to perform at this stage. It would also be necessary to investigate whether there are changes in the expression of other isoforms of PMCA for example, PMCA1 and 3 in PMCA4 (-/-) mice that partially

explain the observed platelet phenotype. Therefore, at present, it is only possible to conclude that PMCA4 has an important role in platelet function.

In vivo studies

To identify whether the use of CE exhibited any anti-thrombotic properties, further investigations to look at the effect of systemic infusion of CE on platelet function *in vivo* were performed.

Our group has established a real time *in vivo* mouse model of pulmonary embolism (Section 2.2.5.) and upon systemic infusion of CE at concentrations known to produce inhibitory effects in human platelets, there appeared to be no change in the maximum response (calculated as percentage count) of clot formation in the lungs when compared to a DMSO control. There was also no change in the area under the curve over 10 minutes (i.e dissolution of the clot) when platelets were stimulated with collagen. Therefore, although inhibition of the platelet response was observed *in vitro*, the effects of CE upon platelets when infused systemically could not be detected. One could suggest that the ability of CE to interact with platelet PMCA could have been compromised. CE may have been taken up by the RBC, which is another main source of PMCA therefore limiting the availability of CE to act directly on the platelets themselves and produce an inhibitory response. Further investigation and experiments are warranted to establish whether CE has uses as an anti thrombotic agent. These would include experiments with higher concentrations of CE and *in vivo* experiments in PMCA4 (-/-) mice. The described mouse

experiments provide strong evidence that the observed impairment of platelet function i.e. aggregation, occurs because of loss of PMCA4 activity. This is the first demonstration of a functional role of the PMCA4 isoform in platelets.

Platelet desensitisation

Partial mechanisms by which PMCA inhibition could have reduced platelet aggregation include partial activation and subsequent desensitisation of platelet function. This may well have been as a result of elevated $[Ca^{2+}]_i$ levels observed in preliminary experiments. Another theory is that PMCA could be coupling to a negative regulator that affects platelet function which is Ca^{2+} dependent, such as NO. The former hypothesis was explored first. It has been reported that a mutation of stromal interaction molecule (STIM1), a Ca^{2+} sensor situated in the ER, leads to elevation of $[Ca^{2+}]_i$ in platelets, resulting in a pre-activation state and reduced response to collagen (Grosse et al. 2007). Therefore the pre-activation states of isolated human platelets in the presence of thapsigargin, which has been shown to elevate basal $[Ca^{2+}]_i$ and CE were investigated. From these results, evidence of pre-activation was not identified, this was measured by observing dense granule 5-HT secretion when platelets were incubated with CE and thapsigargin for comparison, and also by assessing changes in light transmission during pre-incubation with CE. Elevations of $[Ca^{2+}]_i$ through SERCA inhibition (i.e. with thapsigargin), despite stimulating dense granule secretion and partial aggregation, did not desensitise platelets to collagen whereas CE caused no detectable pre-activation using either

assay. Therefore, a background elevation of $[Ca^{2+}]_i$ did not *per se* contribute towards the subsequent inhibition of agonist induced aggregation. To further these studies, molecular analysis was conducted to identify the alternative hypothesis stated previously that PMCA acts to regulate negative signalling events such as VASP phosphorylation.

Molecular studies

It has been demonstrated that following thrombin mediated platelet activation, PMCA is phosphorylated on tyrosine residue 1176 , resulting in a 40% reduction in ATPase activity compared to resting platelets (Redondo et al. 2005; Dean et al. 1997; Wan et al. 2003; Bozulic et al. 2007), however unlike most calmodulin regulated enzymes, PMCA's are constitutively active with substantial un-stimulated basal activity.

In addition, very little PMCA is associated with the cytoskeleton under resting conditions, upon stimulation however approximately 80% of the total PMCA is redistributed to the cytoskeleton, where it retains Ca^{2+} ATPase activity (Zabe & Dean 2001), this potentially enables Ca^{2+} and signalling events to be regulated locally. It is possible therefore, that differential regulation of PMCA occurs with phosphorylation and inhibition of a localised subset of the PMCA population.

This PMCA subset may be both isoform specific and/or spatially determined. During platelet activation, phosphorylated PMCA is inhibited to allow activated platelets to raise the global $[Ca^{2+}]_i$, however one can suggest that a distinct subset of

these ATPases, associate with the cytoskeleton and maintain function allowing them to act as signalling molecules by maintaining microenvironments with low Ca^{2+} concentration. Thus, the application of CE in these experiments inhibits platelet aggregation by inhibiting PMCA's and raising Ca^{2+} in these microenvironments.

To assess whether PMCA4 can modulate inhibitory signalling events in activated platelets VASP-Ser^{239} phosphorylation was assessed. VASP-Ser^{239} phosphorylation was clearly increased in stimulated platelets in the presence of CE, indicating that PMCA may aid platelet activation by preventing the phosphorylation of VASP. One of the major mechanisms of Ser-239 VASP phosphorylation in platelets is increased cGMP production by NO. NO is an important negative regulator of platelet function (Section 1.4.1), which can inhibit platelet secretion, aggregation and intracellular Ca^{2+} mobilisation through the stimulation of SGC and the production of cGMP.

Platelets come into contact with NO produced in endothelial cells by eNOS, it is also believed that platelets contain eNOS and can produce NO, however this is now highly controversial and although there is large body of data to support platelet derived NO production (Randriamboavonjy & Fleming 2005), difficulties in detecting eNOS in human platelets have lead to suggestions that it is not present (Gambaryan et al. 2008; Tymvios et al. 2009; for review see Naseem 2008).

In this study, the inhibition of platelet aggregation by CE was not reversed by the SGC inhibitor ODQ (10 μM) suggesting that CE inhibits platelet function independently of cGMP. Although Ser^{239} is considered the preferred phosphorylation site for cGMP/PKG and Ser^{157} is thought to be the preferred

phosphorylation site for cAMP/PKA, the sites are not exclusive and PKA has been demonstrated to phosphorylate VASP-Ser^{239} (Sudo et al. 2003) indicating that PMCA inhibition may potentially facilitate the cAMP/PKA pathway in platelets. Conversely, PMCA may regulate the signalling of an as yet unidentified calcium dependent inhibitory pathway in platelets. As research continues into the role of PMCA4 as a signalling molecule, more proteins capable of interacting with PMCA *via* PDZ domains have emerged. PMCA associates with Ras-associated factor 1 (RASSF1), which in turn inhibits activation of the Erk pathway (Armesilla et al. 2004) as well as the Ca^{2+}/calmodulin dependent phosphatase calcinuerin (Buch et al. 2005; Holton et al. 2007) . In platelets, PMCA associates with CLP36, which associates with α-actinin (Bozulic et al. 2007). It has been reported that CLP36 is important for the assembly of focal adhesions in BeWo cells (Tamura et al. 2007), raising the question of what other proteins form complexes with PMCA/CLP36 in platelets. Further research is required to fully elucidate the mechanisms by which CE inhibits platelet aggregation and to assess whether there are distinct subpopulations of PMCA in platelets with unique functional roles.

Overall conclusions

In conclusion, the data presented in this chapter demonstrate that PMCA is involved in the regulation of intracellular calcium in both resting and activated platelets. PMCA regulates platelet function with temporal dependence. The effects on platelet function correspond with changes in both global intracellular calcium levels and changes in inhibitory signalling pathways. Further study is required to elucidate fully the mechanisms by which PMCA regulates calcium signalling spatially and temporally although PMCA is clearly a critical regulator of platelet calcium homeostasis, signalling and function and is therefore likely to be a key regulator of haemostasis and thrombosis.

CHAPTER FOUR

4.0. The Sarco-Endoplasmic Reticulum ATPase and the Na^{+}/Ca^{2+} Exchanger

4.1. INTRODUCTION

In non-excitable cells, many agonists increase $[Ca^{2+}]_i$ by inducing inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from the intracellular stores while Ca^{2+} influx from the extracellular medium may then sustain the Ca^{2+} signal. Following this, $[Ca^{2+}]_i$ recovers to its resting level as a consequence of Ca^{2+} removing mechanisms, i.e. plasma membrane Ca^{2+}-ATPase (PMCA), Na^+/Ca^{2+} exchanger (NCX) and sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA).

Previous experiments have shown that an inhibition of PMCA in platelets results in differences in Ca^{2+} homeostasis and function at various stages of platelet activation. To determine whether these functional observations are specific to PMCA, experiments were conducted by inhibiting two major Ca^{2+} transporters in platelets, SERCA and the NCX.

In this chapter, two pharmacological inhibitors of the NCX and SERCA, Bepridil and BHQ, were examined to compare the consequences of inhibiting these two transporters upon Ca^{2+} homeostasis and at the various stages of platelet activation.

4.1.1. Aims of the study

- To investigate the roles of NCX and SERCA in regulating Ca^{2+} homeostasis in platelets.
- To examine the functional consequence of NCX and SERCA inhibition using various functional assays.

4.2. RESULTS

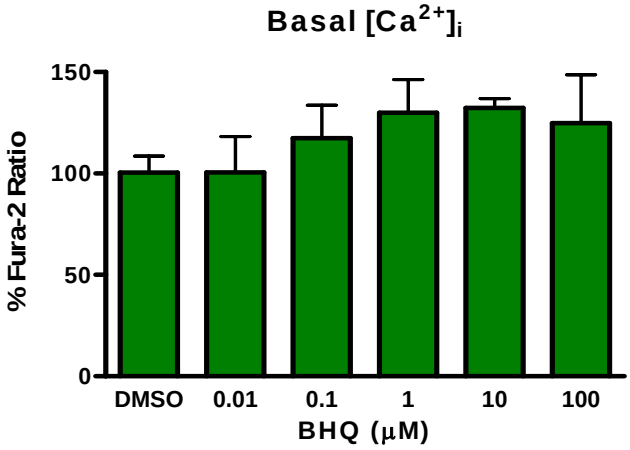
4.2.1. The role of SERCA and the NCX in regulating $[Ca^{2+}]_i$ in resting platelets

To determine whether the NCX or SERCA are able to regulate Ca^{2+} homeostasis, two known inhibitors of these pumps were used to measure $[Ca^{2+}]_i$ in either resting or stimulated platelets.

Washed human platelets were loaded firstly with the Ca^{2+} sensitive loading dye Fura-2 (5 μM) for 40 minutes in the dark (Section 2.2.4.4.) and later with concentrations of either the SERCA inhibitor, BHQ (0.01-100 μM), the NCX inhibitor, Bepridil (1-100 μM) or controls; mTHB, or DMSO (1%) for 30 minutes. Basal Ca^{2+} responses were recorded in the presence of EGTA (100 μM) for 30 seconds as previously described in section 2.2.4.4.

The mean percentage of basal $[Ca^{2+}]_i$ was calculated for both BHQ and Bepridil (Fig. 4.1A and B) and resulted in a non significant effect at these concentrations ($p>0.05$). However, higher concentrations of Bepridil (i.e. 100 μM) displayed a slight increase in basal Ca^{2+} levels, however, this was not significant compared to the control (normalised to 100%): 183±3.5% ($p>0.05$) (Fig. 4.1B).

(A)



(B)

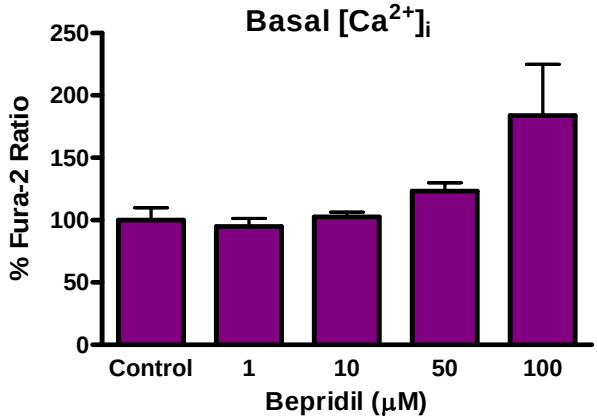


Figure 4.1. The effects of BHQ and Bepridil on basal Ca^{2+} levels in resting platelets
Washed human platelets were loaded with Fura-2 incubated with BHQ (0.01-100 μM), Bepridil (1-100 μM) or controls: mTHB or DMSO (1%). Fura-2AM ratio emission at 340/380 nm was measured for 30 seconds and basal $[Ca^{2+}]_i$ levels recorded. Graphs show the mean percentage of basal $[Ca^{2+}]_i$ in resting platelets for both (A) BHQ and (B) Bepridil relative to controls. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values. No significant values were observed, ($P>0.05$), n=4.

4.2.2. SERCA and the NCX in regulating $[Ca^{2+}]_i$ extrusion in stimulated platelets

To investigate whether the NCX or SERCA elicit an effect in regulating Ca^{2+} homeostasis upon stimulation, Ca^{2+} measurements were recorded in platelets stimulated with thapsigargin plus ionomycin. Washed human platelets were loaded with Fura-2 (5 μM) as described in section 2.2.4.4. and later with concentrations of BHQ (0.01-100 μM), Bepridil (1-100 μM) or controls mTHB or DMSO (1%). Ca^{2+} responses were recorded in the presence of EGTA (100 μM) and stimulated using thapsigargin (1 μM) plus ionomycin (50 nM) which was sufficient to induce a rapid and transient increase in $[Ca^{2+}]_i$ following release from stores. The mean percentage in $[Ca^{2+}]_i$ remaining 3 minutes after stimulation was calculated for each concentration of BHQ and Bepridil. The results indicate a non-significant effect of either BHQ or Bepridil in regulating Ca^{2+} extrusion at these concentrations compared to their controls (normalised to 100%): BHQ: (0.01 μM) 100±0.1%, (0.1 μM) 101±1.0%, (1 μM) 92±0.3%, (10 μM) 97±1.1% and (100 μM) 101±0.1% ($p>0.05$) (Fig. 4.2A). Bepridil: (1 μM) 87±0.99%, (10 μM) 102±0.05%, (50 μM) 93±0.5% and (100 μM) 125±1.8% ($p>0.05$) (Fig. 4.2B). There was also no evidence of an effect of either BHQ or Bepridil on Ca^{2+}store release since $[Ca^{2+}]_{i,}$ (measured as peak minus basal (Fig. 4.2.C and D) was unaffected by this treatment (normalised to 100%): BHQ (0.01μM) 95±0.2%, (0.1μM) 86±0.7%, (1 μM) 92±0.4%, (10 μM) 90±0.5% and (100 μM) 95±0.2% ($p>0.05$). Bepridil: (1 μM) 88±1.7%, (10 μM) 81±2.7%, (50 μM) 82±2.7% and (100 μM) 92±1.0% ($p>0.05$). These results indicate

indicated that neither SERCA nor the NCX play a role in $[Ca^{2+}]_i$ extrusion following stimulation.

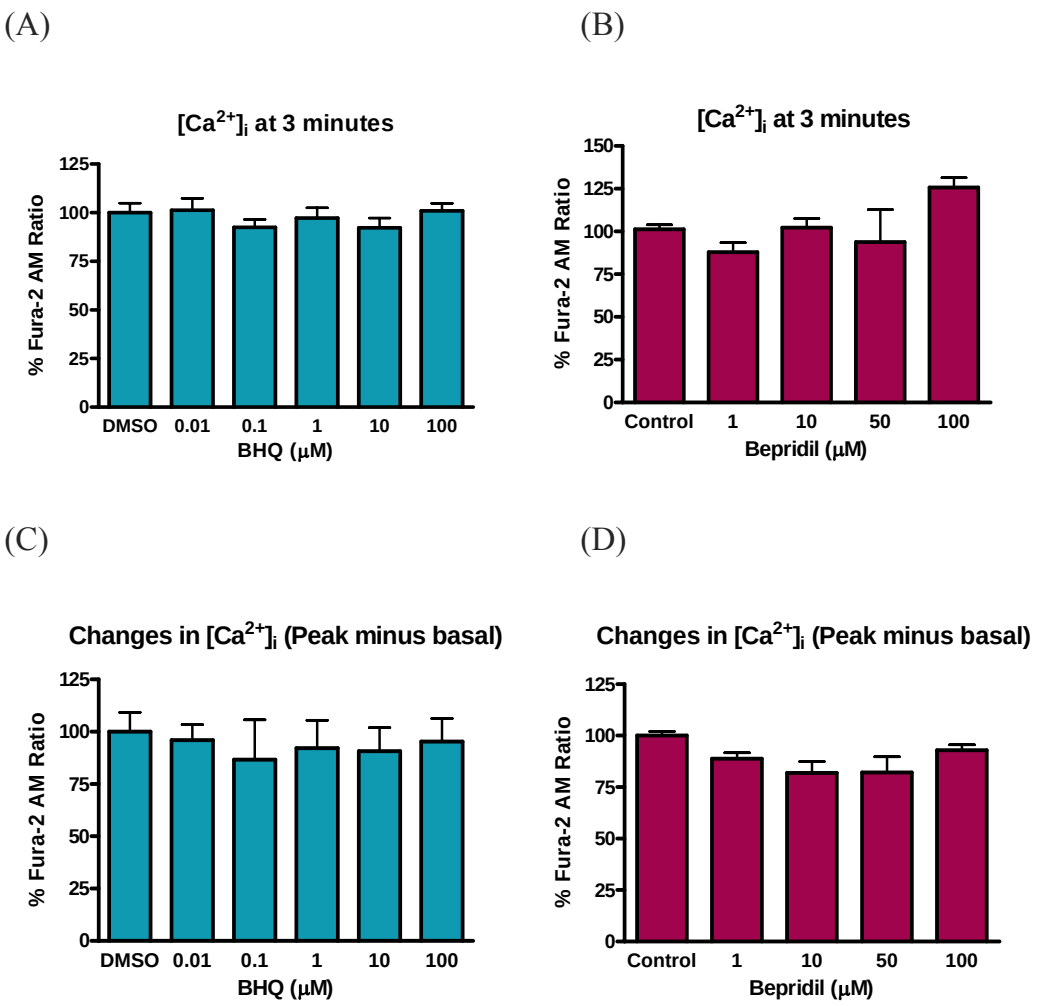


Figure 4.2. The effects of BHQ and Bepridil on thapsigargin plus ionomycin induced $[Ca^{2+}]_i$ release

Washed human platelets were loaded with Fura-2 and incubated with BHQ (0.01-100 μM), Bepridil (1-100 μM) or controls, mTHB or DMSO control (1%). Fura-2AM emission ratio at 340/380nm was measured in response to thapsigargin (1 μM) and ionomycin (50 nM) in the presence of EGTA (100 μM). Figures (A and B) show the % of $[Ca^{2+}]_i$ remaining at 3 minutes post stimulation for both BHQ and Bepridil. Figures (C and D) show the % peak $[Ca^{2+}]_i$ minus the basal $[Ca^{2+}]_i$ post stimulation. Data represents mean percentage ±S.E.M relative to either control. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values. No statistical significance were observed, ($P>0.05$), n=4.

4.2.3. SERCA and the NCX in regulating human platelet aggregation

To investigate whether BHQ or Bepridil elicit a functional effect on washed platelets, cells were pre-incubated with concentrations of BHQ or Bepridil previously used to measure Ca^{2+} extrusion through their associated pumps (Trepakova et al. 1999; Shiraga et al. 1998). Washed human platelets were incubated with BHQ (0.01-100 μM), Bepridil (1-100 μM), or controls, mTHB or DMSO (1%) and stimulated with either collagen (5 μg/ml) or thrombin (0.1 U/ml). Maximal aggregation was achieved at around 3 minutes. In the presence of BHQ, platelets exhibited a concentration dependent inhibition of aggregation upon stimulation. The mean percentage decrease in platelet aggregation was calculated for each concentration of BHQ stimulated firstly with collagen (5 μg/ml) which was significant between 1-100 μM compared to the control (normalised to 100%): (1 μM) 42±4.0% ($p<0.01$), (10 μM) 31±4.8% and (100 μM) 17±5% ($p<0.001$) (Fig. 4.3A), and thrombin (0.1 U/ml) between 1-100 μM compared to the control (normalised to 100%): (1 μM) 52±3.4% ($p<0.05$), (10 μM) 34±4% ($p<0.01$) and (100 μM) 15±6% ($p<0.001$) (Fig. 4.3.B).

With Bepridil, a concentration dependent inhibition of aggregation was observed at 100 μM that was significant when stimulated with collagen (5 μg/ml) compared to the control (normalised to 100%): (100 μM) 17±8% ($p<0.01$) (Fig. 4.3.C) and thrombin (0.1U/ml) which was significant at 70 μM and 100 μM compared to the control (normalised to 100%): (70 μM) 40±1.2% and (100 μM) 28±1.5% ($p<0.01$)

(Fig. 4.3D). The effects BHQ and Bepridil have on isolated human washed platelets demonstrate the functional consequences of the inhibition of Ca^{2+} extrusion through SERCA and the NCX respectively.

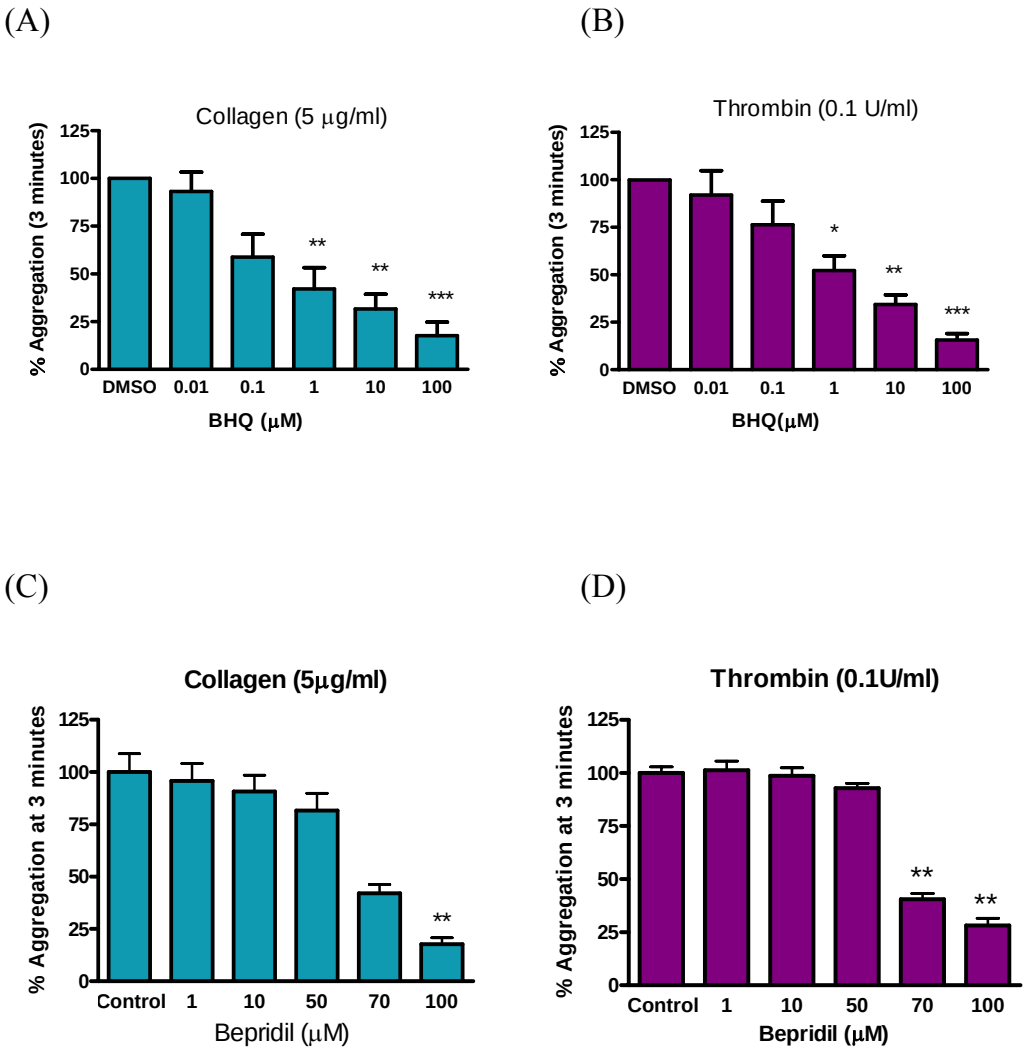


Figure 4.3. The effects of BHQ and Bepridil on collagen and thrombin mediated aggregation

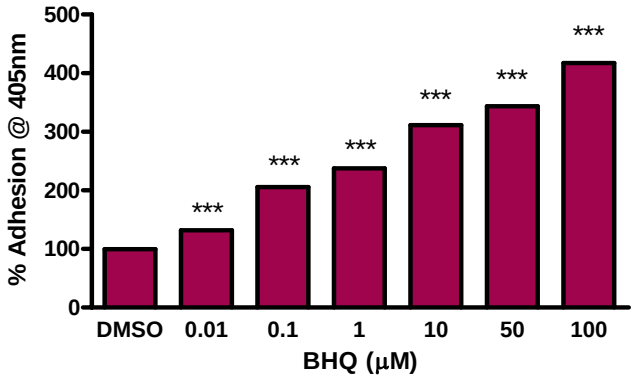
Washed human platelets were pre-incubated with BHQ (0.01-100 μM), Bepridil (1-100 μM) mTHB or DMSO (1%) prior to stimulation. Optical aggregometry was used to measure aggregation in response to thrombin (0.1 U/ml) or collagen (5 μg/ml) for 180 seconds with constant stirring (~1200 rpm). Figures (A and B) show the mean percentage aggregation ±S.E.M of BHQ relative to DMSO, and (C and D) for Bepridil relative to mTHB. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values, *P<0.05, **P<0.01, ***P<0.001, n=5.

4.2.4. The effects of BHQ and Bepridil on platelet adhesion

To investigate another functional consequence of SERCA and NCX inhibition in platelets, platelet adhesion onto fibrinogen (50 μg/ml) coated surfaces was measured spectrophotometrically using the colorimetric platelet phosphatase assay as described by Bellavite and colleagues (Bellavite et al. 1994) (Section 2.2.4.2.) following pre-incubation of platelets with either BHQ (0.01-100 μM), Bepridil (1-100 μM) or controls, mTHB and DMSO (1%).

Platelet adhesion in the presence of BHQ was enhanced in a concentration dependent manner onto fibrinogen coated surfaces (Fig.4.4A) and was significant at all concentrations of BHQ compared to the DMSO control (normalised to 100%): (0.01 μM) 132±0.04%, (0.1 μM) 205±0.3%, (1 μM) 237±0.01%, (10 μM) 311±0.005%, (50 μM) 345±0.001% and (100 μM) 417±0.03% ($p<0.001$). However, platelet adhesion in the presence of Bepridil (Fig.4.4B) was found to be reduced in a concentration dependent manner which was significant at all concentrations compared to the control (normalised to 100%): (1 μM) 95±0.2%, (10 μM) 90±0.9%, (50 μM) 66±0.1% and (100 μM) 47±0.8% ($p<0.001$).

(A)



(B)

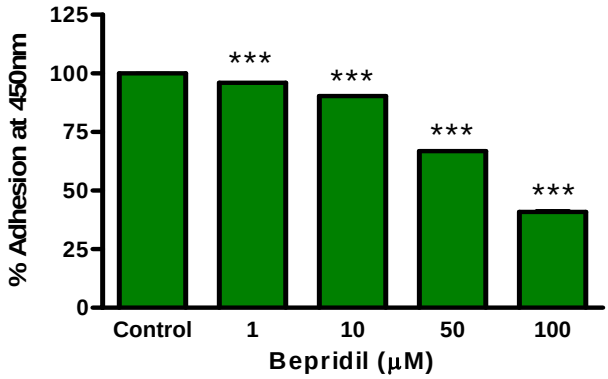


Figure 4.4. Platelet adhesion to fibrinogen coated surfaces

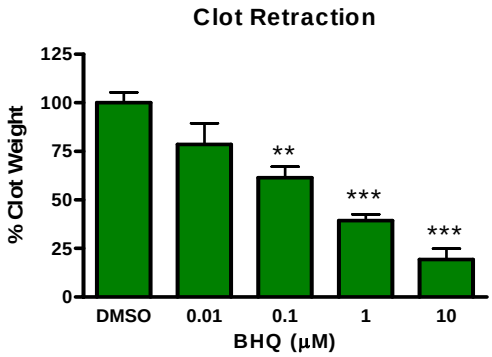
Platelet adhesion onto a fibrinogen (50 μg/ml) coated surface was measured using an acid phosphatase static adhesion assay and absorbance for each well read at 405 nm. Absorbance values for BHQ (0.01-100 μM) and Bepridil (1-100 μM) were normalised to percentage values obtained for non treated cells. Figure (A) Shows the normalised % adhesion of platelets onto fibrinogen coated plates in the presence of BHQ and (B) Bepridil. Data represents mean ±S.E.M in relative units to control responses. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values, ***P<0.001, n=4.

4.2.5. The effects of SERCA and the NCX on clot retraction

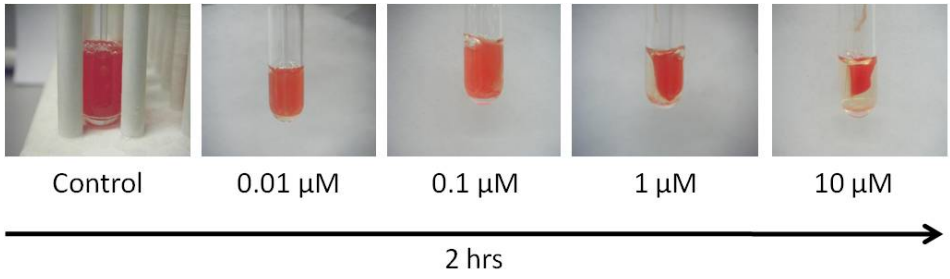
To investigate whether the inhibition of SERCA or the NCX affected the later stages of platelet function, platelet clot retraction was measured. Human PRP was pre-incubated with BHQ (0.01-10 μM), Bepridil (1-100 μM) or controls, mTHB or DMSO (1%) and clot retraction measured as described in section 2.2.4.3. Data was presented as the % clot weight of each sample as shown in Figures (4.5A and B). PRP treated with DMSO (1%) formed a retracted clot within a two hour time frame. Basing each control sample as 100% clot formation, subsequent clots formed in the presence of either BHQ or Bepridil expressed an enhancement i.e. a tighter clot form which was significant for BHQ between 0.1- 10 μM compared to the control (normalised to 100%): (0.1 μM) 61±4% ($p<0.01$), (1 μM) 39±6.2% and (10 μM) 19±8% ($p<0.001$) (Fig. 4.5.A) and Bepridil 10-100 μM compared to the control (normalised to 100%): (10 μM) 64±4% ($p<0.05$), (50 μM) 47±5% and (100 μM) 32±2% ($p<0.001$). In contrast to platelet aggregation, which is suppressed following SERCA and NCX inhibition the enhanced clot retraction demonstrated here suggests that although these pumps positively regulate platelet aggregation they negatively modulate clot retraction.

Figure 4.5. Inhibiting SERCA and the NCX accelerates clot retraction
The effect of SERCA and NCX inhibition by BHQ and Bepridil on clot retraction was measured. Human PRP was incubated with BHQ (0.01-10 μM), Bepridil (1-100 μM) or controls, mTHB or DMSO (1%). Figures (A and C) shows the mean data for the clot weights (normalised to control 100%) ±S.E.M weight in the presence of (0.01-10 μM), Bepridil (1-100 μM) or controls, mTHB or DMSO (1%) and (B and D) are representative clots formed and retracted in the presence of BHQ (0.01-10 μM), Bepridil (1-100 μM) or controls, mTHB or DMSO (1%) at 2 hours. Where statistical comparisons were made a one-way ANOVA followed by a multiple comparison test was used to compare mean values, *P<0.05, **P<0.01, ***P<0.001, n=5.

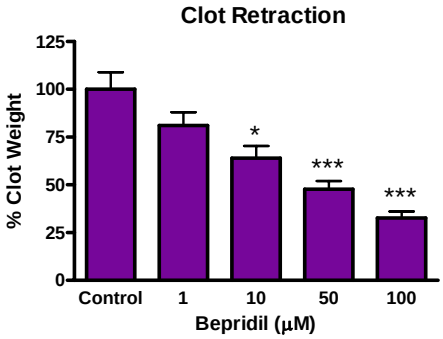
(A)


Clot Retraction
% Clot Weight
125
100
75
50
25
0
**
***
***
DMSO
0.01
0.1
1
10
BHQ (µM)

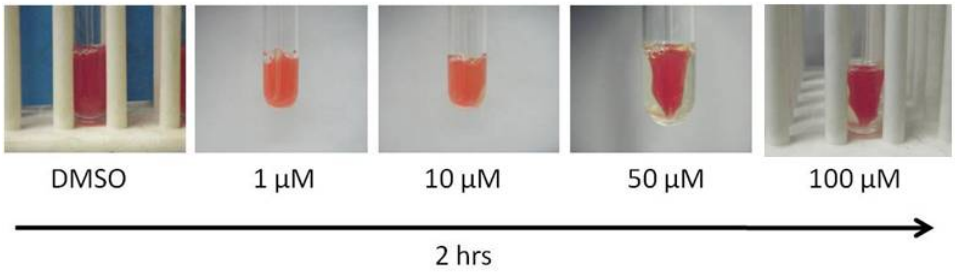
(B)


Control
0.01 µM
0.1 µM
1 µM
10 µM
2 hrs

(C)


Clot Retraction
% Clot Weight
125
100
75
50
25
0
*
***
***
Control
1
10
50
100
Bepridil (µM)

(D)


DMSO
1 µM
10 µM
50 µM
100 µM
2 hrs

4.3. Discussion

The aims of the research carried out in this chapter were to determine the effects of SERCA and NCX inhibition upon Ca^{2+} homeostasis and platelet function. As discussed in chapter three, PMCA plays an important role in Ca^{2+} homeostasis and platelet function which appears to be time dependant. However, to address whether these effects were specific to PMCA, other possible Ca^{2+} pumps that could potentially influence Ca^{2+} regulation and function in platelet were investigated. This was addressed using known inhibitors of SERCA and the NCX, BHQ and Bepridil, to inhibit the activity of these pumps at their respective sites in platelets using a variety of functional assays.

Ca^{2+} regulation

In the resting state, concentrations of Ca^{2+} ions in the cell and its compartments have to be maintained. All ion channels are closed, there being only (uncontrolled) leakage from intracellular stores. These passive ion fluxes have to be countered actively by the fluxes generated by corresponding mechanisms.

The passive flux of Ca^{2+} *via* the plasma membrane is thought to be counteracted by the activities of PMCA and NCX. The NCX in particular, can operate in either direction as described by Juška, 2010 by removing Ca^{2+} ions from cytosol (Juska 2010). The leakage *via* the plasma membrane and the activities of NCX and PMCA, presumably, determine the resting (or background) Ca^{2+} concentration in the cytosol

whereas leakage from the stores is counteracted by the activity of SERCA's, whose fluxes determine Ca^{2+} concentrations within the stores and the leakage from mitochondria is balanced by the activity of NCX and that of uniporters located in the mitochondrial membranes. To maintain the equilibrium of Ca^{2+} fluxes in platelets, the above systems have to operate permanently.

The purpose of these experiments was to determine whether any inhibition of SERCA or the NCX critically affected the regulation of Ca^{2+} homeostasis in comparison to PMCA.

Thapsigargin and BHQ are described as sesquiterpene lactone tumour promoters and have proved useful in the study of Ca^{2+} homoeostasis in a number of different cell types (Thastrup et al. 1990) including platelets (Vostal et al. 1991). Both compounds are membrane permeable and thus block ER Ca^{2+} pumps in intact cells (Thastrup et al. 1990). These compounds have been shown to exert their inhibitory effects by stabilising the E2 conformational state of the ATPase (see section 1.6.3.) and potentially affect PLA_2 activation due to the elevations in $[Ca^{2+}]_i$ (Wictome et al. 1992).

Both compounds are known to elevate cytosolic Ca^{2+} levels *via* the release of Ca^{2+} from intracellular stores, followed by influx from the extracellular medium. This is achieved by inhibiting Ca^{2+} uptake into inositol 1,4,5-trisphosphate releasable pools by inhibiting SERCA without affecting plasma membrane Ca^{2+} transport. Therefore, the increase in $[Ca^{2+}]_i$ in intact cells is due to the release of Ca^{2+} from stores resulting from prevention of reuptake (Brüne & Ullrich 1991).

The results presented here indicate that whilst SERCA is responsible for the elevations in $[Ca^{2+}]_i$ upon activation *via* the release of Ca^{2+} from stores, it does not play a critical role in the maintenance of basal Ca^{2+} levels in resting platelets. This could partly be due to passive Ca^{2+} leakage from stores into areas such as the DTS to maintain Ca^{2+} in a steady state and regulate $[Ca^{2+}]_i$. As previous data indicated a role for PMCA in maintaining basal Ca^{2+} levels, the actions of both SERCA and PMCA in a resting state may prevent inappropriate activation.

Upon stimulation with thapsigargin which has been shown to deplete Ca^{2+} from stores (also demonstrated by Rosado and colleagues (Rosado & Sage 2000)), there appeared to be no change in Ca^{2+} release into the cytosol when SERCA was inhibited. It has also been reported that both thapsigargin and BHQ exert their effects at different isoforms of SERCA within platelets, one sensitive to BHQ (SERCA3) and the other to thapsigargin (SERCA2b) (Cavallini et al. 1995; Redondo et al. 2008). This might suggest that whilst BHQ may inhibit SERCA3, the sequestration of Ca^{2+} by SERCA2a might compensate and still function to sequester some but maybe not all cytosolic Ca^{2+} back into stores.

The capability of SERCA to lower Ca^{2+} concentration in the cytosol, presumably, is rather limited (if any) unless the stores are empty or depleted considerably by thapsigargin. This was evident as Ca^{2+} extrusion through PMCA was unaffected post-stimulation since the Ca^{2+} transient returned to baseline levels normally.

There is increasing evidence the NCX is able to regulate basal Ca^{2+} in platelets (Bose et al. 2002) and can easily reverse its direction and bring Ca^{2+} into the cell if

the Na^+ concentration gradient decreases and/or the membrane potential becomes less negative (Harper & Sage 2007). However, studies to determine whether the NCX plays a role in the maintenance of resting Ca^{2+} have yet to be investigated. The results reported in this chapter demonstrate that whilst the NCX is present within platelets to functionally regulate Na^+ gradients across the membrane, it appeared to play a negligible role in the maintenance of Ca^{2+} in resting platelets, and did not affect Ca^{2+} release from stores or extrusion under the experimental conditions here. This is consistent with studies performed by Rosado and colleagues who demonstrated that in platelets suspended in medium where all Na^+ had been replaced by N-methyl-D-glucamine, $[Ca^{2+}]_i$ was elevated to similar levels as control and then fell back to basal levels after stimulation with thapsigargin (Rosado & Sage 2000). Whilst PMCA and the NCX are the only transporters capable of extruding Ca^{2+} from the cell, the results here indicate that the NCX has a negligible, if any effect on the restoration of $[Ca^{2+}]_i$ to low resting levels when a rise in $[Ca^{2+}]_i$ is induced by depletion of the intracellular Ca^{2+} stores using thapsigargin and a low concentration of ionomycin. There was a slight increase in basal Ca^{2+} levels in the presence of high concentrations of Bepridil (100 μM), however, this was not significant and could possibly be attributed to a non-specific effect. This demonstrates a comparative difference of the NCX in its responsibility for the maintenance of low resting $[Ca^{2+}]_i$ and active Ca^{2+} release in these cells compared to PMCA.

Aggregation

To determine whether the inhibition of SERCA or the NCX elicit a role in platelet function, platelet aggregation was investigated.

Thapsigargin has been shown to induce aggregation and secretion (Harper et al. 2009; Marumo & Wakabayashi 2010), and responses have been shown to be inhibited by cyclo-oxygenase inhibitors (Murthy et al. 1995). BHQ however, appears not to promote aggregation or secretion, but has been shown to inhibit the formation of thromboxane B_2 (Brune & Ullrich 1987).

Our initial hypothesis after preliminary Ca^{2+} experiments were performed was that any impairment of SERCA function would not affect platelet aggregation as extrusion of cytosolic Ca^{2+} remained intact i.e. PMCA is still functional. However, early experiments indicated a concentration dependant inhibition of aggregation in response to collagen and thrombin stimulation. One possible mechanism by which SERCA inhibition could impair aggregation would be its association of local negative regulators upon activation. Studies in myocytes have suggested that cGMP signalling affects function through phosphorylation of phospholamban which regulates SERCA activity by increasing its affinity to Ca^{2+} (Zhang et al. 2005). Another study by Redondo and colleagues demonstrated that the inhibition of SERCA by BHQ accelerated Ca^{2+} store discharge and subsequently enhanced the extent of SOCE stimulated by thrombin in human platelets indicating a negative role for SERCA's in the regulation of Ca^{2+} entry (Redondo et al. 2008). One would expect an increases in $[Ca^{2+}]_i$ following agonist stimulation and subsequent depletion

of stores to promote this event. It could be suggested that prior to stimulation, platelet desensitisation occurs during the incubation process, as studies previously showed an increase in dense granule secretion in platelet pre-treated with thapsigargin (Fig. 3.9.2A). The reasons as to why aggregation is impaired following SERCA inhibition in the presence of BHQ requires further investigation and the role it plays in the regulation of platelet function in this instance, cannot be fully elucidated without investigating the underlying mechanisms associated with this pump. The purpose of these experiments was to directly compare the functional consequence of SERCA inhibition to that of PMCA.

Our initial hypothesis suggested that any impairment of the NCX would not affect cytosolic Ca^{2+} release, as the presence of PMCA would not perturb Ca^{2+} extrusion *via* PMCA. Early experiments confirmed this, as Ca^{2+} extrusion was unaffected by the treatment of NCX inhibitor Bepridil. This would support studies performed by Shiraga and colleagues who demonstrated that Bepridil concentration dependently inhibited platelet aggregation induced by ADP or thrombin. They attributed this to a possible elevation in $[Ca^{2+}]_i$ and subsequent calpain activation which is known to be a potential negative regulator of signalling processes associated with the NCX (Shiraga et al. 1998).

Adhesion and clot retraction

Platelet adhesion and clot retraction were investigated to identify whether the inhibition of SERCA or the NCX play a role in regulating these processes. As observed previously, platelet adhesion and clot retraction were elevated in the presence of the PMCA inhibitor CE due to elevated $[Ca^{2+}]_i$ which is consistent with the activity of both fibrinogen and downstream outside in signalling. Therefore, as initial experiments indicated no change in basal Ca^{2+} levels in resting platelets when SERCA was inhibited, it was surprising to see an elevation of adhesion to fibrinogen along with enhanced clot retraction as both integrin expression and the activation of myosin requires raised Ca^{2+} as a prerequisite. One explanation could be that there are different types of Ca^{2+} sensitive pools located in platelets as described by Cavallini and colleagues, and that expression of different isoforms of SERCA could be more or less sensitive to BHQ inhibition (Cavallini et al. 1995). It could be suggested that in the presence of BHQ, which has a higher affinity for SERCA isoform 3, other isoforms of SERCA, for example SERCA2 may still be functional and deplete stores, raise cytosolic Ca^{2+} levels and potentially activate inside-out signalling to facilitate fibrinogen binding and downstream PLC_β activation required for cytoskeletal actin contraction.

To determine whether or not the NCX plays a role during platelet adhesion or clot retraction, platelet NCX was inhibited using Bepridil. As previous experiments showed no change in resting Ca^{2+} levels along with Ca^{2+} extrusion operating normally through PMCA, there appeared to be a decrease in adhesion to fibrinogen.

One explanation could be that passive leakage of Ca^{2+} from stores is not enough to elicit a change in cytosolic Ca^{2+} levels required for integrin expression. This supports our findings that PMCA acts to regulate Ca^{2+} levels upon activation during the early and later stages of Ca^{2+} release. Another explanation could be that the inhibition of the NCX causes a rise in intracellular Na^{+} concentration, which may be linked to increase refilling of stores to regulate the cytosolic Na^{+}/Ca^{2+} balance within the cell. As there is increasing evidence that the NCX operating in reverse mode may be directly involved in inside-out signalling that activates alpha αIIbβIII, it is possible that the NCX has more of a functional role in platelets than is currently understood (Shiraga et al. 1998). Despite there being an inhibition of adhesion during NCX inhibition, clot retraction was enhanced, presumably due to passive leakage of Ca^{2+} from stores and raising cytosolic Ca^{2+} levels over time during the course of these experiments.

The interpretation of data in this chapter relies on the specificity of the inhibitors used. Concentrations were selected on the basis of published data which showed selective inhibition of the NCX and SERCA at these concentrations. The possibility remains, however, that some of the effects presented could have been due to non-selective effects. Further studies are required to confirm this selectivity. These could involve the use of alternative inhibitors and knock-out mice as well as validatory studies such as those reported in Chapter 3 for CE.

Previous experiments in this thesis outlined the various roles of PMCA in platelets and in contrast, the functional properties of other platelet Ca^{2+} pumps were

investigated. Although the mechanistic profiles of SERCA and the NCX were not explored, the purpose of these experiments were to identify whether the functional changes observed during PMCA inhibition, were specific, in comparison to the inhibition of other Ca^{2+} pumps present, i.e. the inhibition of SERCA and the NCX using BHQ and Bepridil respectively. These results, taken with previous finding for PMCA indicate that all three pumps play a pivotal but contrasting role in regulating platelet Ca^{2+} homeostasis and platelet function (See Table 4.3.).

Table 4.3. Comparative functional differences for inhibition of PMCA, SERCA and the NCX

PMCA	SERCA	NCX
↑ Basal Ca^{2+}	↔ No change in basal Ca^{2+}	↔ No change in basal Ca^{2+}
↓ Ca^{2+} extrusion	↔ Normal Ca^{2+} extrusion	↔ Normal Ca^{2+} extrusion
↔ No change in Ca^{2+}store release	↔ No change in Ca^{2+} store release	↔ No change in Ca^{2+} store release
↓ Platelet aggregation	↓ Platelet aggregation	↓ Platelet aggregation
↑ Platelet adhesion	↑ Platelet adhesion	↓ Platelet adhesion
↑ Enhanced clot retraction	↑ Enhanced clot retraction	↑ Enhanced clot retraction

CHAPTER FIVE

5.0. General Discussion

Introduction

The role of platelet function in the development of thrombotic events has been studied extensively for many years as platelet and coagulation factor-dependent thrombus formation is critical to limit posttraumatic blood loss at sites of vascular injury. Under pathological conditions like rupture of an atherosclerotic plaque, thrombus formation may lead to vessel occlusion causing myocardial infarction or stroke. Therefore, antithrombotic treatment is the prime therapeutic option in the prophylaxis and treatment of ischaemic cardio- and cerebrovascular diseases

The use of existing antithrombotic agents is, however, limited by their inherent effect on primary haemostasis. In recent years, major advances have been made in understanding the mechanisms of thrombus formation in haemostasis and thrombosis and some studies raised the interesting possibility that thrombus formation and haemostasis may involve partially different mechanisms (Hagedorn et al. 2010).

Objectives of this thesis

The main objective of this study was to investigate the role of PMCA in platelet function and Ca^{2+} homeostasis. This was achieved using a pharmacological inhibitor of PMCA, carboxyeosin. Following this, isomer specific roles were investigated using PMCA4 knock-out mice and by investigating the consequence of inhibition in comparison to other Ca^{2+} regulators in platelets, SERCA and the NCX.

Lastly, the molecular signalling pathways of PMCA were also explored. The results presented in this thesis indicate that PMCA is involved in the regulation of intracellular Ca^{2+} in both resting and activated platelets and differentially regulates the various stages of platelet activation with both stimulatory and inhibitory roles. The effects on platelet function correspond with changes in both $[Ca^{2+}]_i$ and inhibitory signalling pathways. These roles were specific to PMCA when compared to other Ca^{2+} pumps located within platelets suggesting that all three Ca^{2+} pumps play pivotal but contrasting roles in regulating platelet function and Ca^{2+} homeostasis.

Ca^{2+} regulation

Ca^{2+} signalling modulates some of the most important processes, including cell origin at fertilisation, stimulus–contraction and stimulus–secretion coupling, intracellular communication, gene transcription, cell growth and apoptotic cell death. The correct functioning of these processes demands that the concentration of Ca^{2+} in the cytoplasm and in the different intracellular compartments must be tightly controlled. Alterations in this control may lead to various cell dysfunctions and diseases.

The first indication that the plasma membrane of eukaryotic cells contains a Ca^{2+}-dependent ATPase was provided almost 50 years ago by Dunham & Glynn (Dunham & Glynn 1961). Five years later Schatzmann showed that the ATPase actually transported Ca^{2+} out of erythrocytes (Schatzmann 1966), but it took a

number of years before it became clear that the pump was, in fact, present in the plasma membranes of all eukaryotic cells. In 1988, the complete amino acid sequence of the pump was deduced by Shull & Greeb from complementary DNA isolated from rat brain (Shull & Greeb 1988) and by Verma and colleagues from human teratoma (Verma et al. 1988) but it wasn't until the mid 1990's when Bokkala and colleagues investigated PMCA in highly purified human platelet membranes using a monoclonal antibody, 5F10, raised to the red-cell membrane Ca^{2+}ATPase (Bokkala et al. 1995).

To date, role of PMCA and its importance in platelet function has yet to be characterised.

PMCA's importance in the cardiovascular system

Platelets do not only have a profound impact within the cardiovascular system but may also be involved in the pathogenesis of other conditions such as diabetes (Chaabane et al. 2007; Jardín et al. 2006; Tempel & Shilling 2007). Work by Blankenship and colleagues has shown that PMCA function/expression in platelets of hypertensive individuals is impaired, resulting in increased platelet intracellular Ca^{2+} and hyperactive platelets which could lead to increased risk of heart attack and stroke (Blankenship et al. 2000) providing a justification for the investigation of PMCA in platelets as a potential therapeutic target in this condition. In addition, work carried out by Jardin and colleagues have demonstrated PMCA as a potential target for the development of therapeutic strategies to palliate cardiovascular

disorders in non-insulin-dependent diabetes mellitus (Jardín et al. 2006) which also demonstrates PMCA's importance as a therapeutic target.

Inhibitors

In recent years, considerable progress has been made in understanding the mechanisms of thrombus formation and studies in mice provided the first experimental evidence that pathological thrombus formation and primary haemostasis may be two mechanistically distinct processes (Hagedorn et al. 2010).

There have been many studies which focus on platelet Ca^{2+} regulation, (Kouvelas et al. 2009) (Kouvelas et al. 2009)(Kouvelas et al. 2009)in which PMCA has been investigated alongside other Ca^{2+} pumps such as SERCA, SOCE and the NCX. However, there have been no studies to fully clarify the functional role of PMCA in platelets.

In terms of targeting platelets in the treatment of thrombosis, the COX inhibitor, aspirin, is the most widely used over-the-counter drugs available. It has low (but useful) therapeutic activity, but is stigmatised by severe adverse side effects such prolonged inhibition of blood coagulation (Brune et al. 2009).

Due to its effects at other ATPases, CE is unlikely to have value as a therapeutic target in platelets. Nevertheless, it is still an important tool to investigate the role of PMCA in other cell types as already demonstrated in various cell types, for example in endothelial cells (Moccia et al. 2002) and mesenchymal stem cells (Kawano et al. 2003). In this thesis, CE has been applied to investigate platelet Ca^{2+} homeostasis and more importantly platelet functional responses.

The application of CE in the *in vivo* model of pulmonary embolism could be extended to include haemodynamic analysis in mouse models as described by Tymvios et al. (Tymvios et al. 2008). This would look at the direct relation CE has on the heart and its effects in blood pressure.

Recently, the identification and use of a more specific extracellular inhibitor of PMCA has emerged. The use of Caloxins to inhibit PMCA was first demonstrated by Chaudhary and colleagues in 2001, who screened a random peptide phage display library to select for peptides binding to the second extracellular domain sequence of PMCA which links transmembrane domains 3 and 4, which is shown to be inhibitory (Guerini et al. 1996). This discovery was the first of its kind to identify a specific extracellular inhibitor of PMCA (Chaudhary et al. 2001).

Future experiments with these novel compounds may be useful in platelet functional studies both in vitro and *in vivo*.

Other models

Here we demonstrated the use of CE as a specific PMCA4 inhibitor by applying it not only to human platelets *in vitro*, but carrying it forward in models of *in vivo* thromboembolism. Other applications would be to examine platelet inhibition in models of vascular disease, for example in vascular flow models already used in areas of endothelial research. By observing platelets in such settings, one could gain a better understanding of the mechanisms by which hemodynamics influences platelets within susceptible regions of the vasculature.

This could be further extended by introducing models of flow in conjunction with microscopic image sequence analysis as described by Macchin and colleagues who have developed a unique way of quantifying adhesion properties of platelets under flow conditions, over time (Machin et al. 2005). Other experimental approaches would be to look at vascular injury models such as endothelial laser injury as described by Atkinson and colleagues, who monitored Ca^{2+} mobilisation to assess activation of the laser-targeted cells (Atkinson et al. 2010; Hechler et al. 2010), or Hechler and colleagues who looked at transient thrombus formation in a model of laser-induced thrombosis in mesenteric arterioles (Hechler et al. 2010).

Other vascular injury models such as ferric chloride induced thrombosis, endothelial denudation and the use of rose bengal in photochemical induced thrombosis may also be of interest by observing the effects of platelet inhibition using CE these settings.

Signalling

Studies to identify PMCA inhibition in human platelets using a variety of *in vitro* assays demonstrated that the inhibition of PMCA with CE displayed an impairment of intracellular Ca^{2+} extrusion coupled with a diminished response in platelet aggregation upon stimulation. Furthermore, an increase in basal Ca^{2+} levels was apparent. Although many of the observations are in fitting with PMCA's role as a Ca^{2+} extruder, its role in positively driving platelet aggregation was somewhat an unanticipated response as one would expect platelets to activate giving the rise in cytosolic $[Ca^{2+}]_{i.}$ Additional studies demonstrated that in the presence of CE,

platelets were not desensitised prior to stimulation owing to the background elevation of $[Ca^{2+}]_i$. Therefore, to further characterise the profile of PMCA in platelets and address other possible mechanisms behind the inhibitory effects observed upon platelet aggregation with CE, inhibitory molecular signalling pathways associated with PMCA were investigated.

A major negative regulator of platelets is the phosphorylation of VASP. Therefore, molecular signalling studies were conducted to identify VASP phosphorylation in platelets pre-treated with CE. From these studies, an increase in VASP-Ser^{239} phosphorylation was apparent suggesting that PMCA may be negatively regulating signalling events in platelets. Similar studies have identified an association of PMCA with negative signalling molecules in other cell types. For example, PMCA has been shown to regulate inhibitory signalling within cardiomyocytes (Oceandy, Cartwright et al. 2007) and vascular smooth muscle where it associates with nNOS (Schuh et al. 2003) and eNOS in endothelial cells (Moccia et al. 2002). Therefore, it was suggested that perhaps the inhibitory effects observed with CE on platelet aggregation could be mediated by the association of PMCA with eNOS and subsequently phosphorylate VASP-Ser^{239} downstream of the NO-cGMP pathway.

One of the major mechanisms of VASP-Ser^{239} phosphorylation in platelets is increased cGMP production by NO (Gambaryan et al. 2008). However, the question as to whether platelets contain NOS enzymes that directly activate the NO-cGMP pathway is somewhat controversial as there is still much debate as to whether NOS enzymes, in particular eNOS, are present in platelets (Naseem & Riba 2008).

Despite this controversy surrounding the expression of NOS enzymes in platelets, further investigations as to whether PMCA could regulate pathways downstream of NO were carried forward to look at the link between NO and SGC which is a downstream effector of this pathway.

In studies carried out here, the inhibition of platelet aggregation by CE occurred independently of cGMP. Therefore, it left the question open as to what could be phosphorylating VASP. Although Ser^{239} is considered to be the preferred phosphorylation site for cGMP/protein kinase G (PKG), the site has also been shown to be both PKG-sensitive and PKA-sensitive (Li et al. 2003; Riba et al. 2006) indicating that PMCA inhibition may stimulate alternative Ca^{2+}-dependent inhibitory pathways in platelets.

These regulatory sites are not exclusive, and as the inhibition of platelet aggregation appeared to be independent of the cGMP/PKG pathway, it could be suggested that PMCA inhibition may potentially facilitate the cAMP/PKA pathway as PKA has also been shown to phosphorylate VASP-Ser^{239} (Sudo et al. 2003) It would also interesting to investigate the phosphorylation of VASP at Ser^{157} by protein kinase C (PKC) which correlates with reduced activation of integrin aIIbβ3 and an inhibition of platelet aggregation (Horstrup et al. 1994). To characterise a signalling pathway that is unique to one particular protein would require an immense amount of research. Nevertheless, to further investigate potential protein kinase pathways aside from the cGMP/PKG which are all Ca^{2+} dependent that may be involved in the

phosphorylation of VASP, experiments using commercially available inhibitors which are selective for PKC and PKA perhaps, would be of interest.

Conversely, PMCA may also be involved in the regulation of signalling molecules of an as yet unidentified Ca^{2+} dependent inhibitory pathway in platelets such as those by acidic phospholipids (Carafoli 1994). It may also be interesting to investigate interaction partners of PMCA as demonstrated by various groups who have showed PMCA's association with syntrophin (Williams et al. 2006), calcineurin (Buch et al. 2005; Holton et al. 2007) and Ras association domain-containing protein 1 (RASSF1) (Armesilla et al. 2004) all of which regulate a number of processes in the cardiovascular system. The recently identified PDZ-binding domain present within PMCA as described by Schuh and colleagues (Schuh et al. 2001) may also be of great interest to determine further interaction molecules that may regulate PMCA in platelets.

Another approach would be to investigate the signalling interactions of another important isoform of PMCA, PMCA1 as this isoform, is essential as a housekeeping regulator of intracellular Ca^{2+} in cells and also for embryonic development (M. Brini 2009).

As research continues into the role of PMCA, novel interactions between PMCA and signalling molecules continually emerge, and this may ultimately clarify the role of PMCA in regulating signalling events in platelets.

Association of other Ca^{2+} pumps

As well as PMCA, the inhibition of other Ca^{2+} pumps located in platelets was investigated. It was originally hypothesised that, perhaps, other Ca^{2+} pumps, notably SERCA and the NCX may be involved in the regulation of Ca^{2+}. However, it was identified that neither of the two play a role in the maintenance of resting Ca^{2+} levels nor do they contribute towards a difference in the extrusion of Ca^{2+} upon stimulation. This was surprising, in the case of the NCX which has been shown to regulate basal Ca^{2+} levels in platelets (Bose et al. 2002). One would expect the NCX to contribute towards Ca^{2+} extrusion should intracellular concentrations become elevated. However, this was not the case as inhibition of PMCA but not the NCX resulted in an inhibition of the decay of Ca^{2+} transients. The observation of an impairment of aggregation was observed was also unexpected. It could be suggested that both pumps associate with local inhibitory signalling molecules that are involved in platelet activation but are independent from changes in cytosolic Ca^{2+} concentration. Alternatively, Ca^{2+} store release may have been distributed to areas within the platelet such as the DTS as a protective means to prevent inappropriate activation. It could also be suggested that the inhibition of SERCA and the NCX using the inhibitors employed may have desensitised platelets during the experimental incubation processes leading to a subsequent reduction in aggregation prior to stimulation. It would be interesting to investigate whether there are any changes in Ca^{2+} homeostasis and function in platelets in the presence of two or more inhibitors in combination.

Knock-out mice

A major breakthrough in studies of PMCA was the affinity purification of the enzyme 25 years ago by Niggli and colleagues (Niggli et al. 1979). Since then, four PMCA isoforms 1-4, and several dozen splice variants have been identified in mammalian tissues (Strehler & Zacharias 2001). PMCA1 and PMCA4 are expressed in all tissues, suggesting that they are necessary for housekeeping functions (Greeb & Shull 1989; Stauffer et al. 1994), although they undoubtedly serve tissue-specific functions as well.

Ideally experiments in which Ca^{2+} homeostasis and platelet function *in vitro* and *in vivo* are observed in PMCA1 knock-out mice and compared to PMCA4 knock-out mice would determine the specific role of PMCA4 and identify its characteristics further. However, as PMCA1 is essential during embryogenesis, reflecting a major housekeeping function, knock-out mice are not available. PMCA's role as a major housekeeping role is further demonstrated by the observation that loss of one copy of the *PMCA1* gene in a PMCA4 null background can lead to apoptosis of vascular smooth muscle cells (Okunade et al. 2004), presumably due to Ca^{2+} overload.

As an alternative approach, knock-down studies could be performed where by the PMCA1 gene is reduced, either through genetic modification of the DNA of one chromosome or by treatment with a reagent such as a short DNA or RNA oligonucleotide with a sequence complementary to either an mRNA transcript or a gene. However, it would take several years to establish such a technique and other factors such cell viability would need to be considered.

These experiments would however, provide further insight into the distinct role of PMCA 1 and 4. In addition, it would be interesting to investigate the functional consequence of any compensatory expression of PMCA1 in PMCA4$^{-/-}$ mice.

Diabetes, hypertension and type 2B von Willebrand disease have all been associated with altered expression of PMCA and all of these conditions are associated with platelet dysfunction through mechanisms that are incompletely understood.

In characterising the integrative role of PMCA in regulating platelet function, this thesis has provided potential mechanisms by which PMCA may modulate platelet function and hence the development and progression of these diseases.

5.1 Conclusions

In conclusion, PMCA is involved in the regulation of $[Ca^{2+}]_i$ in both resting and activated platelets. PMCA differentially regulates the various stages of platelet activation with both stimulatory and inhibitory roles which contrast with other Ca^{2+} pumps such as SERCA and the NCX. The effects on platelet function correspond with changes in both $[Ca^{2+}]_i$ and inhibitory signalling pathways.

The data presented in this thesis demonstrates that PMCA is clearly a critical regulator of platelet Ca^{2+} homeostasis, signalling and function, is a key regulator of haemostasis and thrombosis, and may be a therapeutic target in the treatment of platelet driven disorders such as myocardial infarction.

REFERENCES

Alevriadou, B.R. et al., 1993. Real-time analysis of shear-dependent thrombus formation and its blockade by inhibitors of von Willebrand factor binding to platelets. *Blood*, 81(5), 1263-76.

Altimimi, H.F. & Schnetkamp, P.P.M., 2007. Examining Ca2+ extrusion of Na+/Ca2+-K+ exchangers. *Annals of the New York Academy of Sciences*, 1099, 29-33.

Andersohn, F., Hagmann, F.G. & Garbe, E., 2004. Thrombotic thrombocytopenic purpura/haemolytic uraemic syndrome associated with clopidogrel: report of two new cases. *Heart (British Cardiac Society)*, 90(9), e57.

Andrews, R.K. & Berndt, M.C., 2004. Platelet physiology and thrombosis. *Thrombosis Research*, 114(5-6), 447-453.

Antman, E.M., DeMets, D. & Loscalzo, J., 2005. Cyclooxygenase inhibition and cardiovascular risk. *Circulation*, 112(5), 759-770.

Armesilla, A.L. et al., 2004. Novel functional interaction between the plasma membrane Ca2+ pump 4b and the proapoptotic tumor suppressor Ras-associated factor 1 (RASSF1). *J Biol Chem*, 279(30), 31318-28.

Aszódi, A. et al., 1999. The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. *The EMBO Journal*, 18(1), 37-48.

Atkinson, B.T. et al., 2010. Laser-induced endothelial cell activation supports fibrin formation. *Blood*, 116(22), 4675-4683.

Ayhan, A. et al., 2006. The value of preoperative platelet count in the prediction of cervical involvement and poor prognostic variables in patients with endometrial carcinoma. *Gynecologic Oncology*, 103(3), 902-905.

Barnes, M.J. & Farndale, R.W., 1999. Collagens and atherosclerosis. *Experimental Gerontology*, 34(4), 513-525.

Bartfai, T. & Conti, B., 2010. Fever. *TheScientificWorldJournal*, 10, 490-503.

Batlouni, M., 2010. [Nonsteroidal anti-inflammatory drugs: cardiovascular, cerebrovascular and renal effects]. *Arquivos Brasileiros De Cardiologia*, 94(4), 556-563.

Becker, R.P. & De Bruyn, P.P., 1976. The transmural passage of blood cells into myeloid sinusoids and the entry of platelets into the sinusoidal circulation; a scanning electron microscopic investigation. *Am J Anat*, 145(2), 183-205.

Behnke, O., 1967. Electron microscopic observations on the membrane systems of the rat blood platelet. *The Anatomical Record*, 158(2), 121-137.

Bellavite, P. et al., 1994. A colorimetric method for the measurement of platelet adhesion in microtiter plates. *Analytical Biochemistry*, 216(2), 444-450.

Bensalah, K. et al., 2006. Prognostic value of thrombocytosis in renal cell carcinoma. *The Journal of Urology*, 175(3 Pt 1), 859-863.

Berridge, M.J., 1995. Capacitative calcium entry. *The Biochemical Journal*, 312 (Pt 1), 1-11.

Berridge, M.J., Bootman, M.D. & Roderick, H.L., 2003. Calcium signalling: dynamics, homeostasis and remodelling. *Nature Reviews. Molecular Cell Biology*, 4(7), 517-529.

Best, L.C. et al., 1977. Prostacyclin increases cyclic AMP levels and adenylate cyclase activity in platelets. *Nature*, 267(5614), 850-852.

Bhatt, D.L., 2004. Aspirin resistance: more than just a laboratory curiosity. *Journal of the American College of Cardiology*, 43(6), 1127-1129.

Blankenship, K.A. et al., 2000. Tyrosine phosphorylation of human platelet plasma membrane Ca(2+)-ATPase in hypertension. *Hypertension*, 35(1 Pt 1), 103-7.

Blann, A.D. et al., 2001. Increased soluble P-selectin in patients with haematological and breast cancer: a comparison with fibrinogen, plasminogen activator inhibitor and von Willebrand factor. *Blood Coagulation & Fibrinolysis: An International Journal in Haemostasis and Thrombosis*, 12(1), 43-50.

Blaustein, M.P. & Lederer, W.J., 1999. Sodium/calcium exchange: its physiological implications. *Physiological Reviews*, 79(3), 763-854.

Bobe, R. et al., 1994. The rat platelet 97-kDa Ca2+ATPase isoform is the sarcoendoplasmic reticulum Ca2+ATPase 3 protein. *J Biol Chem*, 269(2), 1417-24.

Bokkala, S. et al., 1995. Localization and identification of Ca2+ATPases in highly purified human platelet plasma and intracellular membranes. Evidence that the monoclonal antibody PL/IM 430 recognizes the SERCA 3 Ca2+ATPase in human platelets. *Biochem J*, 306 (Pt 3), 837-42.

Boring, L. et al., 1998. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature*, 394(6696), 894-897.

Born, G.V., 1962a. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*, 194, 927-9.

Born, G.V., 1962b. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*, 194, 927-9.

Born, G.V., Mills, D.C. & Roberts, G.C., 1967. Potentiation of platelet aggregation by adrenaline. *The Journal of physiology*, 191(1), 43P-44P.

Bose, R., Li, Y. & Roberts, D., 2002. Na+/Ca2+ exchange in activated and nonactivated human platelets. *Annals of the New York Academy of Sciences*, 976, 350-353.

Bozulic, L.D., Malik, M.T. & Dean, W.L., 2007. Effects of plasma membrane Ca(2+) -ATPase tyrosine phosphorylation on human platelet function. *J Thromb Haemost*, 5(5), 1041-6.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72, 248-54.

Brandt, P. & Neve, R.L., 1992. Expression of plasma membrane calcium-pumping ATPase mRNAs in developing rat brain and adult brain subregions: evidence for stage-specific expression. *Journal of Neurochemistry*, 59(4), 1566-1569.

Brass, L.F., 1984. Ca2+ homeostasis in unstimulated platelets. *The Journal of Biological Chemistry*, 259(20), 12563-12570.

Brass, L.F. & Joseph, S.K., 1985. A role for inositol triphosphate in intracellular Ca2+ mobilization and granule secretion in platelets. *The Journal of Biological Chemistry*, 260(28), 15172-15179.

Brass, L.F., Shaller, C.C. & Belmonte, E.J., 1987. Inositol 1,4,5-triphosphate-induced granule secretion in platelets. Evidence that the activation of phospholipase C mediated by platelet thromboxane receptors involves a guanine nucleotide binding protein-dependent mechanism distinct from that of thrombin. *The Journal of Clinical Investigation*, 79(4), 1269-1275.

Bray, P.F., 2000. Platelet glycoprotein polymorphisms as risk factors for thrombosis. *Current Opinion in Hematology*, 7(5), 284-289.

Briddon, S.J. & Watson, S.P., 1999. Evidence for the involvement of p59fyn and p53/56lyn in collagen receptor signalling in human platelets. *The Biochemical Journal*, 338 (Pt 1), 203-209.

Brini, M., 2009. Plasma membrane Ca(2+)-ATPase: from a housekeeping function to a versatile signaling role. *Pflugers Arch*, 457(3), 657-64.

Brockmann, M.A. et al., 2007. Preoperative thrombocytosis predicts poor survival in patients with glioblastoma. *Neuro-Oncology*, 9(3), 335-342.

Brodie, T.G. & Russell, A.E., 1897. The Enumeration of Blood-Platelets. *J Physiol*, 21(4-5), 390-5.

Brown, K.M. et al., 2005. Increased preoperative platelet count is associated with decreased survival after resection for adenocarcinoma of the pancreas. *American Journal of Surgery*, 189(3), 278-282.

Bruckdorfer, R., 2005. The basics about nitric oxide. *Molecular Aspects of Medicine*, 26(1-2), 3-31.

Brüne, B. & Ullrich, V., 1991. Calcium mobilization in human platelets by receptor agonists and calcium-ATPase inhibitors. *FEBS Letters*, 284(1), 1-4.

Brune, B. & Ullrich, V., 1991. Calcium mobilization in human platelets by receptor agonists and calcium-ATPase inhibitors. *FEBS Lett*, 284(1), 1-4.

Brune, B. & Ullrich, V., 1987. Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol Pharmacol*, 32(4), 497-504.

Brune, K., Hinz, B. & Otterness, I., 2009. Aspirin and acetaminophen: should they be available over the counter? *Current Rheumatology Reports*, 11(1), 36-40.

Buch, M.H. et al., 2005. The sarcolemmal calcium pump inhibits the calcineurin/nuclear factor of activated T-cell pathway via interaction with the calcineurin A catalytic subunit. *J Biol Chem*, 280(33), 29479-87.

Buensuceso, C.S. et al., 2005. Regulation of outside-in signaling in platelets by integrin-associated protein kinase C beta. *The Journal of Biological Chemistry*, 280(1), 644-653.

Bussel, J.B., Kunicki, T.J. & Michelson, A.D., 2000. Platelets: New Understanding of Platelet Glycoproteins and Their Role in Disease. *Hematology*, 2000(1), 222-240.

Carafoli, E. & Brini, M., 2000. Calcium pumps: structural basis for and mechanism of calcium transmembrane transport. *Current Opinion in Chemical Biology*, 4(2), 152-161.

Carafoli, E., 1991. The calcium pumping ATPase of the plasma membrane. *Annu Rev Physiol*, 53, 531-47.

Carafoli, E. et al., 2001. Generation, control, and processing of cellular calcium signals. *Crit Rev Biochem Mol Biol*, 36(2), 107-260.

Cartwright, E.J., Schuh, K. & Neyses, L., 2005. Calcium transport in cardiovascular health and disease--the sarcolemmal calcium pump enters the stage. *J Mol Cell Cardiol*, 39(3), 403-6.

Catella-Lawson, F. et al., 2001. Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. *The New England Journal of Medicine*, 345(25), 1809-1817.

Cattaneo, M. et al., 2002. Adenosine diphosphate(ADP) does not induce thromboxane A2 generation in human platelets. *Blood*, 99(10), 3868-9; author reply 3869-70.

Cavallini, L. & Alexandre, A., 1994. Ca2+ efflux from platelets. Control by protein kinase C and the filling state of the intracellular Ca2+ stores. *European Journal of Biochemistry / FEBS*, 222(2), 693-702.

Cavallini, L., Coassin, M. & Alexandre, A., 1995. Two classes of agonist-sensitive Ca2+ stores in platelets, as identified by their differential sensitivity to 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin. *The Biochemical Journal*, 310 (Pt 2), 449-452.

Cavallini, L. et al., 1996. Prostacyclin and sodium nitroprusside inhibit the activity of the platelet inositol 1,4,5-trisphosphate receptor and promote its phosphorylation. *The Journal of Biological Chemistry*, 271(10), 5545-5551.

Cavallini, L., Coassin, M. & Alexandre, A., 1995. Two classes of agonist-sensitive Ca2+ stores in platelets, as identified by their differential sensitivity to 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin. *Biochem J*, 310 (Pt 2), 449-52.

Chaabane, C. et al., 2007. Platelet PMCA- and SERCA-type Ca2+ -ATPase expression in diabetes: a novel signature of abnormal megakaryocytopoiesis. *J Thromb Haemost*, 5(10), 2127-35.

Chaudhary, J. et al., 2001. Caloxin: a novel plasma membrane Ca2+ pump inhibitor. *Am J Physiol Cell Physiol*, 280(4), C1027-30.

Chen, L.Y. & Mehta, J.L., 1996. Further evidence of the presence of constitutive and inducible nitric oxide synthase isoforms in human platelets. *J Cardiovasc Pharmacol*, 27(1), 154-8.

Choi, H.S. & Eisner, D.A., 1999. The role of sarcolemmal Ca2+-ATPase in the regulation of resting calcium concentration in rat ventricular myocytes. *J Physiol*, 515 (Pt 1), 109-18.

Clemetson, J.M. et al., 1999. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors. *The Journal of Biological Chemistry*, 274(41), 29019-29024.

Collen, D., 1980. On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. *Thrombosis and Haemostasis*, 43(2), 77-89.

Collins, T. et al., 1995. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 9(10), 899-909.

Conley, P.B. & Delaney, S.M., 2003. Scientific and therapeutic insights into the role of the platelet P2Y12 receptor in thrombosis. *Current opinion in hematology*, 10(5), 333-8.

Costantini, V. et al., 1990. The platelet count in carcinoma of the lung and colon. *Thrombosis and Haemostasis*, 64(4), 501-505.

Cutler, L., Rodan, G. & Feinstein, M.B., 1978. Cytochemical localization of adenylate cyclase and of calcium ion, magnesium ion-activated ATPases in the dense tubular system of human blood platelets. *Biochim Biophys Acta*, 542(3), 357-71.

Davis, S.M. & Branch, D.W., 2010. Thromboprophylaxis in Pregnancy: Who and How? *Obstetrics and Gynecology Clinics of North America*, 37(2), 333-343.

Dean, W.L. et al., 1997. Regulation of platelet plasma membrane Ca2+-ATPase by cAMP-dependent and tyrosine phosphorylation. *J Biol Chem*, 272(24), 15113-9.

Dean, W.L. & Whiteheart, S.W., 2004. Plasma membrane Ca(2+)-ATPase (PMCA) translocates to filopodia during platelet activation. *Thromb Haemost*, 91(2), 325-33.

Denis, M.M. et al., 2005. Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. *Cell*, 122(3), 379-91.

Doni, M.G., Cavallini, L. & Alexandre, A., 1994. Ca2+ influx in platelets: activation by thrombin and by the depletion of the stores. Effect of cyclic nucleotides. *The Biochemical Journal*, 303 (Pt 2), 599-605.

DUNHAM, E.T. & GLYNN, I.M., 1961. Adenosinetriphosphatase activity and the active movements of alkali metal ions. *The Journal of Physiology*, 156, 274-293.

Ebbeling, L. et al., 1992. Rapid ultrastructural changes in the dense tubular system following platelet activation. *Blood*, 80(3), 718-723.

Eberhardt, R.T. et al., 2000. Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. *The Journal of Clinical Investigation*, 106(4), 483-491.

El-Daher, S.S. et al., 2000. Distinct localization and function of (1,4,5)IP(3) receptor subtypes and the (1,3,4,5)IP(4) receptor GAP1(IP4BP) in highly purified human platelet membranes. *Blood*, 95(11), 3412-3422.

Enouf, J. et al., 1992. Human platelets express the SERCA2-b isoform of Ca(2+)-transport ATPase. *The Biochemical Journal*, 286 (Pt 1), 135-140.

Erdemir, F. et al., 2007. Clinical significance of platelet count in patients with renal cell carcinoma. *Urologia Internationalis*, 79(2), 111-116.

Evangelista, V. et al., 2006. De novo synthesis of cyclooxygenase-1 counteracts the suppression of platelet thromboxane biosynthesis by aspirin. *Circ Res*, 98(5), 593-5.

Falati, S. et al., 2002. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat Med*, 8(10), 1175-81.

Falck, J.R. et al., 2003. 11,12-epoxyeicosatrienoic acid (11,12-EET): structural determinants for inhibition of TNF-alpha-induced VCAM-1 expression. *Bioorganic & Medicinal Chemistry Letters*, 13(22), 4011-4014.

Fang, J. et al., 2005. Therapeutic expression of the platelet-specific integrin, alphaIIbbeta3, in a murine model for Glanzmann thrombasthenia. *Blood*, 106(8), 2671-2679.

Farndale, R.W. et al., 2004. The role of collagen in thrombosis and hemostasis. *Journal of Thrombosis and Haemostasis: JTH*, 2(4), 561-573.

Ferris, C.D. et al., 1991. Inositol trisphosphate receptor: phosphorylation by protein kinase C and calcium calmodulin-dependent protein kinases in reconstituted lipid vesicles. *Proceedings of the National Academy of Sciences of the United States of America*, 88(6), 2232-2235.

Fitzpatrick, F.A. et al., 1986. Inhibition of cyclooxygenase activity and platelet aggregation by epoxyeicosatrienoic acids. Influence of stereochemistry. *The Journal of Biological Chemistry*, 261(32), 15334-15338.

Fox, J.E., 2001. Cytoskeletal proteins and platelet signaling. *Thrombosis and Haemostasis*, 86(1), 198-213.

Freedman, J.E. et al., 1997. Nitric oxide released from activated platelets inhibits platelet recruitment. *J Clin Invest*, 100(2), 350-6.

Freedman, J.E. et al., 1999. Deficient platelet-derived nitric oxide and enhanced hemostasis in mice lacking the NOSIII gene. *Circ Res*, 84(12), 1416-21.

Gaarder, A. et al., 1961. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature*, 192, 531-2.

Gachet, C., 2001a. ADP receptors of platelets and their inhibition. *Thromb Haemost*, 86(1), 222-32.

Gachet, C., 2001b. Identification, characterization, and inhibition of the platelet ADP receptors. *Int J Hematol*, 74(4), 375-81.

Gachet, C., 2006. Regulation of platelet functions by P2 receptors. *Annu Rev Pharmacol Toxicol*, 46, 277-300.

Gambaryan, S. et al., 2008. NO-synthase-/NO-independent regulation of human and murine platelet soluble guanylyl cyclase activity. *J Thromb Haemost*, 6(8), 1376-84.

Gatto, C. et al., 1995. Eosin, a potent inhibitor of the plasma membrane Ca pump, does not inhibit the cardiac Na-Ca exchanger. *Biochemistry*, 34(3), 965-972.

Gatto, C. & Milanick, M.A., 1993. Inhibition of the red blood cell calcium pump by eosin and other fluorescein analogues. *The American Journal of Physiology*, 264(6 Pt 1), C1577-1586.

Gatto, C. & Milanick, M.A., 1993. Inhibition of the red blood cell calcium pump by eosin and other fluorescein analogues. *Am J Physiol*, 264(6 Pt 1), C1577-86.

Gawaz, M. et al., 2000. Platelets induce alterations of chemotactic and adhesive properties of endothelial cells mediated through an interleukin-1-dependent mechanism. Implications for atherogenesis. *Atherosclerosis*, 148(1), 75-85.

Gibbins, J., 2004. Platelet signalling and the regulation of thrombus formation.

Gibbins, J. et al., 1996. Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets. *J Biol Chem*, 271(30), 18095-9.

Gibbins, J.M., 2002. The negative regulation of platelet function: extending the role of the ITIM. *Trends Cardiovasc Med*, 12(5), 213-9.

Gibbins, J.M. et al., 1997. Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain. *FEBS Lett*, 413(2), 255-9.

Gill, A. et al., 1992. Pharmacology of bepridil. *The American Journal of Cardiology*, 69(11), 11D-16D.

Govindan, S., Taylor, E.J.A. & Taylor, C.W., 2010. Ca(2+) signalling by P2Y receptors in cultured rat aortic smooth muscle cells. *British Journal of Pharmacology*, 160(8), 1953-1962.

Greeb, J. & Shull, G.E., 1989. Molecular cloning of a third isoform of the calmodulin-sensitive plasma membrane Ca2+-transporting ATPase that is expressed predominantly in brain and skeletal muscle. *The Journal of Biological Chemistry*, 264(31), 18569-18576.

de Groot, P.G., IJsseldijk, M.J. & Sixma, J.J., 1999. Platelet adhesion to the subendothelium under flow. *Methods in Molecular Biology (Clifton, N.J.)*, 96, 159-170.

Grosse, J. et al., 2007. An EF hand mutation in Stim1 causes premature platelet activation and bleeding in mice. *The Journal of Clinical Investigation*, 117(11), 3540-3550.

Grover, A.K. & Samson, S.E., 1986. Pig coronary artery smooth muscle: substrate and pH dependence of the two calcium pumps. *The American Journal of Physiology*, 251(4 Pt 1), C529-534.

Gryglewski, R.J. et al., 1976. Arterial walls are protected against deposition of platelet thrombi by a substance (prostaglandin X) which they make from prostaglandin endoperoxides. *Prostaglandins*, 12(5), 685-713.

Guerini, D. et al., 1996. Mutation of conserved residues in transmembrane domains 4,6 and 8 causes loss of Ca2+ transport by the plasma membrane Ca2+ pump. *Biochemistry*, 35(10), 3290-3296.

Hagedorn, I., Vögtle, T. & Nieswandt, B., 2010. Arterial thrombus formation. Novel mechanisms and targets Novel mechanisms and targets. *Hämostaseologie*, 30(3), 127-135.

Hammes, A. et al., 1994. Differentiation-specific isoform mRNA expression of the calmodulin-dependent plasma membrane Ca(2+)-ATPase. *FASEB J*, 8(6), 428-35.

Harbeck, B. et al., 2000. Phosphorylation of the vasodilator-stimulated phosphoprotein regulates its interaction with actin. *The Journal of Biological Chemistry*, 275(40), 30817-30825.

Hardy, A.R. et al., 2005. P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood*, 105(9), 3552-3560.

Harper, A.G.S., Mason, M.J. & Sage, S.O., 2009. A key role for dense granule secretion in potentiation of the Ca2+ signal arising from store-operated calcium entry in human platelets. *Cell Calcium*, 45(5), 413-420.

Harper, A.G.S. & Sage, S.O., 2007. A key role for reverse Na+/Ca2+ exchange influenced by the actin cytoskeleton in store-operated Ca2+ entry in human platelets: evidence against the de novo conformational coupling hypothesis. *Cell Calcium*, 42(6), 606-617.

Harper, C. et al., 2005. Secretory pathway Ca(2+)-ATPase (SPCA1) Ca(2)+ pumps, not SERCAs, regulate complex [Ca(2+)](i) signals in human spermatozoa. *Journal of Cell Science*, 118(Pt 8), 1673-1685.

Harrison, P. & Cramer, E.M., 1993. Platelet alpha-granules. *Blood Rev*, 7(1), 52-62.

Hartley, P.S., 2007. Platelet senescence and death. *Clin Lab*, 53(3-4), 157-66.

Hartwig, J.H. & Italiano, J.E., 2006. Cytoskeletal mechanisms for platelet production. *Blood Cells Mol Dis*, 36(2), 99-103.

Hashimoto, T. et al., 2002. CLAC: a novel Alzheimer amyloid plaque component derived from a transmembrane precursor, CLAC-P/collagen type XXV. *The EMBO Journal*, 21(7), 1524-1534.

Hathaway, D.R. & Adelstein, R.S., 1979. Human platelet myosin light chain kinase requires the calcium-binding protein calmodulin for activity. *Proceedings of the National Academy of Sciences of the United States of America*, 76(4), 1653-1657.

Hawrylowicz, C.M., Howells, G.L. & Feldmann, M., 1991. Platelet-derived interleukin 1 induces human endothelial adhesion molecule expression and cytokine production. *The Journal of Experimental Medicine*, 174(4), 785-790.

Hechler, B. et al., 2010. Arterial thrombosis: relevance of a model with two levels of severity assessed by histologic, ultrastructural and functional characterization. *Journal of Thrombosis and Haemostasis: JTH*, 8(1), 173-184.

Hechler, B., Cattaneo, M. & Gachet, C., 2005. The P2 receptors in platelet function. *Semin Thromb Hemost*, 31(2), 150-61.

Heemskerk, J.W. et al., 1994. Indirect regulation of Ca2+ entry by cAMP-dependent and cGMP-dependent protein kinases and phospholipase C in rat platelets. *European Journal of Biochemistry / FEBS*, 223(2), 543-551.

Heinrich, A. et al., 2008. Modulation of neurotransmitter release by P2X and P2Y receptors in the rat spinal cord. *Neuropharmacology*, 54(2), 375-386.

Hers, I. et al., 2000. Evidence against a direct role of the integrin alpha2beta1 in collagen-induced tyrosine phosphorylation in human platelets. *Eur J Biochem*, 267(7), 2088-97.

Hirata, M. et al., 1991. Cloning and expression of cDNA for a human thromboxane A2 receptor. *Nature*, 349(6310), 617-620.

Hirata, T. et al., 1996. Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. *The Journal of Clinical Investigation*, 97(4), 949-956.

Hollopeter, G. et al., 2001. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature*, 409(6817), 202-7.

Holmsen, H., 1994. Significance of testing platelet functions in vitro. *Eur J Clin Invest*, 24 Suppl 1, 3-8.

Holton, M. et al., 2007. The interaction between endogenous calcineurin and the plasma membrane calcium-dependent ATPase is isoform specific in breast cancer cells. *FEBS Lett*, 581(21), 4115-9.

Homoncik, M. et al., 2000. Monitoring of aspirin (ASA) pharmacodynamics with the platelet function analyzer PFA-100. *Thrombosis and Haemostasis*, 83(2), 316-321.

Horstrup, K. et al., 1994. Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. *European Journal of Biochemistry / FEBS*, 225(1), 21-27.

HUME, R. et al., 1964. QUANTITATIVE OBSERVATIONS OF CIRCULATING MEGAKARYOCYTES IN THE BLOOD OF PATIENTS WITH CANCER. *The New England Journal of Medicine*, 270, 111-117.

Inoue, O. et al., 2003. Integrin alpha2beta1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLCgamma2. *J Cell Biol*, 160(5), 769-80.

Ishihara, H. et al., 1997. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature*, 386(6624), 502-6.

Italiano, J.E. et al., 1999. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol*, 147(6), 1299-312.

Italiano, J.E. & Shivdasani, R.A., 2003. Megakaryocytes and beyond: the birth of platelets. *J Thromb Haemost*, 1(6), 1174-82.

Jackson, T.R. et al., 1988. A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free Ca2+ without generation of inositol phosphates in NG115-401L neuronal cells. *The Biochemical Journal*, 253(1), 81-86.

Jardín, I. et al., 2006. Endogenously generated reactive oxygen species reduce PMCA activity in platelets from patients with non-insulin-dependent diabetes mellitus. *Platelets*, 17(5), 283-288.

Jefferson, B.K. et al., 2005. Aspirin resistance and a single gene. *The American Journal of Cardiology*, 95(6), 805-808.

Jin, J. & Kunapuli, S.P., 1998. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci U S A*, 95(14), 8070-4.

Joyce, J.A. & Pollard, J.W., 2009. Microenvironmental regulation of metastasis. *Nature Reviews. Cancer*, 9(4), 239-252.

Jung, S.M. & Moroi, M., 1998. Platelets interact with soluble and insoluble collagens through characteristically different reactions. *The Journal of biological chemistry*, 273(24), 14827-37.

Juska, A., 2010. Plasma membrane calcium pump and sodium-calcium exchanger in maintenance and control of calcium concentrations in platelets. *Biochemical and Biophysical Research Communications*, 392(1), 41-46.

Kahn, M.L. et al., 1998. A dual thrombin receptor system for platelet activation. *Nature*, 394(6694), 690-694.

Kahn, M.L. et al., 1999. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest*, 103(6), 879-87.

Kahn, M.L. et al., 1998. A dual thrombin receptor system for platelet activation. *Nature*, 394(6694), 690-4.

Kaplanski, G. et al., 1994. Interleukin-1 induces interleukin-8 secretion from endothelial cells by a juxtacrine mechanism. *Blood*, 84(12), 4242-4248.

Kass, G.E. et al., 1989. 2,5-Di-(tert-butyl)-1,4-benzohydroquinone rapidly elevates cytosolic Ca2+ concentration by mobilizing the inositol 1,4,5-trisphosphate-sensitive Ca2+ pool. *The Journal of Biological Chemistry*, 264(26), 15192-15198.

Kauffenstein, G. et al., 2010. The ecto-nucleotidase NTPDase1 differentially regulates P2Y1 and P2Y2 receptor-dependent vasorelaxation. *British Journal of Pharmacology*, 159(3), 576-585.

Kaushansky, K., 1995. Thrombopoietin: the primary regulator of platelet production. *Blood*, 86(2), 419-31.

Kawano, S. et al., 2003. Ca(2+) oscillations regulated by Na(+)-Ca(2+) exchanger and plasma membrane Ca(2+) pump induce fluctuations of membrane currents and potentials in human mesenchymal stem cells. *Cell Calcium*, 34(2), 145-56.

Kessler, P. et al., 1999. Proinflammatory mediators chronically downregulate the formation of the endothelium-derived hyperpolarizing factor in arteries via a nitric oxide/cyclic GMP-dependent mechanism. *Circulation*, 99(14), 1878-1884.

Khakh, B.S., 2001. Molecular physiology of P2X receptors and ATP signalling at synapses. *Nat Rev Neurosci*, 2(3), 165-74.

Khorana, A.A., 2003. Malignancy, thrombosis and Trousseau: the case for an eponym. *Journal of Thrombosis and Haemostasis: JTH*, 1(12), 2463-2465.

Khorana, A.A. & Connolly, G.C., 2009. Assessing risk of venous thromboembolism in the patient with cancer. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, 27(29), 4839-4847.

Kiedrowski, L. et al., 1994. Glutamate impairs neuronal calcium extrusion while reducing sodium gradient. *Neuron*, 12(2), 295-300.

King, S.M. & Reed, G.L., 2002. Development of platelet secretory granules. *Seminars in Cell & Developmental Biology*, 13(4), 293-302.

Klages, B. et al., 1999. Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol*, 144(4), 745-54.

Komalavilas, P. & Lincoln, T.M., 1994. Phosphorylation of the inositol 1,4,5-trisphosphate receptor by cyclic GMP-dependent protein kinase. *The Journal of Biological Chemistry*, 269(12), 8701-8707.

Kouvelas, D. et al., 2009. PDE5 inhibitors: in vitro and in vivo pharmacological profile. *Current Pharmaceutical Design*, 15(30), 3464-3475.

Krötz, F. et al., 2004. Membrane-potential-dependent inhibition of platelet adhesion to endothelial cells by epoxyeicosatrienoic acids. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 24(3), 595-600.

Kumar, T.S. et al., 2010. Structure-activity relationship of (N)-Methanocarba phosphonate analogues of 5'-AMP as cardioprotective agents acting through a cardiac P2X receptor. *Journal of Medicinal Chemistry*, 53(6), 2562-2576.

Kunicki, T.J. et al., 1988. The human fibroblast class II extracellular matrix receptor mediates platelet adhesion to collagen and is identical to the platelet glycoprotein Ia-IIa complex. *J Biol Chem*, 263(10), 4516-9.

Lagarde, M. et al., 2010. Fatty acid-derived lipid mediators and blood platelet aggregation. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, 82(4-6), 227-230.

Landesberg, R. et al., 2005. Activation of platelet-rich plasma using thrombin receptor agonist peptide. *Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons*, 63(4), 529-535.

Lau, W.C. et al., 2004. Contribution of hepatic cytochrome P450 3A4 metabolic activity to the phenomenon of clopidogrel resistance. *Circulation*, 109(2), 166-171.

Leger, A.J. et al., 2006. Blocking the protease-activated receptor 1-4 heterodimer in platelet-mediated thrombosis. *Circulation*, 113(9), 1244-54.

Lerner, D.J. et al., 1996. Agonist recognition by proteinase-activated receptor 2 and thrombin receptor. Importance of extracellular loop interactions for receptor function. *The Journal of Biological Chemistry*, 271(24), 13943-13947.

Leung, L.L. & Gibbs, C.S., 1997. Modulation of thrombin's procoagulant and anticoagulant properties. *Thrombosis and Haemostasis*, 78(1), 577-580.

Li, Y. et al., 2003. Effects of pulsatile shear stress on nitric oxide production and endothelial cell nitric oxide synthase expression by ovine fetoplacental artery endothelial cells. *Biol Reprod*, 69(3), 1053-9.

Li, Z. et al., 2003. A stimulatory role for cGMP-dependent protein kinase in platelet activation. *Cell*, 112(1), 77-86.

Lian, L. et al., 2005. The relative role of PLCbeta and PI3Kgamma in platelet activation. *Blood*, 106(1), 110-117.

Liang, W. et al., 2004. Vectorial Ca2+ release via ryanodine receptors contributes to Ca2+ extrusion from freshly isolated rabbit aortic endothelial cells. *Cell Calcium*, 36(5), 431-443.

Libby, P., 2000. Coronary artery injury and the biology of atherosclerosis: inflammation, thrombosis, and stabilization. *The American Journal of Cardiology*, 86(8B), 3J-8J; discussion 8J-9J.

Libby, P., Egan, D. & Skarlatos, S., 1997. Roles of infectious agents in atherosclerosis and restenosis: an assessment of the evidence and need for future research. *Circulation*, 96(11), 4095-4103.

Lipskaia, L. & Lompré, A., 2004. Alteration in temporal kinetics of Ca2+ signaling and control of growth and proliferation. *Biology of the Cell / Under the Auspices of the European Cell Biology Organization*, 96(1), 55-68.

Liu, Y. et al., 2005. The antiinflammatory effect of laminar flow: the role of PPARgamma, epoxyeicosatrienoic acids, and soluble epoxide hydrolase. *Proceedings of the National Academy of Sciences of the United States of America*, 102(46), 16747-16752.

London, F.S., Marcinkiewicz, M. & Walsh, P.N., 2006. PAR-1-stimulated factor IXa binding to a small platelet subpopulation requires a pronounced and sustained increase of cytoplasmic calcium. *Biochemistry*, 45(23), 7289-7298.

Lu, B. et al., 1998. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *The Journal of Experimental Medicine*, 187(4), 601-608.

Macchi, L. et al., 2003. Resistance in vitro to low-dose aspirin is associated with platelet PlA1 (GP IIIa) polymorphism but not with C807T(GP Ia/IIa) and C-5T Kozak (GP Ibalpha) polymorphisms. *Journal of the American College of Cardiology*, 42(6), 1115-1119.

Machin, M. et al., 2005. Characterization of platelet adhesion under flow using microscopic image sequence analysis. *The International Journal of Artificial Organs*, 28(7), 678-685.

Maree, A.O. et al., 2005. Platelet response to low-dose enteric-coated aspirin in patients with stable cardiovascular disease. *Journal of the American College of Cardiology*, 46(7), 1258-1263.

Martin, J.F. et al., 1982. The relationship between platelet and megakaryocyte volumes. *Thromb Res*, 28(4), 447-59.

Martin, V. et al., 2000. Platelet Ca(2+)ATPases : a plural, species-specific, and multiple hypertension-regulated expression system. *Hypertension*, 35(1 Pt 1), 91-102.

Marumo, M. & Wakabayashi, I., 2010. Diverse effects of ethanol on Ca(2+) entry and subsequent aggregation of platelets. *Alcohol (Fayetteville, N.Y.)*, 44(4), 343-350.

May, A.E. et al., 2002. Urokinase receptor surface expression regulates monocyte adhesion in acute myocardial infarction. *Blood*, 100(10), 3611-3617.

McCarthy, M.W. & Kockler, D.R., 2003. Clopidogrel-associated leukopenia. *The Annals of Pharmacotherapy*, 37(2), 216-219.

Meldolesi, J. et al., 1991. Ca2+ influx following receptor activation. *Trends in Pharmacological Sciences*, 12(8), 289-292.

Mercer, J.C. et al., 2006. Large store-operated calcium selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor, Stim1. *The Journal of Biological Chemistry*, 281(34), 24979-24990.

Meyer, B., Staudinger, T. & Lechner, K., 2001. Clopidogrel and aplastic anaemia. *Lancet*, 357(9266), 1446-1447.

Mitchell, J.A. et al., 2006. Stronger inhibition by nonsteroid anti-inflammatory drugs of cyclooxygenase-1 in endothelial cells than platelets offers an explanation for increased risk of thrombotic events. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 20(14), 2468-2475.

Moake, J.L. et al., 1988. Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. *Blood*, 71(5), 1366-74.

Moccia, F. et al., 2002. Ca2+ uptake by the endoplasmic reticulum Ca2+-ATPase in rat microvascular endothelial cells. *The Biochemical Journal*, 364(Pt 1), 235-244.

Moncada, S., Higgs, E.A. & Vane, J.R., 1977. Human arterial and venous tissues generate prostacyclin (prostaglandin x), a potent inhibitor of platelet aggregation. *Lancet*, 1(8001), 18-20.

Moncada, S. et al., 1976. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, 263(5579), 663-5.

Moore, G.A. et al., 1987. 2,5-Di(tert-butyl)-1,4-benzohydroquinone--a novel inhibitor of liver microsomal Ca2+ sequestration. *FEBS Letters*, 224(2), 331-336.

Morel, D.W., Hessler, J.R. & Chisolm, G.M., 1983. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *Journal of Lipid Research*, 24(8), 1070-1076.

Moroi, M. et al., 1997. Analysis of the involvement of the von Willebrand factor-glycoprotein Ib interaction in platelet adhesion to a collagen-coated surface under flow conditions. *Blood*, 90(11), 4413-4424.

Mullis, K.B. & Faloona, F.A., 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol*, 155, 335-50.

Murakami, M. et al., 2010. Efficacy of Low-dose Bepridil for Prevention of Ventricular Fibrillation in Brugada Syndrome Patients with and without SCN5A Mutation. *Journal of Cardiovascular Pharmacology*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20625312 [Accessed August 14, 2010].

Murthy, M. et al., 1995. Influx of extracellular calcium and agonist-coupling appear essential for the activation of thromboxane A2-dependent phospholipase A2 in human platelets. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, 53(1), 31-39.

Nakanishi-Matsui, M. et al., 2000. PAR3 is a cofactor for PAR4 activation by thrombin. *Nature*, 404(6778), 609-13.

Naseem, K.M., 2008. eNOS, iNOS or no NOS, that is the question! *Journal of Thrombosis and Haemostasis: JTH*, 6(8), 1373-1375.

Naseem, K.M. & Riba, R., 2008. Unresolved roles of platelet nitric oxide synthase. *J Thromb Haemost*, 6(1), 10-9.

Nawada, T. et al., 1995. Mechanism of inhibition of the sodium current by bepridil in guinea-pig isolated ventricular cells. *British Journal of Pharmacology*, 116(2), 1775-1780.

Neumann, F.J. et al., 1997. Induction of cytokine expression in leukocytes by binding of thrombin-stimulated platelets. *Circulation*, 95(10), 2387-2394.

Ng, L.C. et al., 2009. TRPC1 and STIM1 mediate capacitative Ca2+ entry in mouse pulmonary arterial smooth muscle cells. *The Journal of Physiology*, 587(Pt 11), 2429-2442.

Nieswandt, B. & Watson, S.P., 2003. Platelet-collagen interaction: is GPVI the central receptor? *Blood*, 102(2), 449-461.

Niggli, V., Penniston, J.T. & Carafoli, E., 1979. Purification of the (Ca2+-Mg2+)-ATPase from human erythrocyte membranes using a calmodulin affinity column. *The Journal of Biological Chemistry*, 254(20), 9955-9958.

Node, K. et al., 1999. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science (New York, N.Y.)*, 285(5431), 1276-1279.

Node, K. et al., 2001. Activation of Galpha s mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids. *The Journal of Biological Chemistry*, 276(19), 15983-15989.

Nurden, A.T., 2005. Qualitative disorders of platelets and megakaryocytes. *J Thromb Haemost*, 3(8), 1773-82.

Nystedt, S. et al., 1994. Molecular cloning of a potential proteinase activated receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 91(20), 9208-12.

Oberprieler, N.G., Roberts, W., Graham, A.M. et al., 2007. Inhibition of ADP-induced platelet adhesion to immobilised fibrinogen by nitric oxide: evidence for cGMP-independent mechanisms. *Biochemical Pharmacology*, 73(10), 1593-1601.

Oberprieler, N.G., Roberts, W., Riba, R. et al., 2007. cGMP-independent inhibition of integrin alphaIIbbeta3-mediated platelet adhesion and outside-in signalling by nitric oxide. *FEBS Letters*, 581(7), 1529-1534.

Oceandy, D., Cartwright, E.J. et al., 2007. Neuronal nitric oxide synthase signaling in the heart is regulated by the sarcolemmal calcium pump 4b. *Circulation*, 115(4), 483-92.

Oceandy, D., Stanley, P.J. et al., 2007. The regulatory function of plasma-membrane Ca(2+)-ATPase (PMCA) in the heart. *Biochem Soc Trans*, 35(Pt 5), 927-30.

Ogan, J.T. et al., 2007. Kinetic characterization of Na,K-ATPase inhibition by Eosin. *Blood Cells, Molecules & Diseases*, 38(3), 229-237.

Okunade, G.W. et al., 2004. Targeted ablation of plasma membrane Ca2+-ATPase (PMCA) 1 and 4 indicates a major housekeeping function for PMCA1 and a critical role in hyperactivated sperm motility and male fertility for PMCA4. *The Journal of Biological Chemistry*, 279(32), 33742-33750.

Ordinas, A. et al., 1990. [Update on the role of vascular, plasmatic and cellular components in the regulation of the interaction of platelets with the subendothelium]. *Sangre*, 35(6), 425-432.

Orrenius, S., Zhivotovsky, B. & Nicotera, P., 2003. Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol*, 4(7), 552-65.

Osdoit, S. & Rosa, J.P., 2001. Fibrin clot retraction by human platelets correlates with alpha(IIb)beta(3) integrin-dependent protein tyrosine dephosphorylation. *The Journal of Biological Chemistry*, 276(9), 6703-6710.

Oury, C. et al., 2001. The ATP-gated P2X1 ion channel acts as a positive regulator of platelet responses to collagen. *Thromb Haemost*, 86(5), 1264-71.

Pande, J. et al., 2006. Aortic smooth muscle and endothelial plasma membrane Ca2+ pump isoforms are inhibited differently by the extracellular inhibitor caloxin 1b1. *Am J Physiol Cell Physiol*, 290(5), C1341-9.

Pang, Y. et al., 2005. The characterization of plasma membrane Ca2+-ATPase in rich sphingomyelin-cholesterol domains. *FEBS Lett*, 579(11), 2397-403.

Papp, B. et al., 1993. Spontaneously hypertensive rats and platelet Ca(2+)-ATPases: specific up-regulation of the 97 kDa isoform. *The Biochemical Journal*, 295 (Pt 3), 685-690.

Papp, B. et al., 1991. Demonstration of two forms of calcium pumps by thapsigargin inhibition and radioimmunoblotting in platelet membrane vesicles. *The Journal of Biological Chemistry*, 266(22), 14593-14596.

Papp, B. et al., 1992. Simultaneous presence of two distinct endoplasmic-reticulum-type calcium-pump isoforms in human cells. Characterization by radio-immunoblotting and inhibition by 2,5-di-(t-butyl)-1,4-benzohydroquinone. *The Biochemical Journal*, 288 (Pt 1), 297-302.

Parekh, A.B. & Penner, R., 1997. Store depletion and calcium influx. *Physiological Reviews*, 77(4), 901-930.

Pászty, K. et al., 2007. Cleavage of the plasma membrane Ca+ATPase during apoptosis. *Annals of the New York Academy of Sciences*, 1099, 440-450.

Patel, S.R., Hartwig, J.H. & Italiano, J.E., 2005. The biogenesis of platelets from megakaryocyte proplatelets. *The Journal of Clinical Investigation*, 115(12), 3348-3354.

Patrono, C. et al., 2001. Platelet-active drugs : the relationships among dose, effectiveness, and side effects. *Chest*, 119(1 Suppl), 39S-63S.

Pontiggia, L. et al., 2002. Increased platelet-collagen interaction associated with double homozygosity for receptor polymorphisms of platelet GPIa and GPIIIa. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 22(12), 2093-2098.

Poole, A. et al., 1997. The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *Embo J*, 16(9), 2333-41.

Prehoda, K.S.S., Lee, D.J. & Lim, W.A., 1999. Structure of the enabled VASP homology 1 domain-peptide complex: A key component in the spatial control of actin assembly. *Cell*, 97, 417-480.

Putney, J.W., 1990. Capacitative calcium entry revisited. *Cell Calcium*, 11(10), 611-624.

Putney, J.W. et al., 1986. Formation and biological action of inositol 1,4,5-trisphosphate. *Federation Proceedings*, 45(11), 2634-2638.

Quek, L.S. et al., 2000. Fyn and Lyn phosphorylate the Fc receptor gamma chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway. *Blood*, 96(13), 4246-4253.

Quinn, M.J. & Fitzgerald, D.J., 1999. Ticlopidine and clopidogrel. *Circulation*, 100(15), 1667-1672.

Quinn, M.J., Plow, E.F. & Topol, E.J., 2002. Platelet glycoprotein IIb/IIIa inhibitors: recognition of a two-edged sword? *Circulation*, 106(3), 379-85.

Radomski, M.W., Palmer, R.M. & Moncada, S., 1990. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc Natl Acad Sci U S A*, 87(13), 5193-7.

Radomski, M.W., Palmer, R.M. & Moncada, S., 1987. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet*, 2(8567), 1057-8.

Randriamboavonjy, V. & Fleming, I., 2005. Endothelial nitric oxide synthase (eNOS) in platelets: how is it regulated and what is it doing there? *Pharmacol Rep*, 57 Suppl, 59-65.

Redondo, P.C. et al., 2005. Collaborative effect of SERCA and PMCA in cytosolic calcium homeostasis in human platelets. *Journal of Physiology and Biochemistry*, 61(4), 507-516.

Redondo, P.C. et al., 2008. SERCA2b and 3 play a regulatory role in store-operated calcium entry in human platelets. *Cell Signal*, 20(2), 337-46.

Remijn, J.A. et al., 2002. Role of ADP receptor P2Y(12) in platelet adhesion and thrombus formation in flowing blood. *Arterioscler Thromb Vasc Biol*, 22(4), 686-91.

Rhee, S.G. & Bae, Y.S., 1997. Regulation of phosphoinositide-specific phospholipase C isozymes. *The Journal of Biological Chemistry*, 272(24), 15045-15048.

Riba, R. et al., 2006. Von Willebrand factor activates endothelial nitric oxide synthase in blood platelets by a glycoprotein Ib-dependent mechanism. *Journal of Thrombosis and Haemostasis: JTH*, 4(12), 2636-2644.

Roberts, W. et al., 2009. Nitric oxide inhibits von Willebrand factor-mediated platelet adhesion and spreading through regulation of integrin alpha(IIb)beta(3) and myosin light chain. *Journal of Thrombosis and Haemostasis: JTH*, 7(12), 2106-2115.

Roberts, W. et al., 2008. Nitric oxide specifically inhibits integrin-mediated platelet adhesion and spreading on collagen. *Journal of Thrombosis and Haemostasis: JTH*, 6(12), 2175-2185.

Rosado, J.A., Jenner, S. & Sage, S.O., 2000. A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Evidence for conformational coupling. *J Biol Chem*, 275(11), 7527-33.

Rosado, J.A. & Sage, S.O., 2000. Regulation of plasma membrane Ca2+-ATPase by small GTPases and phosphoinositides in human platelets. *J Biol Chem*, 275(26), 19529-35.

Rudnick, A., Modai, I. & Zelikovski, A., 1997. Fluoxetine-induced Raynaud's phenomenon. *Biol Psychiatry*, 41(12), 1218-21.

Sage, S.O., 1997. The Wellcome Prize Lecture. Calcium entry mechanisms in human platelets. *Exp Physiol*, 82(5), 807-23.

Salvador, J.M. et al., 1998. Ca2+ transport by reconstituted synaptosomal ATPase is associated with H+ countertransport and net charge displacement. *The Journal of Biological Chemistry*, 273(29), 18230-18234.

Savage, B., Almus-Jacobs, F. & Ruggeri, Z.M., 1998. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell*, 94(5), 657-666.

Savage, B., Cattaneo, M. & Ruggeri, Z.M., 2001. Mechanisms of platelet aggregation. *Curr Opin Hematol*, 8(5), 270-6.

Savage, B., Ginsberg, M.H. & Ruggeri, Z.M., 1999. Influence of fibrillar collagen structure on the mechanisms of platelet thrombus formation under flow. *Blood*, 94(8), 2704-15.

Schatzmann, H.J., 1966. ATP-dependent Ca++-extrusion from human red cells. *Experientia*, 22(6), 364-5.

Schnetkamp, P.P., 1989. Na-Ca or Na-Ca-K exchange in rod photoreceptors. *Progress in Biophysics and Molecular Biology*, 54(1), 1-29.

Schuh, K. et al., 2004. Plasma membrane Ca2+ ATPase 4 is required for sperm motility and male fertility. *J Biol Chem*, 279(27), 28220-6.

Schuh, K. et al., 2003. Regulation of vascular tone in animals overexpressing the sarcolemmal calcium pump. *J Biol Chem*, 278(42), 41246-52.

Schuh, K. et al., 2001. The plasmamembrane calmodulin-dependent calcium pump: a major regulator of nitric oxide synthase I. *J Cell Biol*, 155(2), 201-5.

Serhan, C.N. et al., 2002. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *The Journal of Experimental Medicine*, 196(8), 1025-1037.

Sharma, G.K. & Talbot, I.C., 1986. Pulmonary megakaryocytes: "missing link" between cardiovascular and respiratory disease? *Journal of Clinical Pathology*, 39(9), 969-976.

Shattil, S.J. & Brass, L.F., 1987. Induction of the fibrinogen receptor on human platelets by intracellular mediators. *The Journal of Biological Chemistry*, 262(3), 992-1000.

Shenker, A. et al., 1991. The G protein coupled to the thromboxane A2 receptor in human platelets is a member of the novel Gq family. *The Journal of biological chemistry*, 266(14), 9309-13.

Shi, Y. et al., 2009. Lanthanum chloride suppresses hydrogen peroxide-enhanced calcification in rat calcifying vascular cells. *Biometals: An International Journal on the Role of Metal Ions in Biology, Biochemistry, and Medicine*, 22(2), 317-327.

Shiraga, M. et al., 1996. Affinity modulation of the platelet integrin alpha IIb beta 3 by alpha-chymotrypsin: a possible role for Na+/Ca2+ exchanger. *Blood*, 88(7), 2594-2602.

Shiraga, M. et al., 1998. Involvement of Na+/Ca2+ exchanger in inside-out signaling through the platelet integrin IIbbeta3. *Blood*, 92(10), 3710-3720.

Shull, G.E. & Greeb, J., 1988. Molecular cloning of two isoforms of the plasma membrane Ca2+-transporting ATPase from rat brain. Structural and functional domains exhibit similarity to Na+,K+- and other cation transport ATPases. *The Journal of Biological Chemistry*, 263(18), 8646-8657.

Sierko, E. & Wojtukiewicz, M.Z., 2007. Inhibition of platelet function: does it offer a chance of better cancer progression control? *Seminars in Thrombosis and Hemostasis*, 33(7), 712-721.

Sierko, E. & Wojtukiewicz, M.Z., 2004. Platelets and angiogenesis in malignancy. *Seminars in Thrombosis and Hemostasis*, 30(1), 95-108.

Soffer, D. et al., 2003. Impact of angina class on inhibition of platelet aggregation following clopidogrel loading in patients undergoing coronary intervention: do we need more aggressive dosing regimens in unstable angina? *Catheterization and Cardiovascular Interventions: Official Journal of the Society for Cardiac Angiography & Interventions*, 59(1), 21-25.

Stauffer, T.P. et al., 1994. Quantitative analysis of alternative splicing options of human plasma membrane calcium pump genes. *The Journal of Biological Chemistry*, 269(50), 32022.

Stevens, J.M. et al., 2004. The regulation of integrin-linked kinase in human platelets: evidence for involvement in the regulation of integrin alpha 2 beta 1. *J Thromb Haemost*, 2(8), 1443-52.

Strehler, E.E. & Zacharias, D.A., 2001. Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. *Physiological Reviews*, 81(1), 21-50.

Strehler, E.E. & Treiman, M., 2004. Calcium pumps of plasma membrane and cell interior. *Current Molecular Medicine*, 4(3), 323-335.

Stuart, M.J., 1975. Inherited defects of platelet function. *Semin Hematol*, 12(3), 233-53.

Sudo, T., Ito, H. & Kimura, Y., 2003. Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) by the anti-platelet drug, cilostazol, in platelets. *Platelets*, 14(6), 381-390.

Sugi, K., 2006. [Strategy for cardiac arrhythmias in acute coronary syndrome]. *Nippon Rinsho. Japanese Journal of Clinical Medicine*, 64(4), 729-733.

Supattapone, S. et al., 1988. Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. *Proceedings of the National Academy of Sciences of the United States of America*, 85(22), 8747-8750.

Suzuki-Inoue, K. et al., 2002. Association of Fyn and Lyn with the proline-rich domain of glycoprotein VI regulates intracellular signaling. *The Journal of Biological Chemistry*, 277(24), 21561-21566.

Tamura, N. et al., 2007. The PDZ-LIM protein CLP36 is required for actin stress fiber formation and focal adhesion assembly in BeWo cells. *Biochemical and Biophysical Research Communications*, 364(3), 589-594.

Taubert, D. et al., 2004. Pharmacokinetics of clopidogrel after administration of a high loading dose. *Thrombosis and Haemostasis*, 92(2), 311-316.

Taucher, S. et al., 2003. Impact of pretreatment thrombocytosis on survival in primary breast cancer. *Thrombosis and Haemostasis*, 89(6), 1098-1106.

Teijeiro, R.G. et al., 1999. Calcium efflux from platelet vesicles of the dense tubular system. Analysis of the possible contribution of the Ca2+ pump. *Molecular and Cellular Biochemistry*, 199(1-2), 7-14.

Tempel, B.L. & Shilling, D.J., 2007. The plasma membrane calcium ATPase and disease. *Sub-Cellular Biochemistry*, 45, 365-383.

Thastrup, O. et al., 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. *Proceedings of the National Academy of Sciences of the United States of America*, 87(7), 2466-2470.

Toescu, E.C. & Petersen, O.H., 1995. Region-specific activity of the plasma membrane Ca2+ pump and delayed activation of Ca2+ entry characterize the polarized, agonist-evoked Ca2+ signals in exocrine cells. *J Biol Chem*, 270(15), 8528-35.

Trepakova, E.S., Cohen, R.A. & Bolotina, V.M., 1999. Nitric Oxide Inhibits Capacitative Cation Influx in Human Platelets by Promoting Sarcoplasmic/Endoplasmic Reticulum Ca2+-ATPase•Dependent Refilling of Ca2+ Stores. *Circ Res*, 84(2), 201-209.

Trivier, J.M. et al., 2001. Fatal aplastic anaemia associated with clopidogrel. *Lancet*, 357(9254), 446.

Turner, N.A., Moake, J.L. & McIntire, L., 2001. Blockade of adenosine diphosphate receptors P2Y12 and P2Y1 is required to inhibit platelet aggregation in whole blood under flow. *Blood*, 98, 3340-3345.

Tymvios, C. et al., 2009. Platelet aggregation responses are critically regulated in vivo by endogenous nitric oxide but not by endothelial nitric oxide synthase. *British Journal of Pharmacology*, 158(7), 1735-1742.

Tymvios, C. et al., 2008. Real-time measurement of non-lethal platelet thromboembolic responses in the anaesthetized mouse. *Thromb Haemost*, 99(2), 435-40.

Vale-González, C. et al., 2006. Role of the plasma membrane calcium adenosine triphosphatase on domoate-induced intracellular acidification in primary cultures of cerebelar granule cells. *Journal of Neuroscience Research*, 84(2), 326-337.

Vanderkooi, J.M. et al., 1987. An optical method for measurement of dioxygen concentration based upon quenching of phosphorescence. *The Journal of Biological Chemistry*, 262(12), 5476-5482.

Verheul, H.M. et al., 2000. Platelet and coagulation activation with vascular endothelial growth factor generation in soft tissue sarcomas. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, 6(1), 166-171.

Verma, A.K. et al., 1988. Complete primary structure of a human plasma membrane Ca2+ pump. *The Journal of Biological Chemistry*, 263(28), 14152-14159.

Verrall, S. et al., 1997. The thrombin receptor second cytoplasmic loop confers coupling to Gq-like G proteins in chimeric receptors. Additional evidence for a common transmembrane signaling and G protein coupling mechanism in G protein-coupled receptors. *The Journal of Biological Chemistry*, 272(11), 6898-6902.

Vostal, J.G., Jackson, W.L. & Shulman, N.R., 1991. Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins. *The Journal of Biological Chemistry*, 266(25), 16911-16916.

Vu, T.K. et al., 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*, 64(6), 1057-68.

Wagner, C.L. et al., 1996. Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood*, 88(3), 907-14.

Wan, T.C., Zabe, M. & Dean, W.L., 2003. Plasma membrane Ca2+-ATPase isoform 4b is phosphorylated on tyrosine 1176 in activated human platelets. *Thromb Haemost*, 89(1), 122-31.

Watanabe, Y., Koide, Y. & Kimura, J., 2006. Topics on the Na+/Ca2+ exchanger: pharmacological characterization of Na+/Ca2+ exchanger inhibitors. *Journal of Pharmacological Sciences*, 102(1), 7-16.

Watson, S.P. et al., 2001. The role of ITAM- and ITIM-coupled receptors in platelet activation by collagen. *Thrombosis and Haemostasis*, 86(1), 276-288.

Webb, T.E. et al., 1993. Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Letters*, 324(2), 219-225.

Weber, A.A. et al., 1999. Cyclooxygenase-2 in human platelets as a possible factor in aspirin resistance. *Lancet*, 353(9156), 900.

Werner, G. & Morgenstern, E., 1980. Three-dimensional reconstruction of human blood platelets using serial sections. *Eur J Cell Biol*, 20(3), 276-82.

Weyrich, A.S. et al., 1996. Activated platelets signal chemokine synthesis by human monocytes. *The Journal of Clinical Investigation*, 97(6), 1525-1534.

White, J.G. & Escolar, G., 1991. The blood platelet open canalicular system: a two-way street. *European Journal of Cell Biology*, 56(2), 233-242.

White, J.G., 1979. Ultrastructural studies of the gray platelet syndrome. *Am J Pathol*, 95(2), 445-62.

Wictome, M., Henderson, I. et al., 1992. Mechanism of inhibition of the calcium pump of sarcoplasmic reticulum by thapsigargin. *The Biochemical Journal*, 283 (Pt 2), 525-529.

Wictome, M., Michelangeli, F. et al., 1992. The inhibitors thapsigargin and 2,5-di(tert-butyl)-1,4-benzohydroquinone favour the E2 form of the Ca2+,Mg(2+)-ATPase. *FEBS Letters*, 304(2-3), 109-113.

Williams, J.C. et al., 2006. The sarcolemmal calcium pump, alpha-1 syntrophin, and neuronal nitric-oxide synthase are parts of a macromolecular protein complex. *J Biol Chem*, 281(33), 23341-8.

Wuytack, F. et al., 1994. A sarco/endoplasmic reticulum Ca(2+)-ATPase 3-type Ca2+ pump is expressed in platelets, in lymphoid cells, and in mast cells. *The Journal of Biological Chemistry*, 269(2), 1410-1416.

Wuytack, F., Raeymaekers, L. & Missiaen, L., 2002. Molecular physiology of the SERCA and SPCA pumps. *Cell Calcium*, 32(5-6), 279-305.

Xu, W.F. et al., 1998. Cloning and characterization of human protease-activated receptor 4. *Proceedings of the National Academy of Sciences of the United States of America*, 95(12), 6642-6646.

Yamamoto, T. et al., 2010. Gastroduodenal mucosal injury in patients taking low-dose aspirin and the role of gastric mucoprotective drugs: possible effect of rebamipide. *Journal of Clinical Biochemistry and Nutrition*, 47(1), 27-31.

Yanaga, F. et al., 1995. Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fc gamma-IIA receptor. *The Biochemical Journal*, 311 (Pt 2), 471-478.

Yanagida, E. et al., 2004. Functional expression of Ca2+ signaling pathways in mouse embryonic stem cells. *Cell Calcium*, 36(2), 135-146.

Zabe, M. & Dean, W.L., 2001. Plasma membrane Ca(2+)-ATPase associates with the cytoskeleton in activated platelets through a PDZ-binding domain. *The Journal of Biological Chemistry*, 276(18), 14704-14709.

Zabe, M. & Dean, W.L., 2001. Plasma membrane Ca(2+)-ATPase associates with the cytoskeleton in activated platelets through a PDZ-binding domain. *J Biol Chem*, 276(18), 14704-9.

Zhang, Q. et al., 2005. Cyclic GMP signaling and regulation of SERCA activity during cardiac myocyte contraction. *Cell Calcium*, 37(3), 259-266.

Zimlichman, R. et al., 1987. Effects of ouabain on cytosolic calcium in lymphocytes, platelets and adrenomedullary cells. *Journal of Hypertension*, 5(5), 605-609.

Zimmermann, N. et al., 2003. Functional and biochemical evaluation of platelet aspirin resistance after coronary artery bypass surgery. *Circulation*, 108(5), 542-547.

Zutter, M.M. & Santoro, S.A., 1990. Widespread histologic distribution of the alpha 2 beta 1 integrin cell-surface collagen receptor. *The American Journal of Pathology*, 137(1), 113-120.

APPENDIX

Journal of Thrombosis and Haemostasis, **8**: 2766–2774 DOI: 10.1111/j.1538-7836.2010.04076.x

ORIGINAL ARTICLE

The plasma membrane calcium ATPase modulates calcium homeostasis, intracellular signaling events and function in platelets

S. JONES,*[1] A. SOLOMON,*[1] D. SANZ-ROSA,† C. MOORE,* L. HOLBROOK,* E. J. CARTWRIGHT,‡ L. NEYSES‡ and M. EMERSON*

**Platelet Biology Group, Molecular Medicine Section, National Heart and Lung Institute, Imperial College London, London, UK; †Atherothrombosis and Cardiovascular Imaging Department, Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain; and ‡School of Biomedicine, Manchester Academic Health Sciences Centre, University of Manchester, Manchester, UK*

To cite this article: Jones S, Solomon A, Sanz-Rosa D, Moore C, Holbrook L, Cartwright EJ, Neyses L, Emerson M. The plasma membrane calcium ATPase modulates calcium homeostasis, intracellular signaling events and function in platelets. *J Thromb Haemost* 2010; **8**: 2766–74.

Summary. *Background:* The plasma membrane calcium ATPase (PMCA) regulates localized signaling events in a variety of cell types, although its functional role in platelets remains undefined. *Objectives:* To investigate the role of PMCA in determining platelet intracellular calcium concentration ($[Ca^{2+}]_i$) at rest and following agonist stimulation, and to define the corresponding effects upon different stages of platelet activation. *Methods:* $[Ca^{2+}]_i$ was continuously measured in Fura-2-loaded platelets and *in vitro* and *in vivo* functional analyses performed in the presence of the PMCA inhibitor carboxyeosin (CE). *Results:* Concentrations of CE that selectively inhibited Ca^{2+} extrusion through PMCA were established in human platelets. $[Ca^{2+}]_i$ was elevated by CE in resting platelets, although collagen-stimulated Ca^{2+} release was reduced. Impaired Ca^{2+} mobilization upon agonist stimulation was accompanied by reduced dense granule secretion and impaired platelet aggregation. Platelet aggregation responses were also reduced in PMCA4$^{-/-}$ mice and in an *in vivo* mouse model of platelet thromboembolism. Conversely, inhibition of PMCA promoted the early and later stages of platelet activation, observed as enhanced adhesion to fibrinogen, and accelerated clot retraction. Investigations into the signaling mechanisms underlying CE-mediated inhibition of platelet aggregation implicated cGMP-independent vasodilator-stimulated phosphoprotein phosphorylation. *Conclusions:* Disruption of PMCA activity perturbs platelet Ca^{2+} homeostasis and function in a time-dependent manner, demonstrating that PMCA differentially regulates Ca^{2+}-dependent signaling events, and hence function, throughout the platelet activation process.

Keywords: calcium, pharmacology, platelet, signaling, thrombosis.

Correspondence: Michael Emerson, Molecular Medicine Section, National Heart and Lung Institute, Imperial College London, Sir Alexander Fleming Building, Exhibition Road, London, SW7 2AZ, UK.
Tel.: +44 207 5941877; fax: +44 207 5943100.
E-mail: m.emerson@imperial.ac.uk

[1]These authors contributed equally to this work.

Received 22 October 2009, accepted 9 September 2010

Introduction

An increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and subsequent stimulation of localized Ca^{2+}-dependent signaling processes is central to platelet activation. Platelet Ca^{2+} homeostasis is therefore tightly regulated to maintain low resting Ca^{2+} levels, which rise with spatiotemporal specificity upon activation [1].

The plasma membrane calcium ATPase (PMCA) has been described as the predominant route of Ca^{2+} extrusion across the platelet plasma membrane [2], although there is increasing evidence of a functional role of the Na^+/Ca^{2+} exchanger. Na^+/Ca^{2+} exchange has been shown to regulate basal $[Ca^{2+}]_i$ [3] and, in reverse mode, can increase $[Ca^{2+}]_i$ and promote platelet aggregation [4,5] by influencing cytoskeletal function [5] and dense granule secretion [6]. Thus, the distinct role of Ca^{2+} extrusion through PMCA in regulating Ca^{2+} homeostasis and function in platelets remains unclear.

PMCAs are encoded by four independent genes. Alternative splicing of the primary constructs of these genes results in approximately 30 PMCA isoforms [7]. Platelets express PMCA4b and, to a lesser extent, PMCA1b [8]. Traditionally, PMCA has been thought to play a 'housekeeping' role in controlling basal Ca^{2+} levels [9], but more recently has been shown to act as a versatile signaling molecule [10], for example by undergoing interactions with PDZ-domain-containing proteins such as neuronal nitric oxide (NO) synthase (nNOS)

to produce functionally important effects, including changes in contractile function in the heart [11].

In platelets, PMCA interacts with the actin cytoskeleton and localizes to the filopodal region during activation [12], suggesting roles that are temporally and spatially dependent. The roles of PMCA in regulating $[Ca^{2+}]_i$ and function during the various stages of platelet activation are not known. In the present study, the role of PMCA in regulating platelet $[Ca^{2+}]_i$ and function was investigated with the PMCA inhibitor carboxyeosin (CE). We established concentrations of CE that inhibited Ca^{2+} extrusion through PMCA in platelets, and confirmed that CE acted selectively at PMCA. The consequences of PMCA inhibition for Ca^{2+} homeostasis and platelet function were then determined, and the validity of our functional observations was confirmed with parallel experiments in PMCA4 knockout ($PMCA4^{-/-}$) mice. Mechanistic studies were initiated as a first step in elucidating the pathways linking PMCA activity with function in platelets.

Materials and methods

Materials

Materials used were as follows: [^{3}H]5-hydroxytryptamine (5-HT) and indium-111 oxine (GE Healthcare, Amersham, UK); collagen (Nycomed, Munich, Germany); CE and *p*-nitrophenyl phosphate (Invitrogen, Paisley, UK); anti-vasodilator-stimulated phosphoprotein (VASP) and anti-phospho-VASP (Ser239) (New England Biolabs, Hitchin, UK). All other materials were purchased from Sigma-Aldrich (Poole, UK). Tyrodes–Hepes buffer contained 134 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 20 mM Hepes, 5 mM glucose and 1 mM $MgCl_2$ (pH 7.3).

Platelet preparation

Blood was taken from aspirin-free human volunteers or terminally anesthetized mice, and washed platelets were prepared by differential centrifugation as previously described [13,14]. Informed consent from all blood donors was obtained, and procedures were approved by the National Research Ethics Service.

$[Ca^{2+}]_i$ measurements

Human platelets were loaded with Fura-2 by incubation with 5 μM Fura-2/AM, and washed in modified Tyrodes–Hepes buffer before resuspension to a density of approximately 4×10^8 cells mL^{-1}. Platelets were incubated with CE for 30 min, and $[Ca^{2+}]_i$ measurements were made with an LS50B fluorescence spectrometer (Perkin Elmer, Cambridge, UK) in platelets stimulated with collagen (5 μg mL^{-1}) or thapsigargin (1 μM) plus ionomycin (50 nM) or in resting platelets. Unless otherwise stated, Ca^{2+} analyses were conducted in Ca^{2+}-free buffer in the presence of 100 μM EGTA.

Platelet aggregation

Platelets were incubated for 30 min with CE (10–40 μM) or dimethylsulfoxide (DMSO) (0.4%) prior to stimulation with collagen (5 μg mL^{-1}) or thrombin (0.1 U mL^{-1}), and aggregation was measured at 37 °C under stirring conditions in an optical aggregometer (Chrono-log Corporation, Havertown, PA, USA). When sodium nitroprusside (SNP) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were used, platelets were incubated with these compounds for 60 s prior to stimulation. Mouse studies were conducted as above by isolating platelets from pooled groups of wild-type and $PMCA4^{-/-}$ mice. Unless otherwise indicated, all aggregation studies were conducted in modified Tyrodes–Hepes buffer containing 1 mM $CaCl_2$.

Dense granule secretion

Washed platelets preloaded with [^{3}H]5-HT (0.5 μCi mL^{-1}) were incubated for 30 min with CE (10–40 μM) or DMSO (0.4%) prior to stimulation with collagen (5 μg mL^{-1}) for 180 s under stirring conditions. [^{3}H]5-HT release was measured in supernatants by scintillation counting.

Platelet adhesion

Ninety-six-well plates were coated with 100 μg mL^{-1} fibrinogen for 1 h at room temperature and blocked with 1% bovine serum albumin (BSA) for 1 h. Washed platelets treated with CE (10–40 μM) were incubated in wells for 1 h at 37 °C, and wells were washed three times with Tyrodes–Hepes buffer prior to incubation with acid phosphatase buffer for 1 h at 37 °C. Then, 2 mM NaOH was added to the wells and absorbance was measured at 405 nm.

Clot retraction

Platelet-rich plasma (200 μL) was incubated with DMSO (0.4%) or CE (20 or 40 μM) for 30 min before being transferred to a glass test tube containing Tyrodes–Hepes buffer (750 μL). Erythrocytes were added to enhance the color for photographic purposes before thrombin (2.5 U mL^{-1}) was added and a glass rod was inserted to initiate clot formation. After 2 h at room temperature, the clots were photographed and weighed.

Immunoblotting

Proteins were separated by SDS-PAGE and transferred to poly(vinylidene difluoride) membranes. Membranes were blocked with 5% (w/v) milk and incubated with anti-phospho-VASP (Ser239) or anti-VASP (1 : 1000 in TBST, 2% BSA) overnight at 4 °C. Blots were washed, and incubated with horseradish peroxidase-conjugated secondary antibody (1 : 5000 in TBST, 2% BSA) prior to detection with an enhanced chemiluminescence detection system.

Mice

Male Balb/c mice (20–30 g) were purchased from Harlan (Bicester, UK). PMCA4$^{-/-}$ mice were bred under a heterozygous breeding program and genotyped according to published protocols [15]. All protocols involving the use of animals were licensed by the UK Home Office, approved by the Ethical Review Panel at Imperial College London, and refined in association with the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs) [16].

In vivo *platelet thromboembolism*

A mouse model of platelet thromboembolism developed in our laboratory was employed [16]. Blood was collected from terminally anesthetized donor mice by cardiac puncture, and platelets were isolated and incubated with 1.8 MBq indium-111 oxine for 10 min. Platelets were then resuspended in modified Tyrodes–Hepes buffer in the presence of CE (20 or 40 μM) or DMSO (0.4%). Anesthetized mice were infused with radiolabeled platelets prior to administration of intravenous collagen (25–75 μg kg^{-1}). Platelet thromboembolic responses were measured in the pulmonary region as changes in platelet-associated counts, using a single point extended area ratio detector (eV Products, Saxonburg, PA, USA), and recorded with the use of custom software (Mumed systems, London, UK), as previously described [16].

Data analysis and statistics

All data are expressed as mean ± standard error of the mean. Where statistical comparisons were made, a Students' *t*-test or one-way analysis of variance followed by a multiple comparison test was used to compare mean values, and a *P*-value < 0.05 was considered to denote statistical significance.

Results

PMCA regulates $[Ca^{2+}]_i$ in resting and stimulated platelets

In control platelets loaded with Fura-2 and monitored in Ca^{2+}-free media in the presence of EGTA to assess release of stored Ca^{2+}, thapsigargin plus ionomycin (Tg + Iono) induced a transient increase in $[Ca^{2+}]_i$ that decayed towards baseline because of Ca^{2+} release from internal stores and extrusion through PMCA, as previously reported [2] (Fig. 1A). Pretreatment of platelets with 10–40 μM CE dose-dependently inhibited Ca^{2+} extrusion through PMCA, shown as impaired Ca^{2+} decay following Tg + Iono treatment (Fig. 1A). There was no evidence of an effect of CE on store loading, as Ca^{2+} release (measured as peak minus basal) was unaffected by this treatment (Fig. 1A and Fig. S1).

CE also dose-dependently increased $[Ca^{2+}]_i$ in resting platelets (Fig. 1B), indicating a role for PMCA in maintaining basal Ca^{2+} levels.

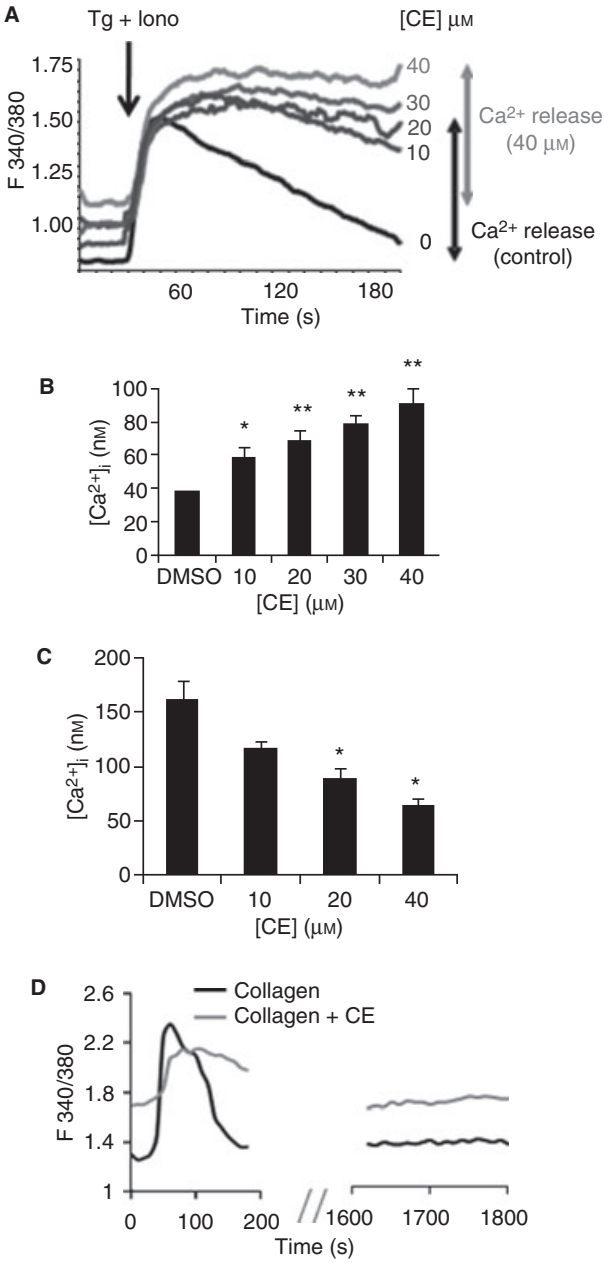


Fig. 1. Carboxyeosin (CE) inhibits Ca^{2+} extrusion through plasma membrane calcium ATPase (PMCA) in platelets and modifies Ca^{2+} homeostasis. Washed platelets loaded with Fura-2 were incubated for 30 min with the PMCA inhibitor CE (10–40 μM) or dimethylsulfoxide (DMSO) (0.4%, control), and the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was measured as the 340/380-nm emission ratio (F 340/380). (A) Platelets were stimulated with thapsigargin (Tg, 1 μM) supplemented with ionomycin (Iono, 50 nM) to induce Ca^{2+} release followed by extrusion through PMCA. CE pretreatment impaired Ca^{2+} extrusion, shown as an impaired return to baseline. Ca^{2+} release from stores is indicated by arrows for the control and 40 μM CE treatments, and shows that Ca^{2+} release is not affected by CE. Typical smoothed traces of $n = 5$ are shown. (B) Mean $[Ca^{2+}]_i$ in resting platelets. (C) Mean maximal $[Ca^{2+}]_i$ following stimulation with collagen (5 μg mL^{-1}) for 180 s. (D) Time course of the 340/380-nm emission ratio in platelets stimulated with collagen for 30 min, showing a typical trace of $n = 5$. Data represent mean ± standard error of the mean, $n = 4$, $^*P < 0.05$, $^{**}P < 0.01$.

The peak $[Ca^{2+}]_i$ following collagen stimulation was reduced in CE-pretreated platelets, indicating reduced collagen-mediated Ca^{2+} release (Fig. 1C). Time-course experiments revealed that $[Ca^{2+}]_i$ in control platelets had returned to basal levels 180 s after stimulation, whereas in platelets incubated with CE, $[Ca^{2+}]_i$ remained elevated beyond this time (Fig. 1D). Thus, CE reduces the amplitude of the initial Ca^{2+} transient following collagen stimulation, but leads to an elevated response at later time points.

Validation of CE as a PMCA inhibitor

As the pharmacologically active, free acid form of CE is eosin, which is fluorescently active, we conducted a series of experiments to determine whether eosin could dose-dependently increase Fura-2 emission independently of $[Ca^{2+}]$ and thus invalidate the data presented in Fig. 1. A broad range of eosin concentrations (0.1–50 μM) caused an apparent suppression of 340/380-nm emission that was neither significant nor dose-dependent (Fig. S2A). Although excess CE was removed from platelets prior to analysis, we could not guarantee its absence from our preparations, and so we additionally demonstrated that CE (0.1–50 μM) did not affect Fura-2 340/380-nm emission (Fig. S2B). Thus, we conclude that although the observed increases in Fura-2 emission in resting platelets containing eosin may have been suppressed (i.e. underestimated) and quantitative studies could not be performed, our finding of a dose-dependent increase in Fura-2 emission with CE and our conclusion that PMCA regulates basal $[Ca^{2+}]_i$ are valid.

We were also able to observe a similar increase in basal $[Ca^{2+}]_i$ to that observed with CE by incubating platelets with the non-selective cation channel antagonist lanthanum (1 mM) (Fig. S3), confirming a comparable increase in Ca^{2+} following inhibition of Ca^{2+} extrusion through an independent mechanism and in the absence of potentially confounding fluorescence issues. In addition, the effect of CE on Ca^{2+} homeostasis did not occur in platelets pretreated with lanthanum, indicating that the effect of CE on Ca^{2+} homeostasis was exerted at the plasma membrane rather than at a location independent of PMCA (Fig. S3).

As CE has been shown to inhibit Na^+/K^+-ATPase pumps [17] as well as PMCA, we investigated the effects of the Na^+/K^+-ATPase inhibitor ouabain on platelet aggregation. Concentrations of ouabain previously shown to inhibit Na^+/K^+-ATPases in platelets (1–10 μM [18,19]) and excess concentrations (200 μM) had no effect on collagen-induced or thrombin-induced platelet aggregation (Fig. S4A,B).

PMCA positively regulates platelet aggregation and secretion

CE dose-dependently inhibited collagen-mediated (Fig. 2A) and thrombin-mediated (Fig. S5A) human platelet aggregation, indicating a positive regulatory role of PMCA in these processes. CE also inhibited collagen-evoked 5-HT secretion (Fig. 2B) and inhibited collagen-evoked aggregation of mouse platelets (Fig. S5B). Collagen-induced platelet aggregation responses were also significantly reduced in platelets from $PMCA4^{-/-}$ mice as compared with those from wild-type controls (Fig. 2C,D). Finally, the inhibitory effect of CE observed in platelets from wild-type mice did not occur in platelets from $PMCA4^{-/-}$ mice, which aggregated normally in the presence of CE (Fig. 2E,F), demonstrating that CE exerted its effects *via* inhibition of PMCA4.

PMCA regulates platelet function in vivo

CE dose-dependently impaired platelet aggregatory responses to collagen *in vivo* (Fig. 3A). The maximal percentage increase in platelet-associated counts in response to collagen was significantly reduced by CE (Fig. 3B), indicating reduced platelet responsiveness *in vivo* following PMCA inhibition.

PMCA negatively regulates early and late stages of platelet activation

Although agonist-induced Ca^{2+} release was suppressed by CE (Fig. 1C), $[Ca^{2+}]_i$ was elevated in resting platelets (Fig. 1B) and in the later stages of activation (Fig. 1D). To establish whether these temporal differences in $[Ca^{2+}]_i$ impacted on platelet function, we determined the consequences of PMCA inhibition for platelet adhesion and clot retraction to assess the early and later stages of platelet activation, respectively.

In contrast to platelet aggregation and secretion, which were suppressed following PMCA inhibition (Fig. 2), CE significantly enhanced platelet adhesion to fibrinogen (Fig. 4A) and dose-dependently reduced clot weight (Fig. 4B–C), indicating enhanced clot retraction. Thus, PMCA positively regulates platelet aggregation but negatively regulates platelet adhesion and clot retraction.

PMCA inhibition does not desensitize platelets

To assess whether prestimulation resulting from raised basal $[Ca^{2+}]_i$ [20] was the mechanism underlying the inhibition of platelet aggregation observed with CE, platelet secretion and aggregation were measured in resting platelets incubated with CE. For contrast, secretion and aggregation were additionally determined in resting platelets incubated with the SERCA inhibitor thapsigargin, which also leads to increased $[Ca^{2+}]_i$ [21].

Thapsigargin dose-dependently increased dense granule secretion (Fig. S6A) and light transmission (Fig. S6B) in unstimulated platelets, indicating prestimulation. When thapsigargin-pretreated platelets were subsequently stimulated with collagen (Fig. S6B), there was no difference in platelet aggregation from that seen in DMSO controls.

In contrast, there was no significant increase in dense granule secretion during incubation with CE at concentrations shown to inhibit aggregation responses (Fig. S6C). Similarly, light transmission in unstimulated platelets was not significantly increased by CE, with the exception of 40 μM, where there was a small but significant increase of 5.3% ± 1.6% (Fig. S6D).

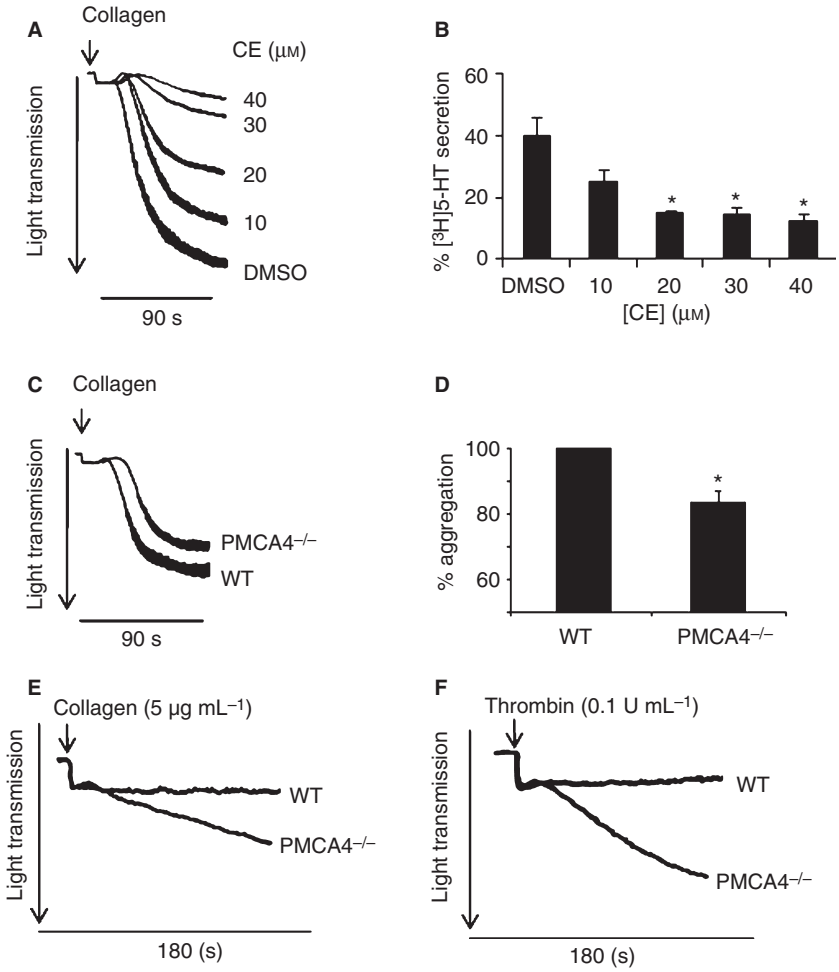


Fig. 2. Inhibition of plasma membrane calcium ATPase (PMCA) inhibits platelet aggregation and secretion. (A, B) Washed human platelets were incubated with the PMCA inhibitor carboxyeosin (CE) (10–40 μM) or dimethylsulfoxide (DMSO) (0.4%) for 30 min prior to stimulation with collagen (5 μg mL^{-1}), and (A) aggregation was measured by optical aggregometry ($n = 4$) or (B) [^{3}H]5-hydroxytryptamine (5-HT) release was measured from [^{3}H]5-HT-preloaded platelets. (C, D) Representative aggregometry traces (C) and mean maximal aggregation responses (D) of platelets from wild-type (WT) and PMCA4$^{-/-}$ mice following stimulation with collagen (0.5 μg mL^{-1}). (E, F) Representative aggregation traces following collagen (E) or thrombin (F) treatment in the presence of CE (40 μM) in platelets from WT and PMCA4$^{-/-}$ mice. Data represent mean ± standard error of the mean, $n = 4$, $^*P < 0.05$.

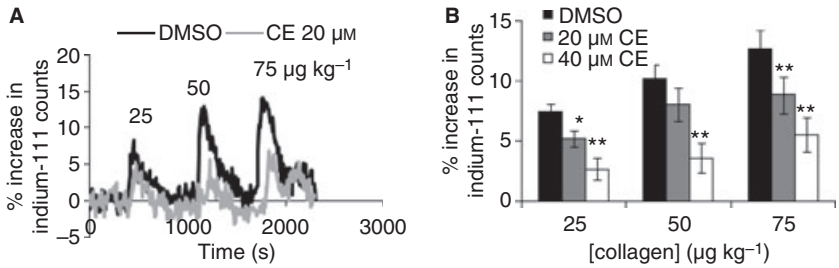


Fig. 3. Plasma membrane calcium ATPase (PMCA) inhibition reduces platelet responses *in vivo*. Washed platelets labeled with indium-111 oxine and incubated with the PMCA inhibitor carboxyeosin (CE) (20 or 40 μM) or dimethylsulfoxide (DMSO) control (0.4%) were infused into anesthetized recipient mice, which were subsequently injected with intravenous collagen (25, 50 and 75 μg kg^{-1}). Radiolabeled platelet aggregation in the pulmonary vasculature was measured *via* external detection probes. Data are shown as (A) changes in counts over time or (B) mean ± standard error of the mean of the maximal % increase in indium-111 counts as compared with basal levels, $n = 5$, $^*P < 0.05$, $^{**}P < 0.01$.

CE enhances VASP phosphorylation independently of cGMP

Western blots clearly demonstrated a dose-dependent increase in VASP (Ser239) phosphorylation in platelets incubated with CE (Fig. 5A), which was normalized to total VASP levels (Fig. 5B).

The NO donor SNP (1 μM) almost completely inhibited collagen-mediated aggregation, and this inhibition was

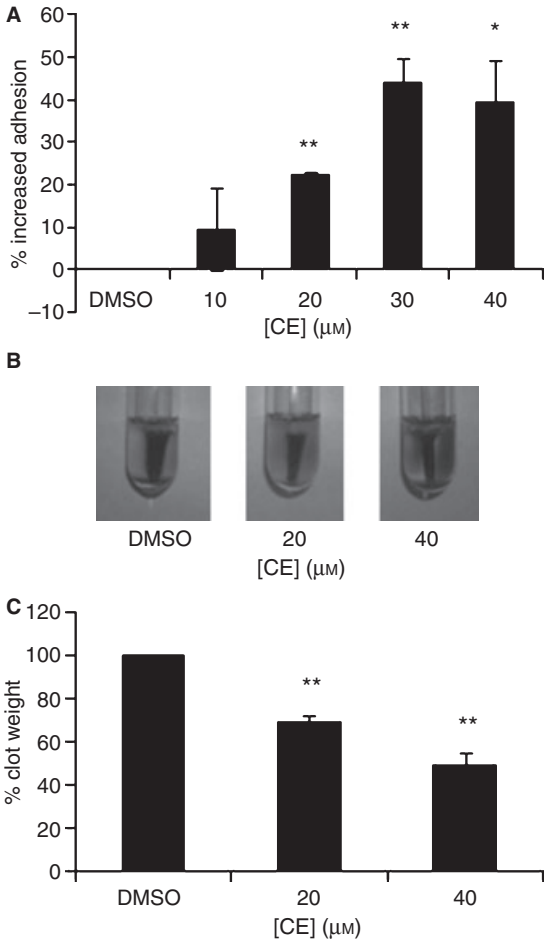


Fig. 4. Plasma membrane calcium ATPase (PMCA) inhibition enhances platelet adhesion and clot retraction. (A) Adhesion of washed platelets to fibrinogen (100 μg mL^{-1}) was measured under static conditions in platelets preincubated for 30 min with the PMCA inhibitor carboxyeosin (CE) (10–40 μM) ($n = 3$). (B-C) Clot retraction induced by thrombin (2.5 U mL^{-1}) was measured in platelet-rich plasma preincubated for 30 min with CE (20 or 40 μM). (B) images of the clots were taken after 2 h, and (C) mean clot weights were expressed relative to dimethylsulfoxide (DMSO) controls. Data are representative of mean ± standard error of the mean, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.

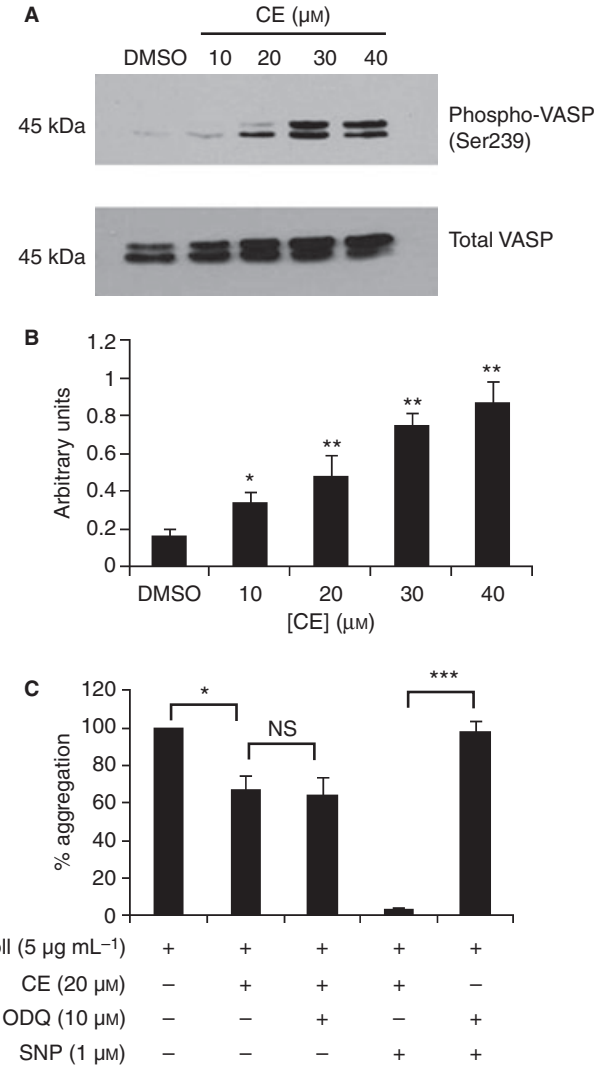


Fig. 5. Plasma membrane calcium ATPase enhances vasodilator-stimulated phosphoprotein (VASP) phosphorylation independently of cGMP. (A, B) Platelet lysates from collagen (5 μg mL^{-1})-stimulated platelets preincubated with carboxyeosin (CE) (10–40 μM) were immunoblotted for phospho-VASP (Ser239) or total VASP (A), and phospho-VASP levels were normalized (B). (C) Platelet aggregation was measured in collagen (Coll) (5 μg mL^{-1})-stimulated platelets preincubated with CE (20 μM) or sodium nitroprusside (SNP) (1 μM) in the presence and absence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM). DMSO, dimethylsulfoxide. Data represent mean ± standard error of the mean, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

reversed by the guanylyl cyclase inhibitor ODQ (10 μM), indicating effective inhibition by ODQ (Fig. 5C). CE-induced inhibition of aggregation, however, was not impaired by ODQ, suggesting that the inhibition caused by CE, although mediated through VASP phosphorylation, was independent of cGMP.

Discussion

The ability of PMCA to regulate Ca^{2+}-dependent signaling events and hence function in a variety of cell types [10] led us to investigate its role in platelet Ca^{2+} homeostasis and relate this to the various stages of platelet activation. This is an essential step in elucidating the signaling and functional events regulated by PMCA in the platelet.

We initially determined concentrations of CE that effectively inhibited Ca^{2+} extrusion through PMCA by showing impaired extrusion under experimental conditions in which PMCA activity predominates [2]. We then used these effective concentrations of CE to ascertain the role of PMCA in regulating Ca^{2+} homeostasis and function in human platelets.

We had originally hypothesized that inhibition of PMCA would impair Ca^{2+} extrusion and elevate $[Ca^{2+}]_i$, leading to platelet activation by subthreshold concentrations of platelet agonists. Early experiments, however, demonstrated that whereas CE elevated basal $[Ca^{2+}]_i$, demonstrating its role in the maintenance of low resting $[Ca^{2+}]_i$, it inhibited agonist-induced intracellular Ca^{2+} release, indicating that PMCA

somehow propagates the rise in $[Ca^{2+}]_i$ following agonist stimulation. Further investigation revealed that reduced Ca^{2+} mobilization following PMCA inhibition was accompanied by impaired dense granule secretion and aggregation, consistent with PMCA being a positive driving force behind these processes. Moreover, inhibition of PMCA resulted in platelets being unable to function normally *in vivo*, confirming the relevance of our observations to physiologic and pathologic processes.

The validity of our data obtained with CE relies on its specificity as an inhibitor of PMCA. CE crosses the plasma membrane and is then cleaved by intracellular esterases to yield eosin. Eosin interacts with amine groups in proteins and inhibits PMCA independently of the ATPase site [22,23]. In terms of specificity, CE inhibits PMCA at the concentrations employed here without inhibiting SERCA or Na^+/Ca^{2+} exchange [22–24], and in line with this we were able to demonstrate normal store loading in the presence of CE, as there was no change in Tg + Iono-induced Ca^{2+} release calculated from data shown in Fig. 1A as peak response minus stable prestimulation values (Fig. S1). CE has been used to determine the function of PMCA in cardiomyocytes [24], sperm [15], embryonic stem cells [25] and vascular endothelial cells [26]. Critical to our argument, the validity of these functional observations has been confirmed by studies in PMCA4$^{-/-}$and transgenic lines [11,15,27,28], suggesting a predominant action of CE at PMCA in a range of cell types. CE does, however, inhibit Na^+/K^+-ATPase activity at micromolar concentrations [17], and effects on transients during Ca^{2+} repletion [19] and procoagulant effects [29] have been reported. We could find no published evidence for a role of this pump in modulating platelet aggregation, and demonstrated here that the Na^+/K^+-ATPase inhibitor ouabain had no effect on platelet aggregation under our experimental conditions. Thus, the effect of CE on platelet aggregation is unlikely to be mediated through inhibition of Na^+/K^+-ATPase activity. As the complete inhibitory profile of CE is unknown, we also conducted experiments in PMCA4$^{-/-}$ mice to determine whether a similar inhibition of platelet aggregation could be observed in the absence of confounding non-specific pharmacologic effects. Platelet aggregation was significantly reduced in PMCA4$^{-/-}$ mice. The occurrence of the same functional observation following both pharmacologic inhibition of PMCA and genetic ablation of PMCA4 provides strong evidence that the observed impairment of platelet aggregation in both situations occurs as a result of loss of PMCA activity. This was further shown by our data indicating that CE inhibited aggregation in wild-type but not PMCA4$^{-/-}$ mice (Fig. 2E–F). These data also suggest that although CE inhibits all isoforms of PMCA, its effects in platelets are mediated *via* inhibition of PMCA4, and that the PMCA4 isoform is a key modulator of platelet function.

In contrast to the inhibitory effect of CE on platelet aggregation, platelet adhesion and clot retraction were enhanced. Increased platelet adhesion to fibrinogen and elevated basal Ca^{2+} prior to this response suggest that, in the early stages of platelet activation, PMCA acts to suppress both $[Ca^{2+}]_i$ and activation, consistent with the Ca^{2+} dependence of the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$, which mediates adhesion. Similarly, enhanced clot retraction occurred against a background of prolonged elevations of $[Ca^{2+}]_i$ (Fig. 1D). These elevations are consistent with the Ca^{2+} dependence of myosin activity, which is essential for the clot retraction process. Thus, PMCA acts to slow the rate at which clots retract, presumably to ensure effective healing of underlying damaged tissue. Our clot retraction data are consistent with results published by Dean *et al.* [12] demonstrating that preventing translocation of PMCA to the filopodia of activated platelets enhanced clot retraction. Together, these two sets of data demonstrate the importance of both the locality and function of PMCA in regulating the clot retraction process.

Possible mechanisms by which PMCA inhibition could reduce platelet aggregation include partial activation and subsequent desensitization of platelets by elevated $[Ca^{2+}]_i$ or coupling of PMCA with a Ca^{2+}-dependent negative regulator of platelet function. The former hypothesis was explored first. It has recently been reported that a mutation of stromal interaction molecule 1, a Ca^{2+} sensor situated in the endoplasmic reticulum, leads to elevated $[Ca^{2+}]_i$ in platelets, resulting in a preactivation state and reduced responses to collagen [20]. We could not, however, find evidence of preactivation in our experiments, as we were unable to detect secretion or aggregation during preincubation with CE. Furthermore, elevation of $[Ca^{2+}]_i$ through SERCA inhibition, despite stimulating dense granule secretion and partial aggregation, did not desensitize platelets to collagen. Thus, we confirmed that our methodologies for detecting platelet preactivation against a background of elevated $[Ca^{2+}]_i$ were effective, and that elevating $[Ca^{2+}]_i$ in platelets did not *per se* lead to inhibition of subsequent agonist-induced aggregation.

Very little PMCA is associated with the platelet cytoskeleton under resting conditions, but upon stimulation approximately 80% of the total PMCA is redistributed by the cytoskeleton and retains ATPase activity [30]. This redistribution potentially enables Ca^{2+} and signaling molecules, potentially inhibitory signaling pathways, to be regulated locally. Such a role of PMCA in regulating inhibitory signaling pathways has already been demonstrated in cardiomyocytes [11] and vascular smooth muscle cells [27], where PMCA exerts its functional effects by regulating nNOS activity. Thus, the application of CE in our study could inhibit platelet aggregation by raising Ca^{2+} in the vicinity of localized inhibitory signaling molecules, such as those regulating VASP activity. VASP (Ser239) phosphorylation was shown to be increased by CE, indicating that PMCA prevents the phosphorylation of VASP.

One of the major mechanisms of VASP (Ser239) phosphorylation in platelets is increased cGMP production by NO and other mediators such as von Willebrand factor [31]. In our study, the inhibition of platelet aggregation by CE occurred independently of cGMP. Although Ser239 is considered to be the preferred phosphorylation site for cGMP/protein kinase G (PKG), the site has also been shown to be both PKG-sensitive

and protein kinase A-sensitive [32,33], indicating that PMCA inhibition may stimulate alternative Ca^{2+}-dependent inhibitory pathways in platelets. As research continues into the role of PMCA, novel interactions between PMCA and signaling molecules continually emerge [34–36], and may ultimately clarify the role of PMCA in regulating signaling events in platelets.

Altered expression of PMCA has been reported in patients with diabetes [37], hypertension [38] and type 2B von Willebrand disease [39]. All of these conditions are associated with platelet dysfunction through mechanisms that are incompletely understood. In defining the role of PMCA in regulating platelet function, we provide potential mechanisms by which PMCA may modulate platelet function and hence the development and progression of these diseases.

In conclusion, PMCA is involved in the regulation of $[Ca^{2+}]_i$ in both resting and activated platelets. PMCA differentially regulates the various stages of platelet activation with both stimulatory and inhibitory roles. The effects on platelet function correspond with changes in both $[Ca^{2+}]_i$ and inhibitory signaling pathways. PMCA is clearly a critical regulator of platelet Ca^{2+} homeostasis, signaling and function, and is therefore a key regulator of hemostasis and thrombosis, and may be a therapeutic target in platelet driven diseases.

Acknowledgements

We thank F. Baudoin for excellent technical assistance. This work was funded by a Research Grant from the Wellcome Trust (085132/Z/08/Z) to M. Emerson. L. Neyses is supported by an MRC Programme Grant and by the NIHR (Manchester Biomedical Research Centre). A. Solomon is supported by a National Heart and Lung Institute Foundation Studentship.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Carboxyeosin does not affect stored Ca^{2+} release.
Fig. S2. Effect of eosin and carboxyeosin on Fura-2 emission spectra.
Fig. S3. Effect of blockade of Ca^{2+} extrusion on platelet Ca^{2+} homeostasis.
Fig. S4. Na^+/K^+-ATPase does not modulate platelet aggregation.
Fig. S5. Carboxyeosin inhibits aggregation of human and mouse platelets.
Fig. S6. Increased $[Ca^{2+}]_i$ following plasma membrane calcium ATPase (PMCA) inhibition does not desensitize platelets.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1 Bobe R, Bredoux R, Corvazier E, Lacabaratz-Porret C, Martin V, Kovacs T, Enouf J. How many Ca(2)+ATPase isoforms are expressed in a cell type? A growing family of membrane proteins illustrated by studies in platelets. *Platelets* 2005; **16**: 133–50.
2 Rosado JA, Sage SO. Regulation of plasma membrane Ca2+-ATPase by small GTPases and phosphoinositides in human platelets. *J Biol Chem* 2000; **275**: 19529–35.
3 Bose R, Li Y, Roberts D. Na+/Ca2+ exchange in activated and nonactivated human platelets. *Ann NY Acad Sci* 2002; **976**: 350–3.
4 Roberts DE, McNicol A, Bose R. Mechanism of collagen activation in human platelets. *J Biol Chem* 2004; **279**: 19421–30.
5 Harper AG, Sage SO. A key role for reverse Na+/Ca2+ exchange influenced by the actin cytoskeleton in store-operated Ca2+ entry in human platelets: evidence against the de novo conformational coupling hypothesis. *Cell Calcium* 2007; **42**: 606–17.
6 Harper AG, Mason MJ, Sage SO. A key role for dense granule secretion in potentiation of the Ca2+ signal arising from store-operated calcium entry in human platelets. *Cell Calcium* 2009; **45**: 413–20.
7 Carafoli E. The calcium pumping ATPase of the plasma membrane. *Annu Rev Physiol* 1991; **53**: 531–47.
8 Martin V, Bredoux R, Corvazier E, Papp B, Enouf J. Platelet Ca(2+)ATPases : a plural, species-specific, and multiple hypertension-regulated expression system. *Hypertension* 2000; **35**: 91–102.
9 Hammes A, Oberdorf S, Strehler EE, Stauffer T, Carafoli E, Vetter H, Neyses L. Differentiation-specific isoform mRNA expression of the calmodulin-dependent plasma membrane Ca(2+)-ATPase. *FASEB J* 1994; **8**: 428–35.
10 Brini M. Plasma membrane Ca(2+)-ATPase: from a housekeeping function to a versatile signaling role. *Pflugers Arch* 2009; **457**: 657–64.
11 Oceandy D, Cartwright EJ, Emerson M, Prehar S, Baudoin FM, Zi M, Alatwi N, Schuh K, Williams JC, Armesilla AL, Neyses L. Neuronal nitric oxide synthase signaling in the heart is regulated by the sarcolemmal calcium pump 4b. *Circulation* 2007; **115**: 483–92.
12 Dean WL, Whiteheart SW. Plasma membrane Ca(2+)-ATPase (PMCA) translocates to filopodia during platelet activation. *Thromb Haemost* 2004; **91**: 325–33.
13 Tymvios C, Moore C, Jones S, Solomon A, Sanz-Rosa D, Emerson M. Platelet aggregation responses are critically regulated in vivo by endogenous nitric oxide but not by endothelial nitric oxide synthase. *Br J Pharmacol* 2009; **158**: 1735–42.
14 Moore C, Tymvios C, Emerson M. Functional regulation of vascular and platelet activity during thrombosis by nitric oxide and endothelial nitric oxide synthase. *Thromb Haemost* 2010; **104**: 342–9.
15 Schuh K, Cartwright EJ, Jankevics E, Bundschu K, Liebermann J, Williams JC, Armesilla AL, Emerson M, Oceandy D, Knobeloch KP, Neyses L. Plasma membrane Ca2+ ATPase 4 is required for sperm motility and male fertility. *J Biol Chem* 2004; **279**: 28220–6.
16 Tymvios C, Jones S, Moore C, Pitchford SC, Page CP, Emerson M. Real-time measurement of non-lethal platelet thromboembolic responses in the anaesthetized mouse. *Thromb Haemost* 2008; **99**: 435–40.
17 Ogan JT, Reifenberger MS, Milanick MA, Gatto C. Kinetic characterization of Na,K-ATPase inhibition by Eosin. *Blood Cells Mol Dis* 2007; **38**: 229–37.
18 Lees AD, Wilson J, Orchard CH, Orchard MA. Ouabain enhances basal and stimulus-induced cytoplasmic calcium concentrations in platelets. *Thromb Haemost* 1989; **62**: 1000–5.
19 Zimlichman R, Goldstein DS, Zimlichman S, Keister HR. Effects of ouabain on cytosolic calcium in lymphocytes, platelets and adrenomedullary cells. *J Hypertens* 1987; **5**: 605–9.

20 Grosse J, Braun A, Varga-Szabo D, Beyersdorf N, Schneider B, Zeitlmann L, Hanke P, Schropp P, Mühlstedt S, Zorn C, Huber M, Schmittwolf C, Jagla W, Yu P, Kerkau T, Schulze H, Nehls M, Nieswandt B. An EF hand mutation in Stim1 causes premature platelet activation and bleeding in mice. *J Clin Invest* 2007; **117**: 3540–50.

21 Redondo PC, Salido GM, Pariente JA, Sage SO, Rosado JA. SERCA2b and 3 play a regulatory role in store-operated calcium entry in human platelets. *Cell Signal* 2008; **20**: 337–46.

22 Gatto C, Milanick MA. Inhibition of the red blood cell calcium pump by eosin and other fluorescein analogues. *Am J Physiol* 1993; **264**: C1577–86.

23 Gatto C, Hale CC, Xu W, Milanick MA. Eosin, a potent inhibitor of the plasma membrane Ca pump, does not inhibit the cardiac Na–Ca exchanger. *Biochemistry* 1995; **34**: 965–72.

24 Choi HS, Eisner DA. The role of sarcolemmal Ca2+-ATPase in the regulation of resting calcium concentration in rat ventricular myocytes. *J Physiol* 1999; **1**: 109–18.

25 Yanagida E, Shoji S, Hirayama Y, Yoshikawa F, Otsu K, Uematsu H, Hiraoka M, Furuichi T, Kawano S. Functional expression of Ca2+ signaling pathways in mouse embryonic stem cells. *Cell Calcium* 2004; **36**: 135–46.

26 Sedova M, Blatter LA. Dynamic regulation of [Ca2+]i by plasma membrane Ca(2+)-ATPase and Na+/Ca2+ exchange during capacitative Ca2+ entry in bovine vascular endothelial cells. *Cell Calcium* 1999; **25**: 333–43.

27 Schuh K, Quaschning T, Knauer S, Hu K, Kocak S, Roethlein N, Neyses L. Regulation of vascular tone in animals overexpressing the sarcolemmal calcium pump. *J Biol Chem* 2003; **278**: 41246–52.

28 Cartwright EJ, Schuh K, Neyses L. Calcium transport in cardiovascular health and disease – the sarcolemmal calcium pump enters the stage. *J Mol Cell Cardiol* 2005; **39**: 403–6.

29 Tomasiak M, Stelmach H, Rusak T, Ciborowski M, Radziwon P. The involvement of Na+/K(+)-ATPase in the development of platelet procoagulant response. *Acta Biochim Pol* 2007; **54**: 625–39.

30 Zabe M, Dean WL. Plasma membrane Ca(2+)-ATPase associates with the cytoskeleton in activated platelets through a PDZ-binding domain. *J Biol Chem* 2001; **276**: 14704–9.

31 Gambaryan S, Kobsar A, Hartmann S, Birschmann I, Kuhlencordt PJ, Muller-Esterl W, Lohmann SM, Walter U. NO-synthase-/NO-independent regulation of human and murine platelet soluble guanylyl cyclase activity. *J Thromb Haemost* 2008; **6**: 1376–84.

32 Li Z, Ajdic J, Eigenthaler M, Du X. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood* 2003; **101**: 4423–9.

33 Riba R, Oberprieler NG, Roberts W, Naseem KM. von Willebrand factor activates endothelial nitric oxide synthase in blood platelets by a glycoprotein Ib-dependent mechanism. *J Thromb Haemost* 2006; **4**: 2636–44.

34 Armesilla AL, Williams JC, Buch MH, Pickard A, Emerson M, Cartwright EJ, Oceandy D, Vos MD, Gillies S, Clark GJ, Neyses L. Novel functional interaction between the plasma membrane Ca2+ pump 4b and the proapoptotic tumor suppressor Ras-associated factor 1 (RASSF1). *J Biol Chem* 2004; **279**: 31318–28.

35 Buch MH, Pickard A, Rodriguez A, Gillies S, Maass AH, Emerson M, Cartwright EJ, Williams JC, Oceandy D, Redondo JM, Neyses L, Armesilla AL. The sarcolemmal calcium pump inhibits the calcineurin/nuclear factor of activated T-cell pathway via interaction with the calcineurin A catalytic subunit. *J Biol Chem* 2005; **280**: 29479–87.

36 Holton M, Yang D, Wang W, Mohamed TM, Neyses L, Armesilla AL. The interaction between endogenous calcineurin and the plasma membrane calcium-dependent ATPase is isoform specific in breast cancer cells. *FEBS Lett* 2007; **581**: 4115–19.

37 Chaabane C, Dally S, Corvazier E, Bredoux R, Bobe R, Ftouhi B, Raies A, Enouf J. Platelet PMCA- and SERCA-type Ca^{2+}-ATPase expression in diabetes: a novel signature of abnormal megakaryocytopoiesis. *J Thromb Haemost* 2007; **5**: 2127–35.

38 Dally S, Chaabane C, Corvazier E, Bredoux R, Bobe R, Ftouhi B, Slimane H, Raies A, Enouf J. Increased expression of plasma membrane Ca(2+)ATPase 4b in platelets from hypertensives: a new sign of abnormal thrombopoiesis? *Platelets* 2007; **18**: 543–9.

39 Nurden P, Debili N, Vainchenker W, Bobe R, Bredoux R, Corvazier E, Combrie R, Fressinaud E, Meyer D, Nurden AT, Enouf J. Impaired megakaryocytopoiesis in type 2B von Willebrand disease with severe thrombocytopenia. *Blood* 2006; **108**: 2587–95.