THE ROLE OF PROTEIN KINASE C IN PLATELET ACTIVATION

A thesis submitted to the Board of the faculty of Biological Sciences, University of Oxford, in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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The Protein kinase C (PKC) superfamily is a key regulator in platelet activation with individual isoforms playing distinct roles. This thesis focuses on the role of the novel PKC isoforms downstream of several agonists using both pharmacological and genetic approaches and human and mouse platelets. Quantification of the protein levels of PKC isoforms identified different levels of the five major PKC isoforms expressed in human platelets and also differences between levels of the same isoform in human and mouse platelets. Use of a selection of broad spectrum and isoform-specific inhibitors, identified both positive and negative novel roles for PKC in the regulation of human and mouse platelets. A net positive role for PKC was found in GPVI, Clec-2, and PAR receptor signalling, with classical isoforms of PKC playing a major role in aggregation and dense granule secretion. A novel negative regulatory role was also identified in the regulation of ADP-induced platelet activation for PKCβ, and both PKCε and PKCβ in human and mouse platelets respectively. Gene knock-out mouse models confirmed a positive regulatory role for PKCθ in αIIbβ3 outside-in signalling but identified no other regulatory role for PKCθ in agonist induced platelet activation. Despite this relatively minor role, functional redundancy was identified between PKCθ and PKCε isoforms in haemostasis, as tail bleeding was significantly increased in mice deficient in both novel isoforms.

The work presented here identifies key roles for the PKC superfamily in the complex regulation of platelet activation, with different isoforms supporting and limiting the process of thrombus formation and haemostasis.

PUBLICATIONS ARISING FROM THIS THESIS

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This thesis has had its highs and its lows, it has had its successes and its struggles. There have been laughs, there have been tears and there have been celebrations. It is the culmination of a lot of hard work, stress and determination, it has been one hell of a journey, thank you for making it with me. I thank you all. *Much love Amanda*

ABBREVIATIONS

5-HT	–	5-hydroxytryptamine (serotonin)
Ab	–	Antibody
ACD	–	Acid citrate dextrose
ADP	–	Adenosine diphosphate
aPKC	–	Atypical protein kinase C isoform
ARC	–	ARC-69931MX/ cangrelor
ATP	–	Adenosine triphosphate
BSA	–	Bovine serum albumin
cAMP	–	Cyclic adenonsine monophosphate
cGMP	–	Cyclic guanosine monophosphate
CLEC-2	–	C -type lectin-like receptor 2
cPKC	–	Conventional/classical protein kinase C isoform
CRP	–	Collagen related peptide
DAG	–	Diacylglycerol
DMSO	–	Dimethylsulphoxide
ECL	–	Enhanced chemiluminescence
ECM	–	Extracellular matrix
EDTA	–	Ethylenediamine tetra-acetic acid
EGTA	–	Ethylene glycol tetra-acetic acid
FAK	–	Focal adhesion kinase
FcR	–	Fc receptor
FURA-2 AM	–	Fura-2-acetoxymethyl ester
Gads	–	Grb2 adaptor downstream of Shc
GDP	–	Guanosine diphosphate
GEF	–	Guanine nucleotide exchange factor
GPCR	–	G protein coupled receptor
GPIb-IX-V	–	Glycoprotein Ib- IX-V
GPVI	–	Glycoprotein VI
GTP	–	Guanosine triphosphate
hemITAM	–	Hemi -Immunoreceptor tyrosine based activation motif
HRP	–	Horse radish peroxidase
IC_{50}	–	Inhibitory concentration required for 50% inhibition
Ig	–	Immunoglobulin
IP_3	–	Inositol-1,4,5-trisphosphate
ITAM	–	Immunoreceptor tyrosine based activation motif
ITIM	–	Immunoreceptor tyrosine based inhibition motif
kDa	–	Kilodalton
LAT	–	Linker for activation of T-cells
mAb	–	Monoclonal antibody

MRS	–	MRS 2179
nPKC	–	Novel protein kinase C isoform
pAb	–	Polyclonal antibody
PAR	–	Protease activated receptor
PBS	–	Phosphate buffered saline
PDGF	–	Platelet derived growth factor
PDK-1	–	Phosphoinositide-dependent kinase-1
PF4	–	Platelet factor 4
PGI_2	–	Prostaglandin I2
PH	–	Pleckstrin homology
PI3K	–	Phosphoinositide 3-kinase
PIP_3	–	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	–	Protein kinase C
PLC	–	Phospholipase C
PMA	–	Phorbol 12-myristate 13-acetate
PMCA	–	Plasma membrane calcium pump
PRP	–	Platelet rich plasma
PS	–	Phosphatidylserine
PTB	–	Phosphotyrosine binding
RACKs	–	Receptors for activated C kinase
RIAM	–	Rap1-GTP-interacting adaptor molecule
SDS-PAGE	–	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	–	Src homology 2
SH3	–	Src homology 3
SLP-76	–	SH2 containing leukocyte protein of 76 kDa
SNARE	–	SNAP (soluble NSF attachment protein) receptor
SOCE	–	Store operated calcium entry
STIM1	–	Stromal interaction molecule 1
TBS-T	–	Tris buffered saline - Tween
TRAP	–	PAR 1 peptide
TRPC6	–	Transient receptor potential cation channel 6
TSB	–	Tris buffered saline
TxA_2	–	Thromboxane A2
VASP	–	Vasodilator-stimulated phosphoprotein
VEGF	–	Vascular endothelial growth factor
vWF	–	Von Willebrand factor
WP	–	Washed platelets
WT	–	Wild type

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CHAPTER 1.

INTRODUCTION

1.1 General Introduction

Cardiovascular diseases are the world's largest killers, claiming 17 million lives a year (Mackay, Mensah et al. 2004). A significant proportion of these deaths are caused by the formation of a thrombus within a blood vessel, which prevents blood flowing to the heart or brain. Platelets play a central role in haemostasis and thrombosis and therefore play a very important role in cardiovascular disease. Anti-platelet therapies, such as aspirin, clopidogrel and glycoprotein IIb/IIIa inhibitors, have proven successful in the treatment of coronary heart disease. Understanding the roles of cellular receptors, adhesive proteins and regulatory proteins involved in platelet-vessel interaction, platelet activation and thrombus formation is crucial for the development of new anti-platelet drugs

1.2 Introduction to Platelets

Platelets, small anucleate cells that are present in the blood, act as the body's first line of defence following injury to the blood vessel wall. Activation of platelets enables the formation of a blood clot which acts to 'plug' the wound and creates a barrier against infection.

Platelets are present in the blood in an inactive or a quiescent state, which is maintained in part by the release of nitric oxide and prostacyclin from the endothelium, which stimulate cGMP and cAMP formation respectively. Due to their small size and the high mass of blood vessels, platelets circulate at the edge of blood–vessel wall interface which places them in a prime location for a rapid response following vessel injury.

1.2.1 Platelet development

Platelets are formed and released into the bloodstream from the bone marrow by mature megakaryocytes (Patel, Hartwig et al. 2005), following a process of cytoplasmic fragmentation (Wendling and Han 1997). Platelet production requires a series of remodelling events that can result in the release of up to 2000-3000 platelets from a single megakaryocyte (Hartwig and Italiano 2003). Megakaryocyte progenitors differentiate (Ebbe 1976), enter into endomitosis, and undergo DNA endoreplication and amplification allowing the megakaryocytes to become polyploid reaching up to 128N (Vitrat, Cohen-Solal et al. 1998; Ravid, Lu et al. 2002). Cytoplasmic maturation, synthesis of platelet-specific proteins, internal membrane systems, granules and organelles occur (Behnke 1968; Nakao and Angrist 1968; Handagama, George et al. 1987) and once completely differentiated, platelets 'bud' or fragment off entering into the bloodstream from the end of thin, elongated megakaryocyte extensions called proplatelets (Italiano, Lecine et al. 1999). Platelets have a relatively short life span of approximately 7-10 days, (Hartley 2007; Dowling, Josefsson et al. 2010) before removal from the blood which involves Kupffer cells in the liver and macrophages in the spleen.

1.2.2 Platelet structure

Platelets, the smallest of the three types of blood cells, with a diameter of approximately 2-3μM, have a complex structure (Figure 1.1), which enables them to fulfil their roles in haemostasis (Born 1972; Lind and Stossel 1982; Karlsson, Lassing et al. 1984). In their resting state, platelets are discoid in shape, maintained by a cytoskeleton consisting of a spectrin skeleton, that adheres to the cytoplasmic side of the plasma membrane and interacts with many surface receptors, a single microtubule ring that is located underneath the plasma membrane, and a rigid actin filament network that fills the cytosol (Boyles, Fox et al. 1985; Kenney and Linck 1985; Hartwig and DeSisto 1991). Invaginations in the platelet plasma membrane link the open canalicular system to a series of internal membranes that increases the surface area of the platelet and facilitates granule secretion. Platelets contain two major types of granule, the α-granules and the dense granules (King and Reed 2002), as well as cellular organelles including lysosomes, peroxisomes, mitochondria, and a dense tubular system, similar to the endoplasmic reticulum which acts as a store for the release of Ca^{2+} and the site of formation of prostaglandin and thromboxane A_2 (TxA_2) synthesis .

The α-granules are the larger and most abundant of the platelet granules, with 50-80 granules present per platelet (Blair and Flaumenhaft 2009). These granules contain many coagulation/adhesive proteins, transmembrane proteins and both growth factors and protease inhibitors that are released upon activation. In contrast there are approximately 5 dense granules per platelet. These granules store high concentrations of the secondary mediators of platelet activation, ADP, as well as 5-hydroxytryptamine (5-HT), divalent cations and polyphosphates

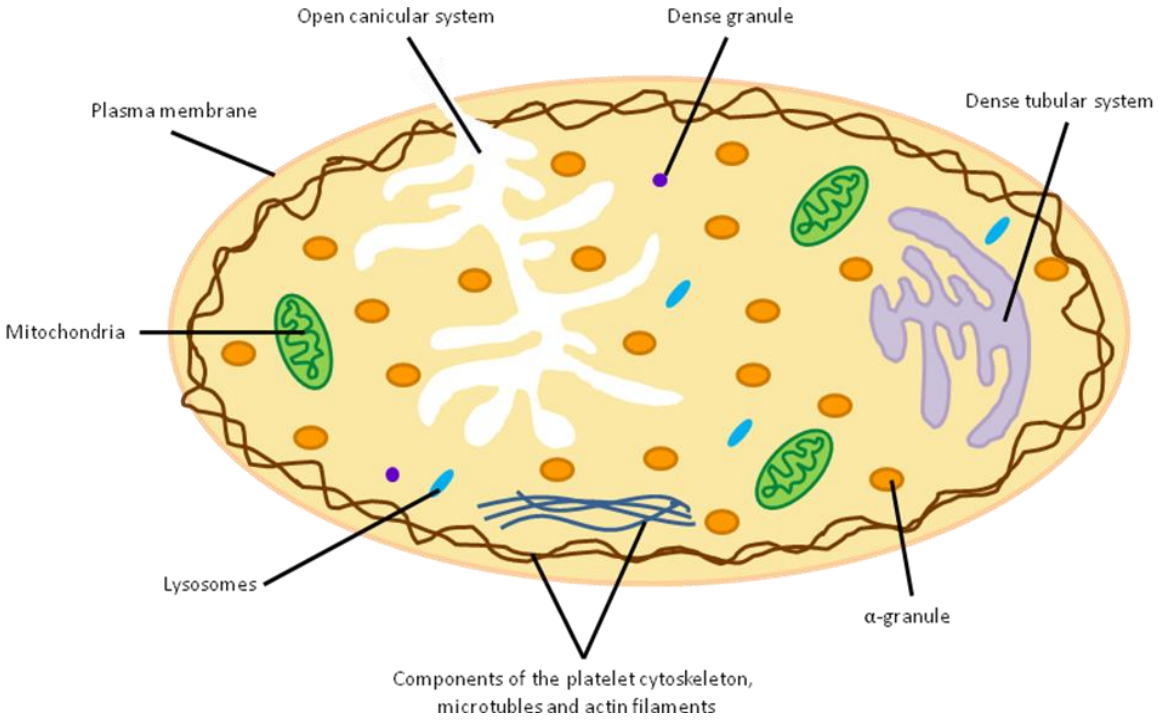


Figure 1.1. Platelet Structure. A schematic of a cross section of a discoid resting plating. Platelets contain several structures essential for their function. The key structural features are shown.

(Meyers, Holmsen et al. 1982; Harrison and Cramer 1993; King and Reed 2002). Release of these granular contents via the open canalicular helps to sustain platelet activation.

Platelets also undergo actin rearrangements leading to shape change and spreading, thereby increasing the platelet surface area and thus facilitating adhesion and aggregation (Hartwig 1992). In suspension, upon activation, the discoid shape of the platelet is lost and the platelet immediately becomes spherical in shape. On a monolayer, filopodia, finger-like projections, form from the cell periphery and are followed by lamellipodia which flatten the platelet and force the granules and organelles into the centre to produce a 'fried egg'-like structure.

1.2.3 Thrombus formation

The process of thrombus formation can be summarised in several stages (Figure 1.2) For more extensive reviews see (Jackson, Nesbitt et al. 2003; Gibbins 2004; Sachs and Nieswandt 2007; Jackson, Nesbitt et al. 2009) (Watson and Harrison, 2010).

1. **Initial adhesion and tethering -** Following injury to the blood vessel wall, components of the extracellular matrix (ECM), such as collagen, become exposed providing sites of adhesion for passing platelets. Under the high shear rates in the arterial system, platelets are able to adhere to the exposed collagen via initial tethering to von Willebrand factor (vWF) which binds to the GPIb-IX-V receptor complex and subendothelial collagen (Savage, Saldivar et al. 1996). This interaction is however, unstable and instead acts to 'slow down' platelets enabling them to bind to collagen via the low affinity GPVI immunoglobulin receptor.
2. **Activation and stable adhesion -** GPVI signalling induces activation of integrins αIIbβ3 and α2β1 which bind to vWF and collagen, respectively, mediating stable

adhesion (Polanowska-Grabowska, Simon et al. 1999). The capture of additional platelets and subsequent cross-linking is mediated by αIIbβ3 which binds vWF (supporting tethering) and fibrinogen which mediates formation of platelet aggregates. The release of ADP and TxA_2 reinforces αIIbβ3 activation in the tethered platelets.

3. **Spreading -** Once stably adhered, the platelets undergo a series of cytoskeletal rearrangements ('spreading') that increases the surface area and strengthens adhesion to the surface and with other platelets.
4. **Secretion -** Platelet shape change supports the process of granule secretion. Release of granular contents including secondary mediators, such as ADP from the dense granules and fibrinogen and vWF from the α-granules and synthesis of TxA_2 *de novo* from arachidonic acid serves to stabilise and recruit platelets to the growing thrombus (Kulkarni, Dopheide et al. 2000).
5. **Aggregation and thrombus formation -** This leads to the production of the primary haemostatic plug which is able to occlude the site of damage, preventing further blood loss. Activation of the coagulation pathway on the procoagulant platelet surface (as a result of phosphatidylserine exposure) generates thrombin which cleaves fibrinogen to form fibrin and stabilise the platelet plug.
6. **Clot retraction and stabilisation –** Further stabilisation of the thrombus occurs via the process of clot retraction which is dependent on the fibrin-fibrinogen bound αIIbβ3 and the actin cytoskeleton.

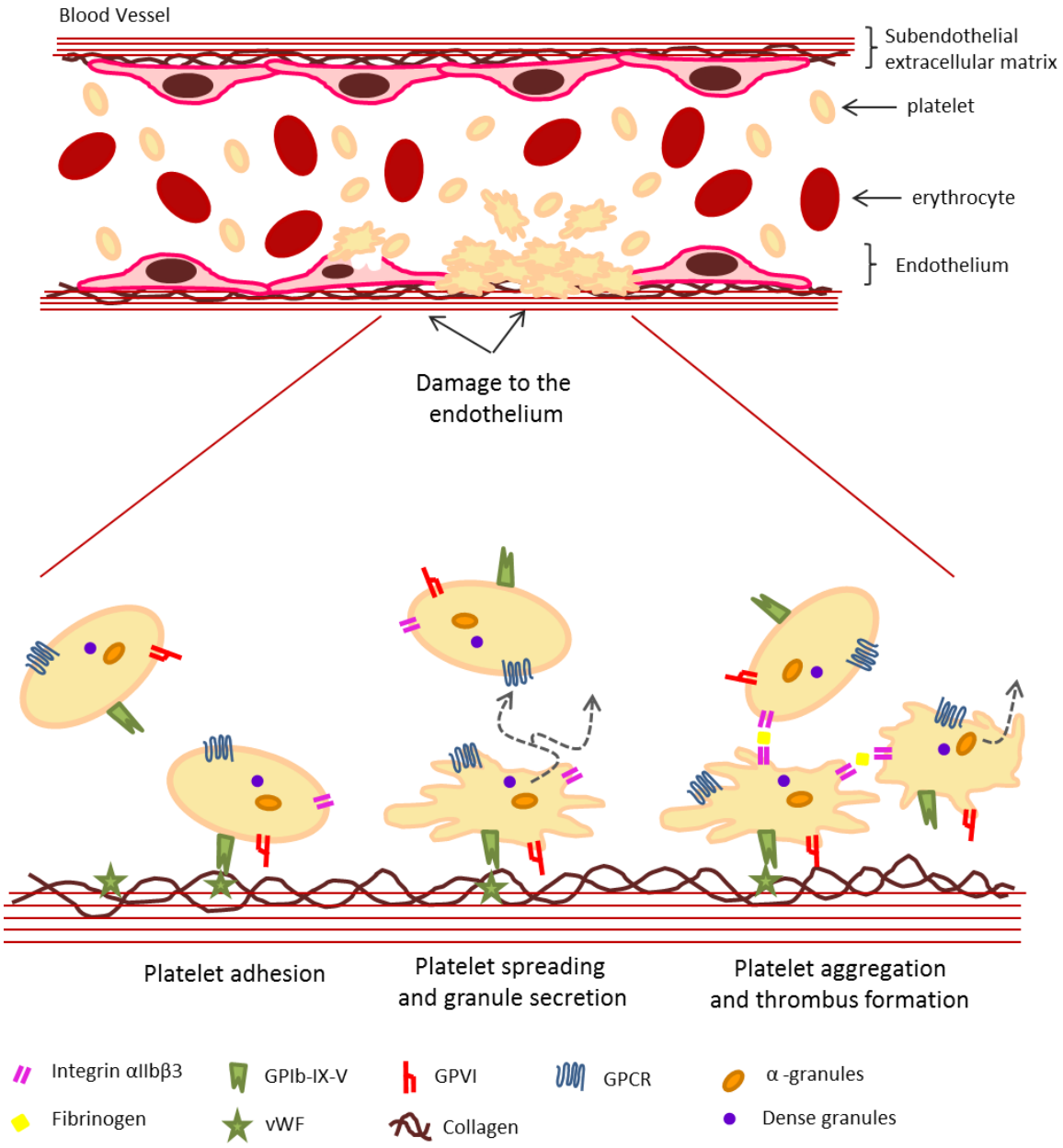


Figure 1.2 Thrombus formation. A schematic of thrombus formation. Following vascular injury, platelets are recruited to the site of damage. The process of thrombus formation can be summarised in several stages, platelet capture and adhesion to the subendothelium, spreading, granule secretion, activation of other platelets, aggregation, coagulation and thrombus formation.

1.3. Platelet Reponses

1.3.1 Granule secretion

Activation of platelet granule secretion is one of the major platelet responses that supports thrombus formation. Secretion from the α-granules, dense granules and lysosomes (Born 1972) enables release of a large assortment of small molecules and proteins that aid and sustain the process of platelet activation and thrombus formation. The dense granules store high concentrations of several small molecules that act as platelet activators, including ADP and ATP, 5-HT and the cations Ca^{2+} and Mg^{2+}. The α-granules secrete several proteins that act to support haemostasis, including adhesion molecules (e.g. fibrinogen and vWF), surface receptors (e.g. $\alpha IIb\beta_3$ and P-selectin), coagulation factors (e.g. Factor V), growth factors (e.g. PDGF and VEGF) that facilitate vascular repair, and chemokines (e.g. platelet factor 4 [PF4]) which recruit leukocytes and stem cells to the site of injury, aiding host defence and vascular repair.

In the resting state the α- and dense granules are distributed throughout the platelet. Following platelet activation the granules are drawn together and fuse with the open canalicular system enabling their release (Reed, Fitzgerald et al. 2000). The process of membrane fusion is orchestrated by SNARE (soluble NEM sensitive attachment protein receptors) proteins and chaperone proteins that bind to and modulate the activity of the SNARE proteins. At the site of membrane fusion, the exocytotic core complex forms which includes SNARE proteins from both the granule (v-SNAREs e.g. VAMP) and target membranes (t-SNAREs e.g. syntaxins 1-9 and SNAP-23) (Ren, Ye et al. 2008). A heterotrimeric complex of SNARE proteins, SNAP-23, syntaxin-4 and VAMP-3, has been shown to be required for α-granule secretion (Flaumenhaft, Croce et al. 1999). Various chaperone proteins play key roles in granule secretion via the activation of the SNARE

proteins. These chaperone proteins including NSF, Munc-18, CDCrel-1 and the Rab family of Ras GTPases, regulate the interaction of SNARE proteins. Others are required to stabilise and sort SNAREs to the appropriate membranes or to promote and select SNARE-SNARE interactions (e.g. Sec1 and Munc19) (Flaumenhaft 2003). Many of these granule secretion regulators are sensitive to second messengers such as diacylglycerol (DAG) and Ca^{2+} and are regulated via phosphorylation by several intracellular kinases including protein kinase C (PKC).

1.3.2 Integrin activation

Integrins make up a family of heterodimeric transmembrane receptors that connect the platelet cytoskeleton to the ECM of the blood vessel wall and act as bidirectional signalling molecules via the processes of inside-out and outside-in signalling (Hynes 2002). Integrins are made up of noncovalently bound α and β subunits. Each subunit has a large extracellular domain that spans the membrane once and has a short cytoplasmic domain. Integrins exist in an inactive low affinity conformation that shifts via a conformational change to a high affinity state with increased ligand affinity following platelet activation and subsequent inside-out signalling (Ginsberg, Partridge et al. 2005). Platelets express β1 and β3 integrins including α2β1 and αIIbβ3.

A wide variety of agonists trigger inside-out integrin activation by increasing levels of Ca^{2+} and DAG which lead to the activation of PKC and the Rap1 exchanger protein CalDAG-GEF1 which subsequently activate and translocate the GTPase Rap1 to the plasma membrane. Rap1 binds and interacts with its effector molecule RIAM at the plasma membrane leading to recruitment and interaction with talin-1. Binding of talin, a key step in integrin activation, disrupts the salt bridge found between the two transmembrane regions of the α and β integrin subunits causing a conformational

change in the extracellular domain converting the integrin from the low to the high affinity state for ligand binding. The final step also requires the recruitment and binding of kindlin-3 to the β tail although the full details of the molecular basis of this are not fully understood (Figure 1.3) (Han, Lim et al. 2006; Hidalgo and Frenette 2009). Once activated and bound to the ligand, integrins cluster at focal adhesion sites where the actin filaments that make up the cytoskeleton meet the membrane. Once bound to a ligand, integrins signal into the cell, a process known as outside-in signalling. In platelets this signalling contributes to the remodelling of the cytoskeleton leading to the formation of filopodia and lamellipodia (Ginsberg, Partridge et al. 2005).

1.3.3 Actin polymerisation

Platelet activation is associated with major changes in actin remodelling (Hartwig 1992) and formation of filopodia, lamellipodia and stress fibres. Filopodia are membrane protrusions that are supported by bundles of extending actin filaments. They are transiently formed and replaced by the formation of sustained lamellipodia (Frojmovic, Longmire et al. 1990). This change in platelet morphology following platelet activation is mediated by the rapid reorganisation of the actin cytoskeleton through a combination of uncapping, severing and nucleation of the actin filaments. Several regulators of these processes have been identified including the Rho GTPases, Rac and Cdc42, the Arp2/3 complex, Rif and VASP (vasodilator-stimulated phosphoprotein) many of which are thought to be regulated by PKC (Pula, Schuh et al. 2006).

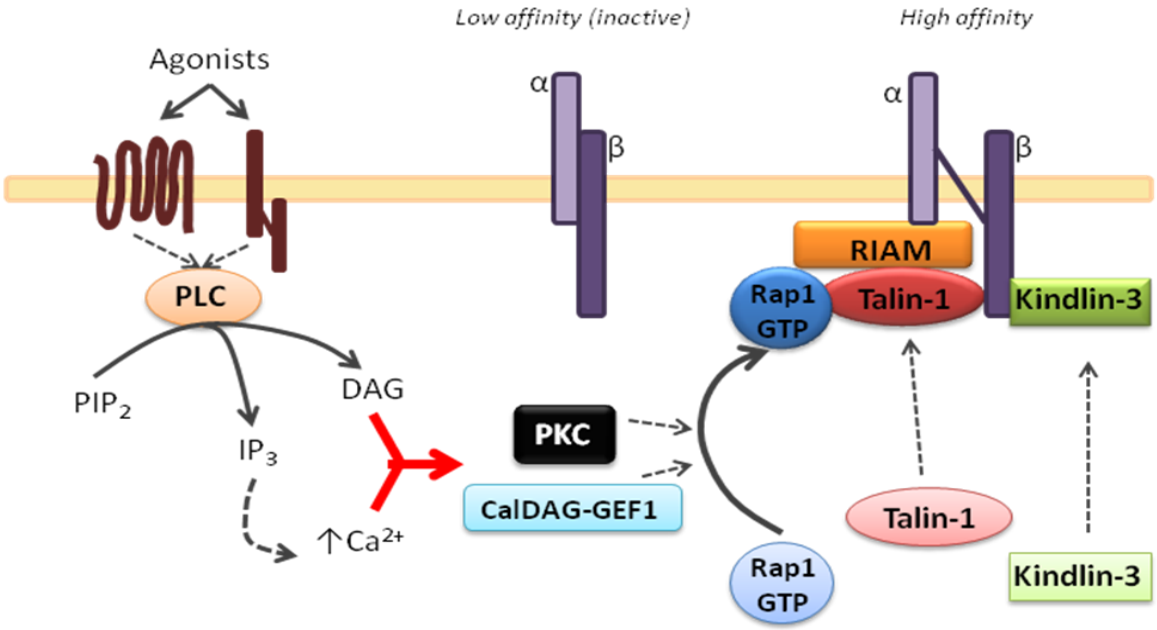


Figure.1.3 Integrin Activation. The signalling pathways that lead from agonist stimulation to αIIbβ3 integrin activation via PKC and Rap1-GTPase mediated pathways through the recruitment of talin-1 and kindlin-3 to the inactive integrin complex. DAG and intracellular Ca^{2+} activate PKC and CalDAG-GEF1 which mediate activation of Rap1 and RIAM, that recruit talin-1, which in addition to kindin-3 enable αIIbβ3 integrin activation. Figure adapted from Han et al, 2006, and Hidalgo et al, 2009.

1.4 Platelet Signalling

Platelet agonists bind to and activate platelets via their specific receptors which initiate a series of signalling cascades. The majority of these signalling pathways converge at the activation of phospholipase C (PLC), resulting in the generation of the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) (Sargeant and Sage 1994). Many receptors also liberate the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3).

1.4.1 Receptor signalling pathways

Platelets receptors in general, can be subdivided on whether they signal primarily through tyrosine kinase-linked or G protein-coupled pathways (Figure 1.4).

1.4.1.1 Tyrosine kinase linked pathways

This class of receptors includes the ITAM linked GPVI, the hemITAM linked CLEC-2, the integrins and the leucine rich repeat proteins such as GPIb-IX-V although their mechanism of regulation and extent of activation do differ. These tyrosine kinase linked pathways lie upstream of PLCγ2 and PI3-kinase activation. Key players in these pathways include a combination of the non-receptor tyrosine kinases, Src, Fyn, Lyn and Syk, and adaptor proteins such as Shc, LAT (linker for activation of T cells), Gads, Grb2, Cbl and SLP-76 (Gibbins 2004). Tyrosine kinases phosphorylate specific tyrosine residues on the receptors and adapter proteins following agonist stimulation. The majority of the proteins involved in these pathways share domains which play key roles in protein-protein and protein-lipid interactions enabling signal transduction. Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains bind directly to phosphorylated tyrosine residues within particular recognition sequences on activated platelet receptor cytoplasmic domain tails or on associated effector proteins. Additional domains

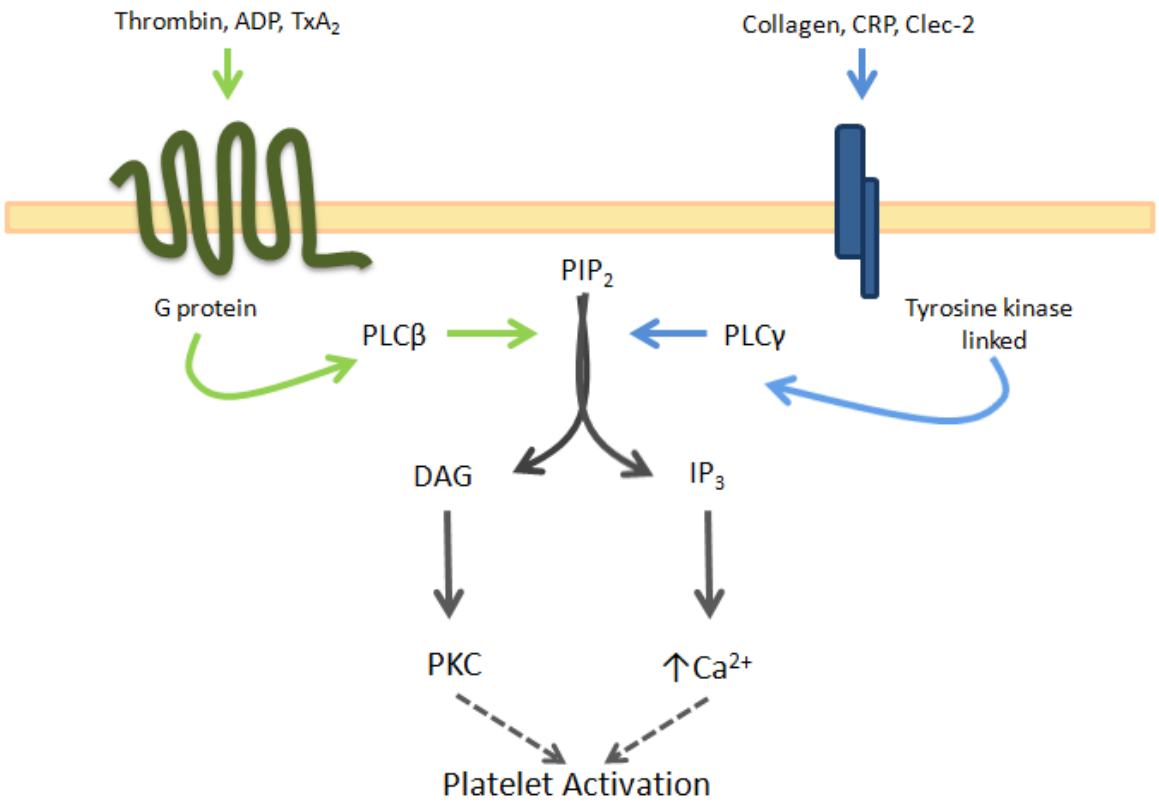


Figure 1.4 Major agonist signalling pathways. Summary of the activatory G protein coupled and tyrosine kinase linked signalling pathways which converge at activation of phospholipase C (PLC), leading to the production of diacylglycerol (DAG) and inositol phosphate (IP_3). These then lead to the activation of key cellular mediators, such as protein kinase C (PKC) and increases in levels of intracellular Ca^{2+}.

including Src homology 3 (SH3) and pleckstrin homology (PH) domains are also involved. Many of the signalling proteins that contain these domains have enzymatic activity, which in combination with several adaptor proteins enable tyrosine kinase linked signal transduction. For more detailed receptor signalling pathways see later receptor signalling sections.

1.4.1.2 G protein-coupled receptor signalling.

G protein-coupled receptors (GPCRs) are seven transmembrane domain signalling molecules that play essential roles in platelet signalling (Offermanns 2006). Several key platelet agonists bind to and stimulate G protein-coupled receptors including, thrombin, ADP, TxA_2 and epinephrine/adrenaline. GPCRs are coupled to heterotrimeric G-proteins containing Gα, Gβ and Gγ subunits. GPCRs are classified according to the type of α-subunit that they associate with: in platelets these are $G\alpha_q$, $G\alpha_{12/13}$, $G\alpha_i$, $G\alpha_z$ and $G\alpha_s$. In their inactive state (GDP bound), the Gα subunit has high affinity for the Gβγ complex. Ligand binding and receptor activation causes a conformational change within the Gα subunit which promotes dissociation from GDP and thereby enabling binding of GTP. This conversion to the GTP bound state favours dissociation from the Gβγ subunit enabling target protein binding to Gα. The dissociated Gβγ is also able to activate effector molecules. GPCR activation involves several pathways:

i) stimulation of PLCβ isoforms via G_q

ii) reorganisation of the actin cytoskeleton via $G_{12/13,}$

iii) suppression of cAMP formation and activation of PI-3K through G_i (Figure 1.5)

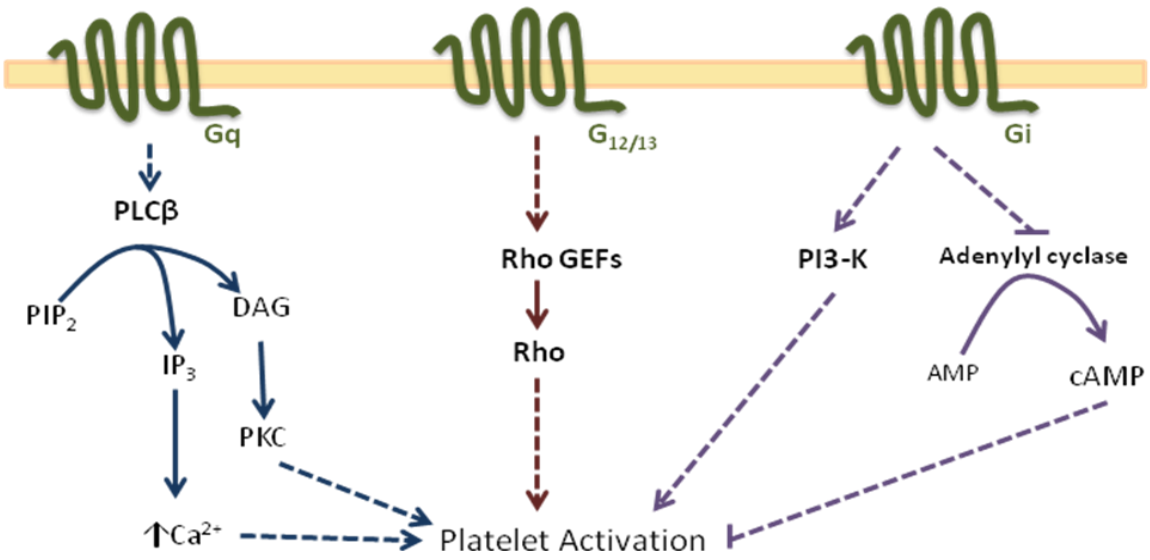


Figure 1.5 G protein coupled receptor signalling pathways. The major signalling pathways downstream of the 3 stimulatory GPCRs classes in platelets. G_q stimulates PLCβ isoforms, increasing in cytosolic Ca^{2+} and activating PKC. $G_{12/13}$ activates cytoskeletal rearrangements. G_i suppresses cAMP formation via inhibition of adenylyl cyclise and activates PI-3K signalling pathways.

1.4.2 Platelet agonists and their receptors

This section focuses on those agonists and receptors that are important for this study.

1.4.2.1 Collagen

There are several types of collagen that are expressed in the blood vessel wall, types I and III are found in the ECM and type IV is found in the subendothelial basement membrane. Collagen consists of a repeated glycine-proline-hydroxyproline, GXY (or GPO) motif, and forms a fibrous structure that usually consists of more than one collagen type in combination with other ECM components. Platelets express two major collagen receptors, GPVI and α2β1 both of which bind to and are activated by the GPO collagen motif (Nieswandt and Watson 2003). The most commonly used collagen in platelet functional studies and the one used in this thesis is 'HORM' collagen, which is made up of fibrils that contain equine collagen type 1 and a small amount of type III in addition to low levels of other ECM proteins. In addition synthetic collagen, CRP (collagen related peptide) which is based on GPO collagen repeats is also used. CRP is a GPVI specific agonist and unlike collagen does not activate platelets through α2β1 (Morton, Hargreaves et al. 1995; Asselin, Gibbins et al. 1997; Asselin, Knight et al. 1999).

GPVI

GPVI is an approximately 60-65kDa platelet specific protein and a member of the Ig receptor superfamily (Clemetson, McGregor et al. 1982; Sugiyama, Okuma et al. 1987; Clemetson, Polgar et al. 1999). GPVI consists of two extracellular Ig domains linked to a mucin-like region, a short peptide linker sequence, a transmembrane domain that enables association with the FcR-γ chain and a short cytoplasmic tail that consists of a number of domains and is able to bind the Src kinases Fyn and Lyn via their SH3 domains (Suzuki-Inoue, Tulasne et al. 2002). The associated FcR γ-chain is a disulphide

homodimer and contains the conserved ITAM (immunoreceptor tyrosine-based activation motif) sequence that acts as the signal-transducing subunit of the receptor. Upon binding to collagen or CRP and the crosslinking of GPVI, the constitutively bound Src tyrosine kinases Fyn and Lyn phosphorylate the ITAM sequence, which allows binding and activation of Syk via its SH2 domain and initiates further downstream signalling involving LAT, SLP-76 and Gads effector proteins. Ultimately PLCγ2 is activated followed by the generation of essential second messengers and platelet activation (Blake, Schieven et al. 1994; Poole and Watson 1995; Yanaga, Poole et al. 1995; Gibbins, Asselin et al. 1996; Poole, Gibbins et al. 1997; Ezumi, Shindoh et al. 1998; Quek, Pasquet et al. 2000; Watson, Auger et al. 2005) (Figure 1.6). Mice deficient in GPVI or the FcR-γ chain show significantly reduced platelet responses to collagen and show drastically impaired thrombus formation, highlighting the major role for this receptor in collagen signalling (Poole, Gibbins et al. 1997; Kato, Kanaji et al. 2003).

α2β1

In addition to GPVI, the integrin α2β1 also plays a role in the adhesion of platelets to collagen and collagen induced platelet activation. Unlike mice deficient in GPVI that show significantly impaired platelet activation and thrombus formation, mice deficient in α2β1 only show minor defects and display normal bleeding times (Holtkotter, Nieswandt et al. 2002; Gruner, Prostredna et al. 2003). α2β1 binds to collagen following activation via inside-out signalling in a mechanism similar to αIIbβ3 (see 1.4.2.3.1) (Cosemans, Iserbyt et al. 2008) and works in combination with GPVI to support stable platelet adhesion and platelet activation (Nieswandt and Watson 2003). Collagen binding to α2β1 initiates an outside-in signalling cascade that is similar to that induced

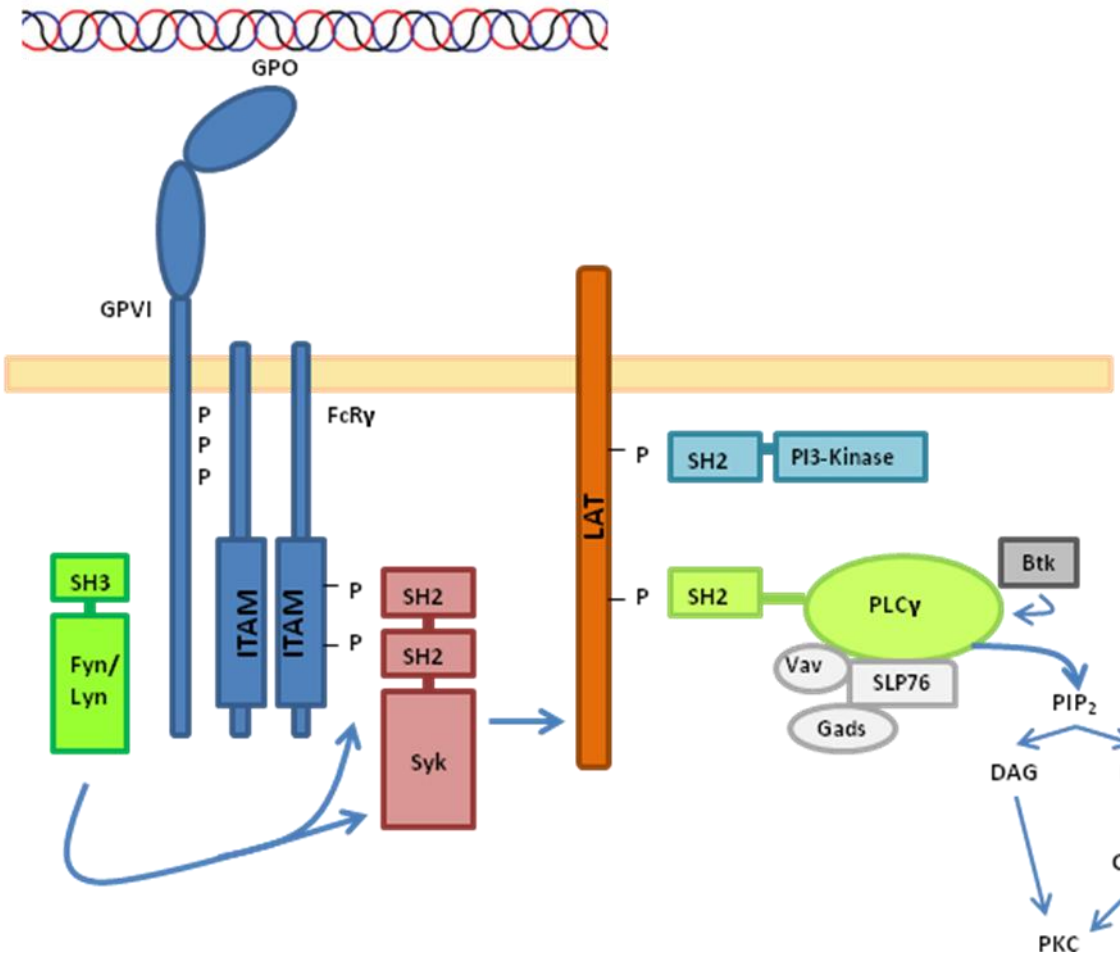


Figure 1.6 GPVI signalling. Binding of collagen induces activation, crosslinking and tyrosine phosphorylation of the GPVI/FcRγ- chain ITAM sequences by the Src kinases Fyn and Lyn. This then initiates a Syk dependent signalling pathways with the activation of several downstream proteins including LAT and SLP-76 and also PLCγ which liberating DAG and IP3, leading to the release of intracellular Ca^{2+} and the activation of PKC.

by GPVI involving Src, Syk, SLP-76 and PLCγ2, although the signalling is far weaker and on its own, α2β1 is unable to support platelet aggregation (Inoue, Suzuki-Inoue et al. 2003; Nieswandt and Watson 2003). The major role of α2β1 is to bring about a net increase in binding of collagen to GPVI. Mice deficient in both α2β1 and GPVI show a complete impairment in the ability to form thrombi unlike the partial defects observed in the single receptor deficient mice, therefore indicating that the two receptors work synergistically to bring about collagen-induced platelet activation and thrombus formation (Sarratt, Chen et al. 2005).

1.4.2.2 Thrombin

Thrombin is a serine protease that is formed via the cleavage of prothrombin during the first stages of the coagulation pathway. Thrombin subsequently mediates the cleavage of fibrinogen to fibrin which is required for stable thrombus formation and also activates platelets via the GPCRs, the protease activated receptors (PAR).

PAR receptors

Thrombin activates platelets through the G protein coupled PAR-1 and PAR-4 receptors in human platelets and PAR-3 and PAR-4 in mouse platelets. In mouse platelets only PAR-4 is able to signal and induce platelet activation with PAR-3 acting as a cofactor for activation of PAR-4 (Nakanishi-Matsui, Zheng et al. 2000). Activation occurs when thrombin cleaves a short inhibitory peptide from the extracellular N terminal of the PAR receptor that binds the receptor initiating a conformational change that causes activation. Synthetic peptides can be made that mimic these short peptide sequences, PAR-1 peptide, SFLLRN (Ser-Phe-Leu-Leu-Arg-Asn) (also known as TRAP peptide is used in these studies) and PAR-4 peptide, GYPGKF (Gly-Tyr-Pro-Gly-Lys-Phe) or AYPGKF (Ala-Tyr-Pro-Gly-Lys-Phe). Activation of PAR-1 requires lower concentrations of thrombin than

activation of PAR-4. All PAR receptors are 7 transmembrane receptors that couple to G_q and G_{12} leading to the activation of PLCβ (Kahn, Zheng et al. 1998; Kahn, Nakanishi-Matsui et al. 1999; Brass 2003), see section '1.4.1.2 G protein-coupled Receptor Signalling' for further signalling details.

1.4.2.3 GPIIbIIIa Ligands - Fibrinogen

Fibrinogen a soluble glycoprotein is synthesised by the liver and phagocytosed by platelets and stored in the α-granules. Fibrinogen binds to the integrin αIIbβ3 receptors on platelets mediating platelet outside-in signalling and supports aggregation. During the process of coagulation fibrinogen is converted into fibrin which plays an essential role in the crosslinking and the formation of a fibrin mesh that enables stable thrombus formation and clot retraction.

Integrin αIIbβ3

αIIbβ3 the dominant integrin on the platelet surface, is expressed at between 50-200 times greater level than that of the other integrins. It is a receptor for a number of ECM proteins including vWF, fibronectin and fibrinogen. αIIbβ3 mediates platelet aggregation through the binding of plasma fibrinogen and serves as the main receptor for platelet adhesion to the ECM (Gruner, Prostredna et al. 2003). Clustering of αIIbβ3 by fibrinogen results in the generation of intracellular signals (outside-in signalling) with the activation of Src and Syk kinases (Miranti, Leng et al. 1998). Src kinase is constitutively associated via its SH3 domain to the β3 tail of αIIbβ3 and following agonist binding and integrin clustering becomes activated via autophosphorylation (Arias-Salgado, Lizano et al. 2003). This activation of Src leads to the activation and recruitment of Syk kinase (via its SH2 domains to the β3 tail) (Woodside, Obergfell et al. 2001; Woodside, Obergfell et al. 2002) although unlike GPVI this regulation of Src and Syk is ITAM independent. Activation of

Syk leads to the activation of several downstream effectors including SLP-76, Vav1/3 and PLCγ2 leading to the activation and release of secondary messengers calcium and PKC (Pelletier, Bodary et al. 1992; Judd, Myung et al. 2000; Giuliano, Nesbitt et al. 2003; Watson, Auger et al. 2005), unlike GPVI signalling, signalling via αIIbβ3 appears to be independent of LAT (Pasquet, Gross et al. 1999; Wonerow, Obergfell et al. 2002) as detailed in Figure 1.7. αIIbβ3 can also activate platelets via a Syk independent outside-in signalling mechanisms in which both αIIbβ3 and Src appear to interact and associate with FAK (the focal adhesion kinase) (Woodside, Obergfell et al. 2001).

1.4.2.4 CLEC-2 ligands

Rhodocytin

Rhodocytin a C-type lectin snake venom toxin was found to be capable of stimulating powerful platelet activation through a novel Src kinase regulated pathway, that was shown to be independent of GPVI (Inoue, Ozaki et al. 1999). Rhodocytin was isolated from the venom of the Malayan pit viper, *Calloselasma rhodostoma* (Huang, Liu et al. 1995; Shin and Morita 1998) and has been shown to mediate platelet activation through the CLEC-2 receptor (Suzuki-Inoue, Fuller et al. 2006; Suzuki-Inoue, Inoue et al. 2011).

Podoplanin

In addition to rhodocytin, podoplanin, a type I transmembrane sialomucin-like glycoprotein, was identified to be the endogenous ligand for CLEC-2 (Suzuki-Inoue, Kato et al. 2007). Podoplanin is found in several human tissues, including the lung type I alveolar cells, kidney podocytes and the lymphatic endothelial cells and is also found to be up regulated in a variety of tumours and is not necessarily thought to be involved in the regulation of haemostasis.

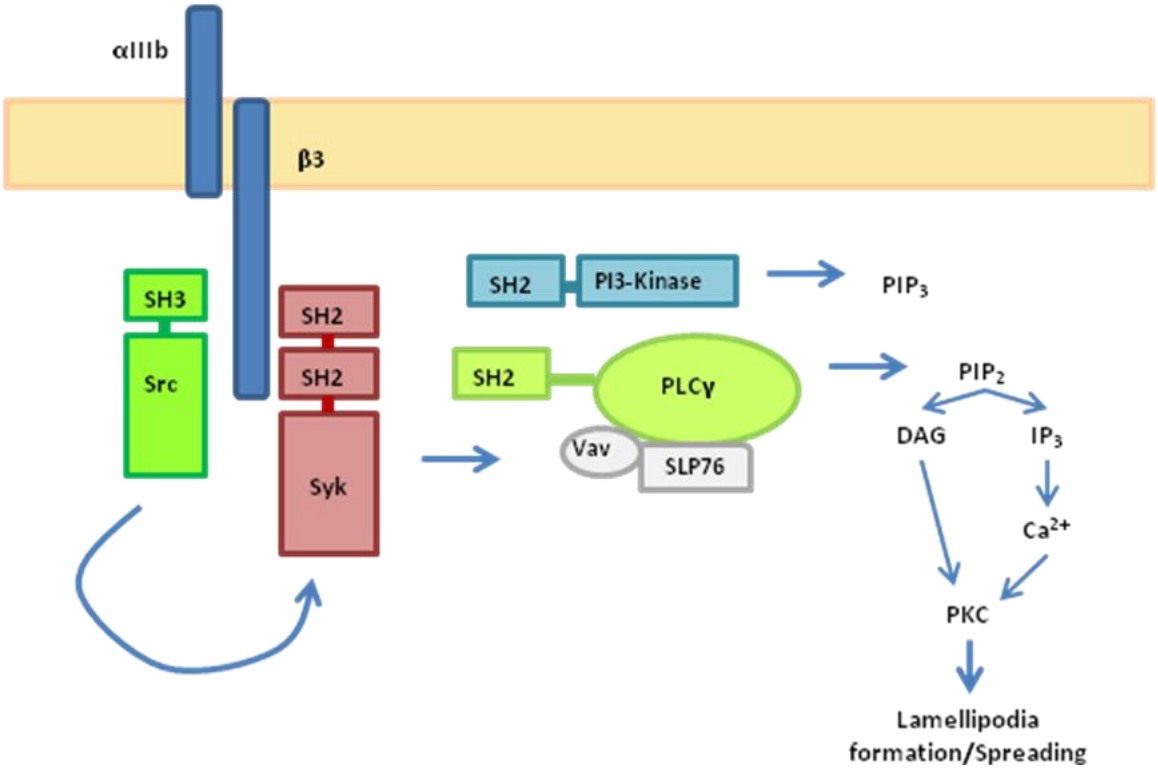


Figure 1.7. αIIbβ3 outside-in signalling via a Syk-dependent cascades. Binding to fibrinogen promotes integrin clustering and activation of Src kinase. Src-dependent activation of Syk then leads to the activation of PLC*γ*2 via activation of SLP-76 and Vav ultimately leading to activation of platelet spreading and generation of filopodia and lamellipodia.

CLEC-2

CLEC-2 is a non-classical C-type lectin receptor that is expressed at high levels in platelets and is activated by binding either rhodocytin or podoplanin. CLEC-2 is a type II membrane protein that consists of an N-terminal cytoplasmic tail containing a single YxxL sequence, termed a hemITAM, that is unique from ITAM, ITIM and ITSM YxxL sequences found in other signalling proteins, a single transmembrane spanning domain, a stalk region and a C terminal carbohydrate-like recognition domain, that is modified in that it does not bind carbohydrates and instead is involved in protein-protein interactions. Following activation CLEC-2 dimerises, translocates to lipid rafts and stimulates Src kinase dependent phosphorylation of the tyrosine of the YxxL sequence in its cytoplasmic tail, which then enables signal transduction through a Syk kinase dependent pathway, in which Syk is thought to bind to the CLEC-2 dimer via its phosphorylated hemITAM to initiate signalling (Watson, Herbert et al. 2010).

Despite the novel mechanism of Syk activation and regulation, CLEC-2 stimulates similar patterns of tyrosine phosphorylation as those seen downstream of GPVI-FcRγ-chain complex, indicative of an ITAM-like signalling pathway in which several adapter proteins are recruited and activated including the Tec family tyrosine kinases which leads to the activation of various effector proteins including PI3-kinase, Vav, Rac1 and PLCγ2. (Suzuki-Inoue, Fuller et al. 2006; Hughes, Pollitt et al. 2010). Interestingly, although required at low agonist stimulation, the need for LAT and Gads (as required by GPVI signalling) is overcome downstream of high stimulation of CLEC-2. CLEC-2 stimulation can also initiate weak platelet activation in the absence of SLP-76, unlike GPVI signalling which appears to be SLP-76 dependent (Suzuki-Inoue, Fuller et al. 2006).

Activation of CLEC-2, unlike GPVI is also dependent on several other factors and several feedback events, including actin polymerisation, release of secondary mediators such as ADP and TxA_2 and also the activation of the small G protein Rac (Pollitt, Grygielska et al. 2010) which all act to enhance CLEC-2 signalling (Figure 1.8).

1.4.2.5 ADP

The nucleotide adenosine diphosphate (ADP) is stored in and released from the dense granules following platelet activation by all stimulatory platelet agonists. Following its release it acts as a secondary mediator of platelet activation, where via a positive feedback mechanism it stimulates and enhances platelet activation so as to sustain the haemostatic response (Mills 1996; Jin, Quinton et al. 2002). Despite its importance as a feedback mediator, ADP is a weak platelet agonist when compared to the other major stimulatory platelet agonists. Studies using pharmacological inhibitors have revealed ADP-induced platelet activation is dependent on TxA_2 synthesis and outside in signalling through αIIbβ3 (Jin, Quinton et al. 2002). ADP is capable of stimulating increases in intracellular Ca^{2+}, TxA_2 synthesis, protein phosphorylation, platelet shape change, granule secretion, integrin αIIbβ3 activation and aggregation (Jin, Quinton et al. 2002). Apyrase, an ADP scavenger, is sometimes used to block the effect of ADP released from platelets to limit observations to those of the agonist and its receptors of interest. ADP, signals through two different GPCRs, $P2Y_1$ and $P2Y_{12}$ (Woulfe, Yang et al. 2001; Rivera, Lozano et al. 2009) (Figure 1.9).

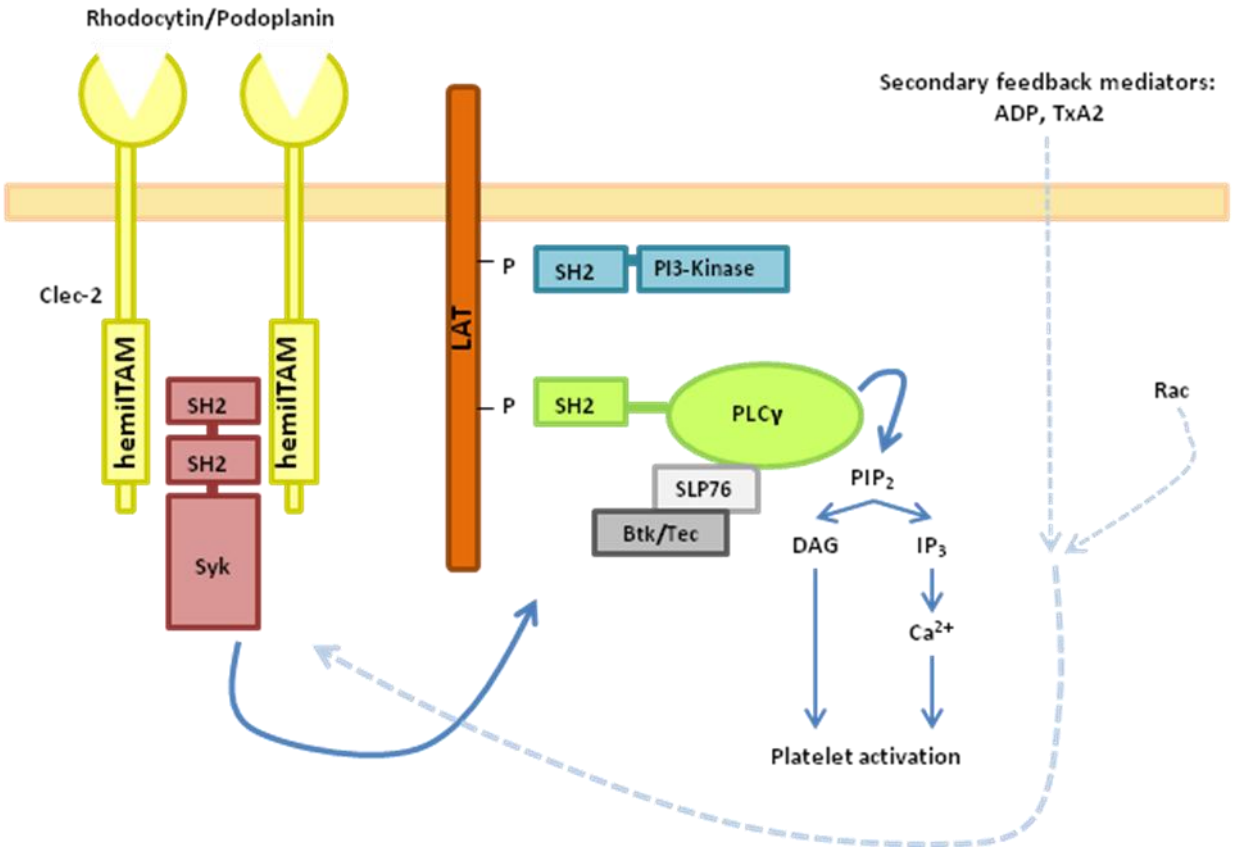


Figure 1.8. CLEC-2 Signalling. On binding to its ligand (exogenous; rhodocytin, endogenous; podoplanin), CLEC-2 dimerises, translocates to lipid rafts and becomes tyrosine phosphorylated. This enables the interaction with and activation of Syk, which then initiates a Syk-dependent signalling cascade leading to the activation of PLCγ2 and the production of DAG and IP_3. CLEC-2 signalling is reinforced and amplified by the secondary mediators ADP and TxA_2 and the activation of the small G protein Rac.

$P2Y_1$

ADP signals through the Gq-protein coupled $P2Y_1$ receptor which stimulates platelet activation through a PLC dependent pathway. Upon activation the $P2Y_1$ receptor activates PLCβ isoforms leading to the formation of the second messengers DAG and IP_3 which results in the mobilisation of Ca^{2+} and subsequent platelet activation (Gachet, Hechler et al. 1997; Murugappa and Kunapuli 2006). Stimulation of the $P2Y_1$ receptor is sufficient for the stimulation of weak responses to ADP, in which shape change and transient aggregation is observed. $P2Y_1$ has also been shown to be involved in the early stages of platelet activation and in the completion and amplification of (via feedback signalling) platelet aggregation by ADP and other platelet agonists. However, the $P2Y_1$ receptor is only expressed at low levels on the platelet surface and although it can initiate platelet aggregation it is not sufficient for stimulation of a full platelet aggregatory response to ADP and requires synergy with the $P2Y_{12}$ receptor for full activation.

$P2Y_{12}$

In addition to the $P2Y_1$ receptor, ADP also signals though the G_i-coupled $P2Y_{12}$ receptor (Gachet, Hechler et al. 1997; Murugappa and Kunapuli 2006). Unlike with the $P2Y_1$ receptor sole stimulation of the $P2Y_{12}$ receptor is not sufficient to trigger platelet activation. However, it does facilitate complete activation in response to ADP (Daniel, Dangelmaier et al. 1998; Hardy, Conley et al. 2005). Stimulation of $P2Y_{12}$ inhibits adenylyl cyclase preventing the production of the inhibitory molecule cAMP, and also activates PI 3'-kinase mediating stabilisation of forming platelet aggregates (Gachet, Hechler et al. 1997; Murugappa and Kunapuli 2006). The latter is believed to underlie the ability of $P2Y_{12}$ to synergise with other Ca^{2+}-mobilising receptors including Gq- and

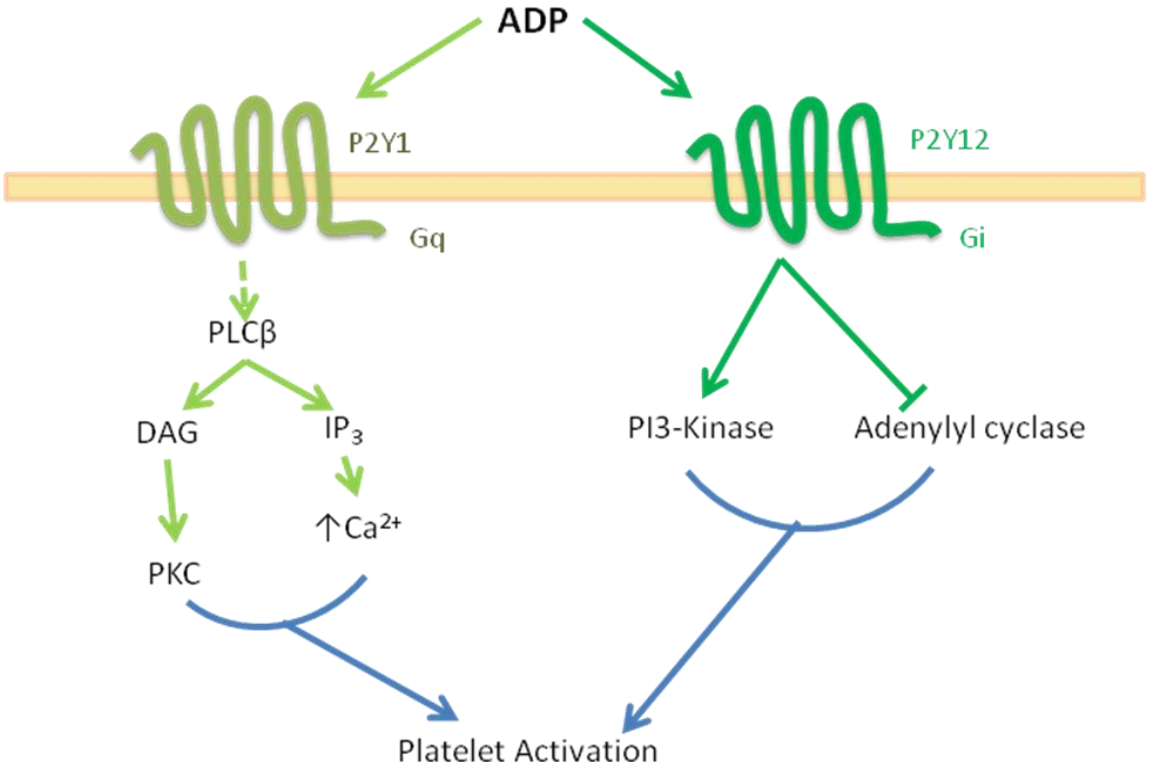


Figure 1.9. ADP signalling through $P2Y_1$ and $P2Y_{12}$. ADP activates both the G_q and G_i protein coupled signalling pathways through the $P2Y_1$ and $P2Y_{12}$ receptors respectively. P2Y1 works via the activation of PLCβ, leading to the production of secondary messengers DAG and IP_3 in contrast $P2Y_{12}$ activates PI3-kinase and inhibits adenylyl cyclise. The synergy of these two pathways leads to full platelet activation in the response to ADP.

tyrosine kinase-receptor regulated pathways, such as the $P2Y_1$ receptor (Jin and Kunapuli 1998; Pulcinelli, Ciampa et al. 1999), the TxA_2 receptor and integrin αIIbβ3 (Cattaneo, Gachet et al. 2002; Kahner, Shankar et al. 2006). Signalling through the $P2Y_{12}$ receptor is therefore involved in all cases where ADP acts as a co-stimulus in the presence of low concentrations of other agonists.

The roles of the two different ADP receptors can be distinguished using specific receptor antagonists, MRS-2179 ($P2Y_1$) and ARC-69931MX/Cangrelor ($P2Y_{12}$).

1.4.2.6 Thromboxane A_2

TxA_2 is a lipid that is synthesised following platelet activation, when platelets liberate arachidonic acid (stored as a phospholipid at the plasma membrane) and convert it to TxA_2 via oxygenation catalysed by cyclooxygenase and TxA_2 synthase (Samuelsson, Goldyne et al. 1978). TxA_2 acts as a vasoconstrictor and is a potent platelet agonist capable of stimulating platelet shape change, granule secretion, aggregation, phosphoinositide hydrolysis, Ca^{2+} mobilisation and protein phosphorylation (Rivera, Lozano et al. 2009). TxA_2 when released by the platelet, like ADP, acts as a secondary mediator of platelet activation, providing a positive feedback mechanism to enable the activation of more platelets, sustaining the process of thrombus formation (FitzGerald 1991; Hourani and Cusack 1991; Nakahata 2008). TxA_2 signals through a G protein-coupled receptor, the TP receptor. TxA_2 release and production can be studied using the TxA_2 analogue U46619 which activates the TP receptor, and can inhibited using inhibitors of the cyclooxygenase, such as aspirin and indomethacin.

TP receptor

The TxA_2 receptor (TP receptor), a G protein coupled receptor, exists in two variants, (TPα and TPβ), biochemical studies have shown that the TP receptor couples to both G_q

and $G_{12/13}$ signalling pathways (although it is thought that this is mainly through G_{13}, with G_{12} mediating secondary event signalling), and could also couple to a G_i pathway although this is controversial. In platelets the TP receptor is therefore thought to have the capacity to couple to different G protein coupled receptor families that enable the initiation of several downstream signalling events, including activation of PLCβ and activation of the Rho/Rho kinase pathways contributing to overall shape change, aggregation and secretion producing a full activation response (Dorn 1989; Takahara, Murray et al. 1990; Huang, Ramamurthy et al. 2004).

1.5 Intracellular Signalling Molecules.

1.5.1 Phospholipase C and the production of DAG and IP_3

The production of DAG and IP_3 via signalling through activation of the phospholipase C isoforms has been identified as a central pathway in the majority of platelet responses, as it is essential for the activation and regulation of many important effectors and mediators of platelet activation such as PKC and increases in cytosolic Ca^{2+}. As previously mentioned, the PLCβ isoform is activated following signalling via GPCRs, including the PAR, TP and $P2Y_1$ receptors whilst PLCγ is activated downstream of the tyrosine kinase linked and integrin signalling pathways, such as GPVI, CLEC-2, GPIb-IX-V and αIIbβ3 (Falati, Edmead et al. 1999; Watson 1999; Nieswandt, Brakebusch et al. 2001; Suzuki-Inoue, Fuller et al. 2006; Varga-Szabo, Braun et al. 2009) (Figure 1.4). The action of DAG can be mimicked by phorbol esters such as PMA (phorbol 12-myristate 13-acetate) which bind to and can activate DAG binding proteins such as the PKCs.

1.5.2 Calcium (Ca^{2+})

The second messenger Ca^{2+} acts as a cofactor for many cellular proteins enabling their activation and regulation, including PKC. Platelet activation is critically controlled by changes in the levels of intracellular Ca^{2+}, with roles for Ca^{2+} in cytoskeleton rearrangements, integrin activation, granule secretion and procoagulant activity (Hathaway and Adelstein 1979; Shattil and Brass 1987; Varga-Szabo, Braun et al. 2009). Platelet agonists increase levels of intracellular Ca^{2+} by stimulating Ca^{2+} release from intracellular stores via the IP_3 receptors (IP_3R) located on the dense tubular system membrane and also by activating entry of extracellular Ca^{2+} through the plasma membrane, a process that has also been shown to be regulated by DAG, although the significance of this latter pathway is uncertain (Bird, Aziz et al. 2004). Entry of extracellular Ca^{2+} occurs mostly through store-operated Ca^{2+} entry (SOCE) which is controlled by the concentration of Ca^{2+} within the intracellular stores/sarcoplasmic reticulum. Following a decrease in intracellular store Ca^{2+} concentration, as a result of IP_3 induced Ca^{2+} release, STIM1 (stromal interaction molecule 1) translocates to the plasma membrane and activates and opens the Orai1 channels stimulating extracellular Ca^{2+} entry (Muik, Frischauf et al. 2008; Varga-Szabo, Braun et al. 2008; Bergmeier, Oh-Hora et al. 2009; Braun, Gessner et al. 2009). Entry of extracellular Ca^{2+} also occurs through non-SOCE mediated by DAG through activation of TRPC6 (transient receptor potential channel 6) (Hassock, Zhu et al. 2002) although whether this has any functional significance in the regulation of thrombosis and haemostasis is uncertain as normal platelet agonist-induced activation responses have been observed in platelets from mice deficient in TRPC6 (Ramanathan, Gupta et al. 2011). In addition the elevation of intracellular calcium is also supported through the activation of $P2X_1$ and a Na^+/Ca^{2+}

exchanger (Vial, Rolf et al. 2002). In order to regulate and alter the levels of intracellular Ca^{2+}, mechanisms exist to remove cytosolic Ca^{2+} and reduce Ca^{2+} concentration. SERCAs (sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases) pump cytosolic Ca^{2+} back into the intracellular stores and PMCAs (plasma membrane Ca^{2+} ATPases) pump Ca^{2+} through the plasma membrane and out of the cell (Enyedi, Sarkadi et al. 1986). Several of these Ca^{2+} channels and pumps are subjected to regulation by intracellular and kinases which can therefore effect changes in the levels of intracellular Ca^{2+} (Dean, Chen et al. 1997; Wan, Zabe et al. 2003). For example it has been shown that Ca^{2+} regulated PKC also regulates levels of intracellular Ca^{2+} (Gilio, Harper et al. 2010; Harper, Molkentin et al. 2010).

1.6 Protein Kinase C

Protein kinase C (PKC) is a family of closely related serine/threonine kinases (Stabel and Parker 1991) that is a member of the AGC family of serine/threonine kinases. PKC consists of several different isoforms that play key regulatory roles in intracellular signalling pathways in a variety of cell types, regulating many cellular processes including cell growth, differentiation, cell survival, neurotransmission and carcinogenesis (Mellor and Parker 1998).

1.6.1 The isoforms of PKC

The term 'isoform' is usually used to describe several different forms or similar proteins that are variants or polymorphisms of the same gene by alternative splicing. However, the convention in the literature in regards to PKC is to use the term 'isoform' although the correct term would be 'isoenzyme/isozyme' as the different PKC family members are products of different genes despite being similar in amino acid sequence and function.

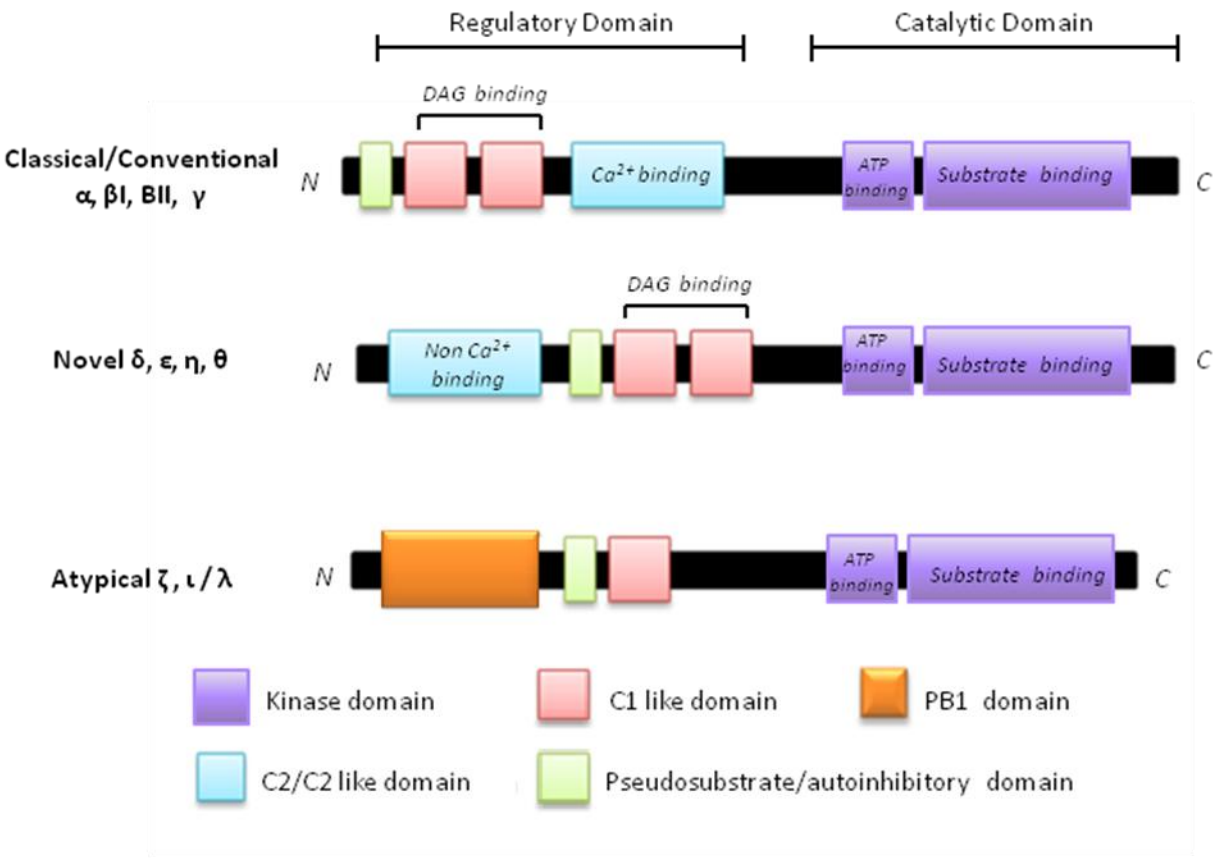
Several isotypes/ isoforms of protein kinase C (PKC) have been identified and then further classified into three different subtypes according to their structure and mechanisms of regulation; the conventional (or classical) PKCs (cPKCs), the novel PKCs (nPKCs) and the atypical PKCs (aPKCs). The cPKCs are PKCα, βI/II and γ, the nPKCs; PKCδ, ε, η and θ and the aPKCs, PKCζ and ι/λ (Newton 1997; Mellor and Parker 1998). PKCμ or as it is otherwise known, PKD, shows high homology to some of the domains in the other PKC's but differs in others. For the purposes of this thesis it is not considered to be a member of PKC family as it is recognised as a distinct family of PKD kinases (Wang 2006).

1.6.2 Structure of PKC

The PKC isoenzymes are made up of a single polypeptide chain that contains an amino terminal regulatory region and a carboxyl-terminal kinase domain: a schematic of PKC structure is outlined in figure 1.10. The regulatory region of PKC contains one or two membrane targeting motifs called the C1 and C2 domains and an autoinhibitory domain. The C1 domain is able to bind to the activators DAG or phorbol esters whilst the C2 domain is capable of binding to Ca^{2+} and acidic phospholipids such as phosphatidylserine (PS). The catalytic domain of PKC contains the ATP-binding site and the substrate binding site, which contains three key sites required for catalytic activity, the activation loop, the turn motif and the hydrophobic motif.

1.6.3. Regulation of the PKC subtypes

There is increasing evidence that the individual isoforms have different and distinct roles in cell signalling and regulation. These distinct roles could be due to different modes of regulation, different substrate specificities and/or the association of the different



Isoform	Number of amino acids	Homology
α	672	≥70% sequence homology is observed between the different conventional PKC isoforms.
βI/βII	671/673	
γ	697	
δ	676	≤40% sequence homology is observed between the different novel PKC isoforms.
θ	706	
ε	737	
η	683	
ζ	592	≥80% sequence homology is observed between these atypical isoforms.
ι / λ	596	

Figure 1.10 Structure of the PKC isoforms. High sequence homology exists between the different isoforms of PKC. Despite this the different classes of isoforms show different modes of regulation. The conventional isoforms (PKCα, ßI, ßII ,γ) via their C2 domains, require both DAG and Ca^{2+} for full activation. In contrast the novel isoforms (PKCδ, ϵ, η, θ) have C2 domains that are unable to coordinate Ca^{2+} and are therefore only DAG responsive. Both are also regulated by phospholipids which helps target these proteins to the membrane. The atypical isoforms (PKCζ, ι/λ) have altered C1 domains and are not DAG or Ca^{2+} sensitive . Figure adapted from Parker and Murry-Rust (2004).

isoforms with distinct binding proteins.

1.6.3.1 Maturation

All of the isoforms of PKC exist in a catalytically inactive state and require phosphorylation on serine, threonine and tyrosine residues at three key regulatory sites for maturation of the enzyme, priming it for activation. For activation, phosphorylation of a threonine near the activation loop occurs first by an upstream kinase phosphoinositide-dependent kinase-1 (PDK-1), this is then followed (for most of the PKC isoenzymes) by constitutive and essential autophosphorylation of the activation loop. To complete the maturation processes, PKC autophosphorylates the hydrophobic motif and the turn motif, which primes PKC for second messenger binding. This mature species of PKC, located in the cytosol, is now catalytically active but remains autoinhibited as the pseudo-substrate occupies the substrate binding pocket (Liu and Heckman 1998; Newton 2003; Steinberg 2008).

1.6.3.2 Activation by second messengers

Second messengers DAG and Ca^{2+} are two of the key regulators of the PKC isoforms. Activation via their C1 and C2 domains increases the affinity of PKC for the negatively charged phospholipids, which then localises the kinases to membranes (reviewed in (Newton 1997; Harper and Poole 2010)). Binding of second messengers causes a conformational change that results in the dissociation of the pseudo substrate sequence, unmasking the substrate binding site enabling PKC substrate binding and subsequent substrate phosphorylation. As a result of their slightly differing structure the different subclasses of PKC are regulated differently.

The classical/conventional PKC isoforms

The cPKCs (PKCα, ßI, ßII ,γ) contain a tandem C1 domain that is capable of binding DAG and are also regulated via their Ca^{2+} binding C2 domains, both DAG and Ca^{2+} are required

for full activation. cPKCs are targeted to the membrane where they are tethered to phosphatidylserine via their C2 domains following Ca^{2+} binding.

The novel PKC isoforms

In contrast the nPKCs (PKCδ, ϵ, η, θ) bind DAG via their C1 domains but have C2 domains that are unable to coordinate Ca^{2+} (classed as C2-like, Ca^{2+} insensitive domains) and are therefore only DAG responsive.

The atypical PKC isoforms

The atypical isoforms (PKCζ, ι/λ) have altered C1 domains and lack a C2 domain and are therefore insensitive to both DAG and Ca^{2+}.

1.6.3.3 Cellular localisation

Despite the high sequence homology within the catalytic domain of the PKC family members, there is thought to be a considerable amount of non redundancy between the different isoforms with each isoform regulating unique cellular functions via the phosphorylation and regulation of Isoform-specific substrates. The activity of the PKC family is therefore further controlled by several molecular mechanisms, including interaction with Isoform-specific binding-partner proteins and different cellular localisation which enables the PKC isoenzymes to colocalise with their substrates and activators (Poole, Pula et al. 2004). PKC Isoform-specific scaffolding proteins enable the selective localisation of the kinase isoforms to different cellular locations, including the plasma membrane, nuclear membrane and subcellular compartments and hence different signalling complexes, cofactors and substrates. It was first proposed by Mochly-Rosen and colleagues (Mochly-Rosen, Khaner et al. 1991; Mochly-Rosen and Kauvar 1998) that isoform-specific (or selective) anchoring proteins RACKs (receptors for activated C-kinase) could selectively bind to PKCs in their active, second messenger

bound states and thereby enhance PKC substrate phosphorylation by bringing multiple proteins together in distinct cellular locations. This provides an extra level of regulation of the members of the PKC family and allows for substrate specificity of the individual PKC isoforms as a unique RACK for each PKC isoform, could translocate the isoforms to different cellular locations with a unique set of substrates.

1.6.3.4 PKC interacting proteins

Many other proteins have also been identified that are capable of associating with PKC. These PKC-interacting proteins have been classified into four categories; 1) proteins that target PKC isoforms to their upstream activators, such as DAG. 2) proteins that direct the isoforms to the different intracellular compartments, 3) substrates of PKC, RACKs are also included in this category as they act to bring together PKC and its substrates and 4) other signalling proteins that do not necessarily fall into any of the above categories (Jaken and Parker 2000).

1.7 Studying PKC Function.

PKC is frequently studied in various cell systems using pharmacological inhibitors and activators of the kinase. Such pharmacological tools are widely used in the study of PKC function and are one of the approaches used in this thesis. Pharmacological inhibitors of PKC, both broad spectrum, which are non-selective between the different PKC isoforms and Isoform-specific inhibitors have been utilised to further understand the role of this family of kinases. The inhibitors that were used in this thesis are listed here and the structures summarised in Figure 1.11. For further details in regard to inhibitor specificity please refer to Chapter 4.

1.7.1 Broad spectrum inhibitors

1.7.1.1 Bisindolylmaleimides

Ro31-8220 and Ro31-8425 are two bisindolylmaleimide derivatives (differing by a single functional group) that act as pan-specific inhibitors of the PKC superfamily, and show no specificity or selectivity for the different isoforms. They are considered to be both potent and selective PKC inhibitors (IC_{50} of approximately 15 nM for rat brain PKC) which compete for the ATP-binding site on PKC (Wilkinson, Parker et al. 1993). Both inhibitors have IC_{50} values in the nM range for the PKC α and β isoforms. Despite their apparent increased specificity for PKC over other serine/threonine specific proteins such as protein kinase A, some non-specific targets do exist, including GSK-3 and MAP kinase for Ro31-8220 (Davis, Hill et al. 1989; Nixon, Bishop et al. 1992; Davies, Reddy et al. 2000; Bain, McLauchlan et al. 2003; Bain, Plater et al. 2007). Studies using human platelets by Gilio et al (2010) showed 10μM Ro31-8425 was sufficient to suppress total platelet PKC kinase activity, as measured by monitoring PKC exogenous substrate peptide phosphorylation to 2.1 ± 0.5% of the control.

1.7.2 Class specific inhibitors

1.7.2.1 Gö6983

Gö6983 is thought to be a potent inhibitor of several PKC isoforms by acting as a reversible ATP-competitive inhibitor. The inhibitor is thought to primarily target the classical isoforms of PKC, PKCα and PKCβ (IC_{50} = 7, 7 nM respectively) but at higher concentrations can also inhibit PKCδ. It is therefore primarily used to differentiate PKCθ function but can also be used to study classical isoform function when used at lower concentrations (Gschwendt, Dieterich et al. 1996).

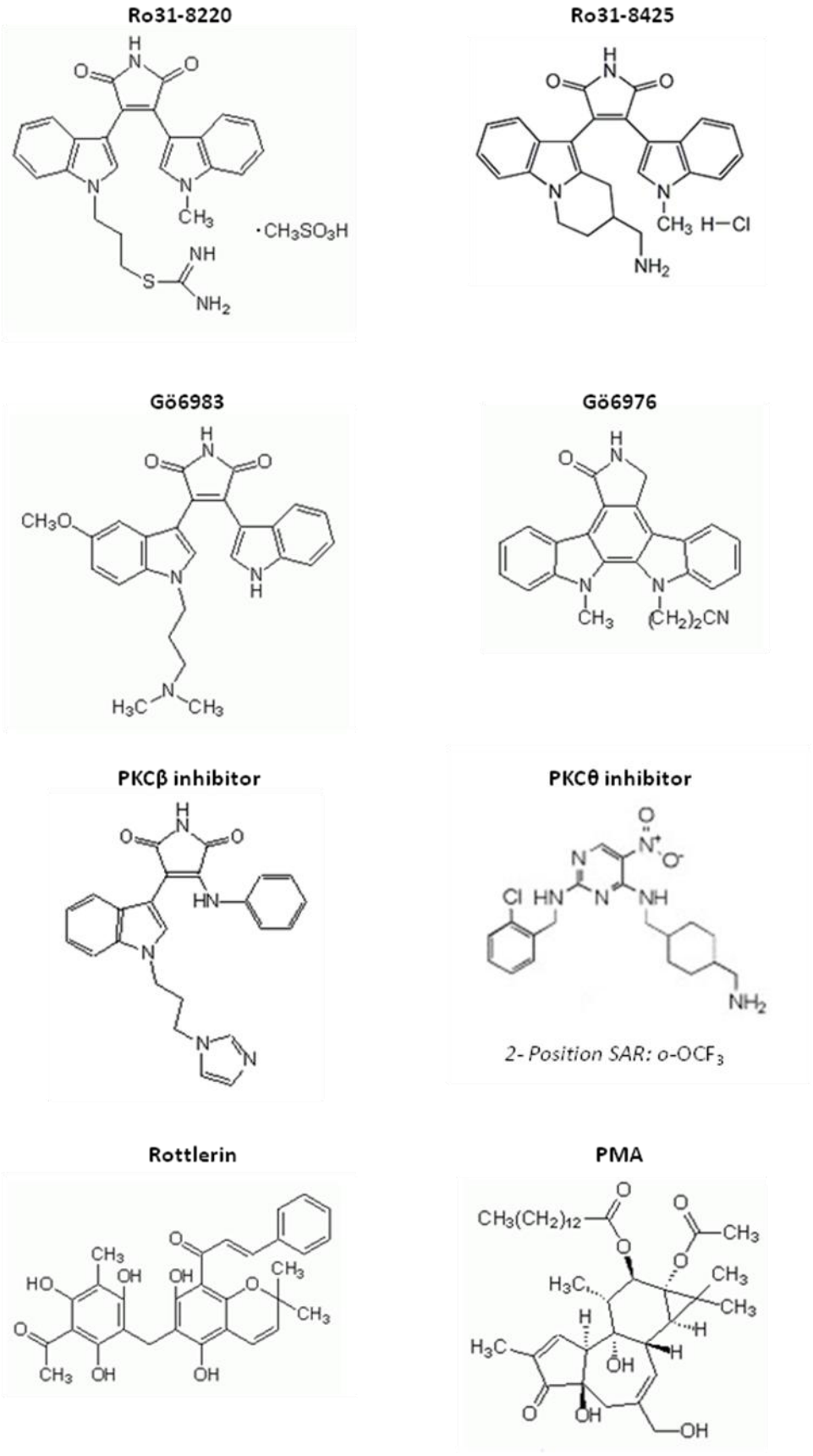


Figure 1.11. Structure of PKC inhibitors. Inhibitors used include bisindolylmaleimide derivatives Ro31-8220, Ro31-8425, Gö6983 and Gö6976, the anilino-

monoindolylmaleimide PKCβ inhibitor, the 2,4-diamino-5-nitropyrimidine PKCθ inhibitor and Rottlerin 5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromene. Also included is the structure of the DAG mimetic and PKC activator, PMA (phorbol 12,13-myristate acetate).

1.7.2.2 Gö6976

Gö6976, an ATP competitive inhibitor, is able to discriminate between the calcium dependent and independent isoforms and therefore can selectively inhibit PKCα and PKCβ over the other isoforms (Martiny-Baron, Kazanietz et al. 1993). Gö6976 has been frequently used to determine the role of the classical PKC isoforms in human platelet activation, however, Getz et al have recently identified that Gö6976 is also able to inhibit Syk kinase activity in human platelets, in particular downstream of the major activating platelet receptor GPVI (Getz, Mayanglambam et al. 2011). Additionally Gö6976 has also been shown to be an effective inhibitor against PKCμ/PKD (Gschwendt, Dieterich et al. 1996) and is therefore no longer an appropriate strategy for investigation of classical PKC isoform function, especially downstream of tyrosine kinase receptor linked signalling pathways.

1.7.3. Isoform-specific inhibitors

1.7.3.1 PKCβ inhibitor

The anilino-monoindolylmaleimide compound, 3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione acts as a potent ATP-competitive inhibitor that selectively inhibits PKCβ at lower concentrations than the other PKC isoforms (effective IC_{50} value of 0.02μM for PKCβ in comparison to 0.33μM for PKCα) (Tanaka, Sagawa et al. 2004). Studies using human platelets by Gilio et al (2010) showed 2.5μM PKCβ inhibitor was sufficient to suppress total platelet PKC kinase activity, as measured by monitoring PKC exogenous substrate peptide phosphorylation to 38 ± 6% of the control.

1.7.3.2 PKCθ inhibitor

The PKCθ used in studies presented here in this thesis is the novel compound 20 (a 2,4-diamino-5-nitropyrimidine) kindly donated by Boehringer Pharmaceuticals (Cywin,

Dahmann et al. 2007) and used by Zanin-Zhorov et al (Zanin-Zhorov, Ding et al. 2010). In the original study in which the inhibitor was developed the different compounds were tested for selectivity to PKCθ against a panel of kinases including, Lyn, Syk, EGFR and JAK, although further testing of the inhibitor in *in vitro* and *in vivo* studies to determine inhibitor specificity is needed.

1.7.3.3. Rottlerin

Rottlerin (5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromene) a supposedly selective inhibitor for the novel isoform PKC (IC_{50} = 3-6 µM), has been used to study PKCδ function in many studies, including many looking at platelet function. However, although the inhibitor is thought to exhibit greater selectivity for PKCδ over the classical isoforms and the other novel isoforms in *in vitro* studies (Gschwendt, Muller et al. 1994), Davies et al (2000) were unable to identify any effect of Rottlerin on PKCδ activity using a kinase assay even at inhibitor concentrations of 20µM (Davies, Reddy et al. 2000). Additionally rottlerin has also been shown to have several non-specific effects, including inhibitory actions on several other kinases including CaM kinase (IC_{50} = 5.3µM), activation of ion channels, uncoupling of mitochondria and has also been shown to reduced intracellular ATP levels which are thought to then inhibit PKCδ as a secondary effect of the inhibitor (Soltoff 2007). Despite these findings Rottlerin is still actively used as a PKCδ inhibitor within the literature and is therefore included here for comparison with other studies and discussion (Crosby and Poole 2003; Murugappan, Tuluc et al. 2004; Pula, Schuh et al. 2006; Yacoub, Theoret et al. 2006; Gilio, Harper et al. 2010). Studies using human platelets by Gilio et al (2010) showed 10µM rottlerin was sufficient to suppress total

platelet PKC kinase activity to 63 ± 8% of the control, as measured by monitoring PKC exogenous substrate peptide phosphorylation.

1.8 Protein Kinase C and Platelets.

Many intracellular signalling pathways are involved in the process of platelet activation, and many of the agonist receptor signalling cascades lead to the downstream activation of PLC which catalyses the production of DAG and IP_3 leading to an increase in intracellular calcium and activation of the PKC superfamily.

1.8.1 PKC isoform expression in platelets.

Both human and mouse platelets have been shown to express some of the isoforms of the PKC family, including the classical isoforms PKCα and PKCβ, and the novel isoforms PKCδ and PKCθ. Additionally, unlike human, mouse platelets also express the novel isoform PKCε. There is also some evidence for the expression of other isoforms, including PKCη and the atypical isoforms (PKCζ and ι/λ) which remain controversial (Hall, Harper et al. 2008; Konopatskaya, Gilio et al. 2009; Nagy, Bhavaraju et al. 2009) (Murugappan, Tuluc et al. 2004; Buensuceso, Obergfell et al. 2005; Pears, Thornber et al. 2008; Bynagari, Nagy et al. 2009; Cohen, Braiman et al. 2009).

1.8.2 PKC and platelet activation

Studies using broad spectrum inhibitors have highlighted an overall and generally positive role for the family of PKC isoforms in platelet granule secretion, integrin activation and aggregation, outside-in signalling, prostaglandin and thromboxane synthesis, Ca^{2+} entry and PS exposure (Walker and Watson 1993; Paul, Jin et al. 1999; Quinton, Kim et al. 2002). Interestingly, broad spectrum PKC inhibitors have also

highlighted several negative roles for the PKC superfamily in receptor desensitisation and Ca^{2+} extrusion (Pollock, Sage et al. 1987; Rosado and Sage 2000; Harper and Sage 2006; Mundell, Jones et al. 2006; Strehl, Munnix et al. 2007; Steinberg 2008; Gilio, Harper et al. 2010). A simplified model for the role of PKC in platelet activation is summarised in Figure 1.12. This highlights a variety of roles for PKC in the complex regulation of platelet activation, suggesting the PKC superfamily could act as a key mediator downstream of several platelet agonists and could act to maintain the balance between positive and negative platelet signalling pathways.

1.8.3 Regulation through tyrosine phosphorylation.

Different patterns of tyrosine phosphorylation of the different PKC isoforms are thought to be an important mechanism of control for kinase activity and hence different isoform functions. All of the major PKC isoforms that are expressed in human and mouse platelets, PKCα, β, δ, θ and ε have all been shown to be tyrosine phosphorylated downstream of several platelet agonists including GPVI and GPIb-V-IX, and in the case of PKCδ downstream of thrombin (Murugappan, Shankar et al. 2005; Pears, Thornber et al. 2008) (Crosby and Poole 2002; Crosby and Poole 2003; Pula, Crosby et al. 2005; Hall, Jones et al. 2007). These findings therefore not only implicate PKC in several platelet agonist induced signalling pathways, but also their phosphorylation as a possible regulatory mechanism, although more in depth analysis is needed to determine whether this is true. Studies have revealed that tyrosine phosphorylation of the PKCα, δ and θ isoforms is regulated by different tyrosine kinases indicating different modes of regulation for the different isoforms. In human platelets, PKCδ has been shown to undergo Src family kinase mediated transient phosphorylation at Tyr311 and Tyr565

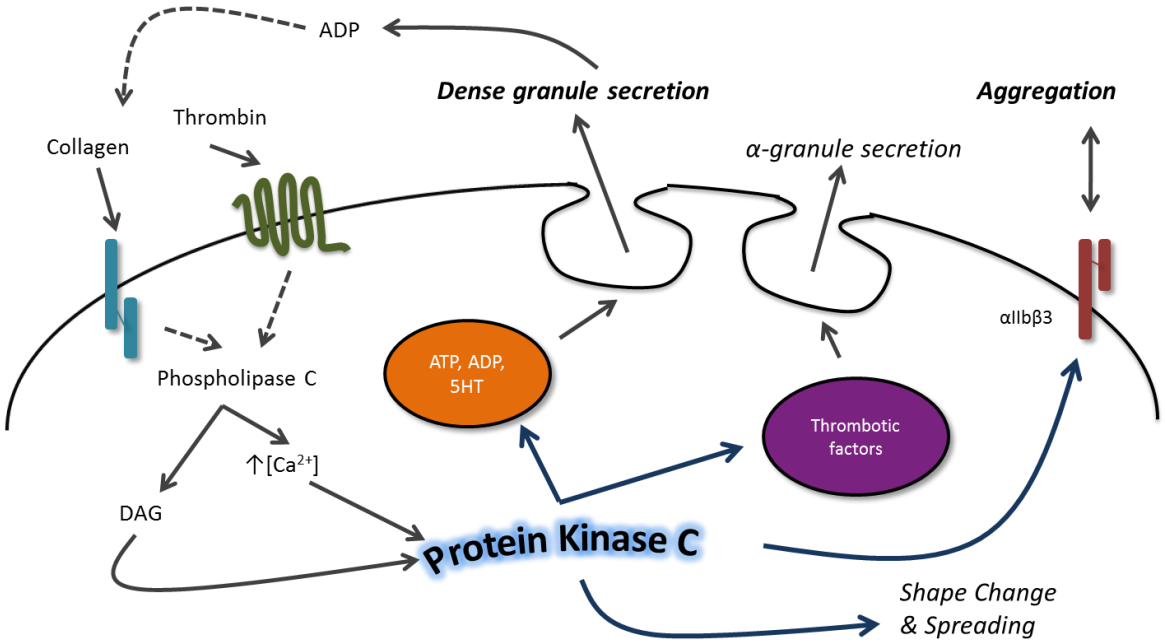


Figure 1.12 PKC is a key regulator of platelet activation. Stimulation by agonists such as thrombin, collagen and ADP activates a variety of intracellular signalling pathways, many converging at the activation of phospholipase C resulting in a rise of intracellular [Ca^{2+}] and activation of the PKC family. PKC regulates many processes involved in platelet activation including granule secretion, integrin activation and aggregation and spreading. Figure adapted from Harper M.T, Poole A.W (2007). Biochem Soc. Trans, 35:1005-1008.

sites following platelet stimulation by collagen (interestingly this is sustained in mouse following GPVI stimulation) and also downstream of thrombin. This phosphorylation has been shown to be essential for kinase activity, but does not appear to regulate the translocation of the isoform from the cytosol to the membrane. PKCε has also been shown to be constitutively tyrosine phosphorylated in mouse platelets. Finally PKCθ in human platelets has been shown to be phosphorylated on Tyr90 (found in the regulatory domain) downstream of GPVI or integrin induced platelet stimulation (Soriani, Moran et al. 2006). The equivalent phosphorylation of PKCθ has been shown in T-cell lymphocytes to be mediated by the Src family kinases (Liu, Witte et al. 2000; Zanin-Zhorov, Ding et al. 2010). These differences in tyrosine phosphorylation of the different novel PKC isoforms may provide the basis for isoform-specific PKC functions in the regulation of platelet activation.

1.8.4 The classical isoforms of PKC and the regulation of platelet activation

Studies using inhibitors specific for the classical isoforms have identified major positive individual roles for the classical isoforms in both GPVI and PAR receptor induced human platelet activation (Murugappan, Tuluc et al. 2004; Gilio, Harper et al. 2010). Gilio et al have used a PKCβ inhibitor to identify a positive role for the isoform in the regulation of several processes including aggregation, granule secretion and integrin activation downstream of GPVI signalling (Gilio, Harper et al. 2010). In addition to the use of pharmacological inhibitors, studies using mice deficient in individual PKC isoforms also provide support for major positive roles for the two classical isoforms in the regulation of platelet activation (Table 1.1). In human and mouse platelets, PKCα is proposed to be the most important of the PKC isoforms in the regulation of the processes of granule

secretion and platelet aggregation downstream of several platelet agonists (Yoshioka, Shirakawa et al. 2001; Tabuchi, Yoshioka et al. 2003; Konopatskaya, Gilio et al. 2009; Gilio, Harper et al. 2010; Konopatskaya and Poole 2010). PKCα deficient mice show reduced dense granule secretion biogenesis and reduced SNAP-23 phosphorylation, which is thought to play a role in SNARE complex formation or recycling, a key component required for granule secretion, thereby providing mechanisms that could explain its importance in the regulation of secretion. In comparison, PKCβ plays a key role in the positive regulation of outside-in signalling though $\alpha IIb\beta_3$, although it does it not play a role in inside-out signalling. Studies using PKC$\alpha^{-/-}$ mice and PKCβ specific pharmacological inhibitors also suggest redundant roles exist between the two isoforms in the processes of aggregation and granule secretion.

Both classical isoforms have been shown to play major positive and non-redundant roles in the regulation of thrombus formation and in several key processes when monitored under flow conditions on collagen It has been shown that whole blood from mice deficient in either of the classical isoforms show marked reductions in α-granule secretion, aggregate formation, calcium signalling and procoagulant activity on collagen under flow conditions (Gilio, Harper et al. 2010). Interestingly despite these apparent major roles in platelet activation and thrombus formation *in vitro*, no significant difference in tail bleeding was observed in PKC$\alpha^{-/-}$ mice (Konopatskaya, Gilio et al. 2009).

1.8.5 The novel isoforms of PKC and the regulation of platelet activation

The novel isoforms in comparison are thought to play minor and in some cases negative roles in the regulation of platelet activation. Studies using the putative PKCδ inhibitor rottlerin in human platelets have identified a major role for PKCδ in GPVI mediated platelet activation, although what this role is, is controversial. Yacoub et al (2006) have

Function	PKCα (Konopatskaya, Gilio et al. 2009; Gilio, Harper et al. 2010; Harper, Molkentin et al. 2010)		PKCβ (Buensuceso, Obergfell et al. 2005; Gilio, Harper et al. 2010)	
	GPVI	**PAR**	**GPVI**	**PAR**
Aggregation	Positive	Positive	-	-
Dense granule secretion.	Positive*	Positive*	-	-
Alpha granule secretion	Positive	Positive	Positive (under flow)	-
Inside-out signalling (αIIbβ3 activation)	Positive	Positive	-	-
Outside-in signalling (Spreading on fibrinogen)	No role		Positive	
Spreading on collagen	No role			
Filopodia development	No role			
Thromboxane A2 formation	-	-		
Intracellular Ca^{2+}	Positive	-	Positive	
Coagulation (PS exposure on collagen under flow)	Positive		Positive	
Thrombus formation on collagen under flow *in vitro*	Positive		Positive	
Thrombus formation in vivo (fecal occlusion)	Positive			
Thrombus formation in vivo (Tail Bleeding)	No role			
Redundancy			Redundancy with PKCα exists for strong agonists.(Konopatskaya, Gilio et al. 2009)	
Platelet development	Positive role in dense granule biogenesis			
Interacting proteins/Substrates	SNAP-23			

*PKCα has a positive role in the regulation of dense granule biogenesis.

Table 1.1 Summary of the roles of the classical isoforms PKCα and PKCβ in platelet activation. Summary of published data using platelets from PKCα-/- and PKCβ-/- mice to determine the role of the individual isoforms in platelet function.

shown a reduction in collagen induced platelet aggregation and a decrease in GPVI mediated αIIbβ3 activation in the presence of the inhibitor in human platelets. In contrast Gilio et al (2010) have identified a negative role for PKCδ in GPVI induced platelet aggregation and show no role for the isoform in αIIbβ3 activation. Rottlerin has also been used to indicate positive roles for PKCδ in thrombin and PAR peptide stimulated regulation of aggregation, dense and α-granule secretion and αIIbβ3 activation (Murugappan, Tuluc et al. 2004; Yacoub, Theoret et al. 2006; Gilio, Harper et al. 2010) .

PKCθ Isoform-specific inhibitors have recently been used in the study of human platelet function by Gilio et al (2010), in an attempt to delineate the role for the isoform in human platelet activation downstream of GPVI. Negative roles for PKCθ in several processes involved in GPVI- induced platelet activation were identified including aggregation, α-granule secretion, αIIbβ3 activation and changes in intracellular levels of Ca^{2+}, all of which were increased in the presence of the inhibitor. As with the classical isoforms, platelets from isoform-deficient mice have also been used in the study of the novel isoforms of PKC, and both positive and negative roles for the novel isoforms in platelet activation and thrombus formation have been described (Pula, Schuh et al. 2006; Pears, Thornber et al. 2008; Chari, Getz et al. 2009; Gilio, Harper et al. 2010). Table 1.2 summarises the key roles for the novel isoforms PKCδ and PKCε in the processes involved in platelet activation, determined using mice deficient in either of the isoforms.

In the case of PKCθ several contradictory reports have been published which makes understanding the role for that PKCθ difficult (for further detail please refer to Chapter 6 for further discussion) (Soriani, Moran et al. 2006; Hall, Harper et al. 2008; Cohen, Braiman et al. 2009; Harper and Poole 2009; Nagy, Bhavaraju et al. 2009; Gilio, Harper et

al. 2010). Table 1.3 summarises the roles for PKCθ that have been reported in the literature.

Studies using mouse models have identified key roles for PKCθ and PKCδ in the regulation of $\alpha IIb\beta_3$ outside-in signalling and filopodia formation on fibrinogen, PKCθ like PKCβ appears to play a positive role in the regulation of spreading on fibrinogen whilst PKCδ plays a negative role in filopodia generation and spreading. The molecular mechanism underlying this negative role is thought to involve a physical interaction between PKCδ and VASP, as PKCδ appears to negatively regulate Ser157 phosphorylation of VASP, reducing filopodial extension and negatively regulating platelet-platelet interaction.

Unlike mice deficient in the classical isoforms PKCα or β which show significantly reduced aggregate formation on collagen under flow, mice deficient in PKCε show no difference in this assay, whilst mice deficient in PKCδ or PKCθ show increased aggregate formation highlighting minor and negative roles for these isoforms respectively. Interestingly thrombus formation *in vivo* remains unaffected in $PKC\delta^{-/-}$ mice (Chari, Getz et al. 2009) and has been reported to be either reduced or not affected in $PKC\theta^{-/-}$ mice, thereby indicating a complex regulation of platelet activation by the PKC superfamily in which the regulation of the different processes involved are regulated in an isotype dependent manner.

Function	PKCδ (Pula, Schuh et al. 2006; Chari, Getz et al. 2009; Gilio, Harper et al. 2010)		PKCε (Pears, Thornber et al. 2008)	
	GPVI	PAR	GPVI	PAR
Aggregation	Negative	Positive	Positive	No role
Dense granule secretion.	Negative[¥]	Positive	Positive	Minor positive
Alpha granule secretion	Negative*	Positive	-	-
Inside-out signalling (αIIbβ3 activation)	No role	Positive	-	-
Outside-in signalling (Spreading on fibrinogen)	-		No role	
Spreading on collagen	-		No role	
Filopodia development (on collagen)	Negative		-	
Actin polymerisation	Negative		-	
Thromboxane A2 formation	Negative	Positive	-	-
Intracellular Ca^{2+}	No role	-	-	-
Coagulation (PS exposure on collagen under flow)	No role		-	
Thrombus formation on collagen under flow *in vitro*	Negative		No role	
Thrombus formation in vivo ($FeCl_3$ injury and occlusion)	No role		-	
Thrombus formation in vivo (Tail Bleeding)	-		-	
Interacting proteins/ Substrates	VASP			

*Gilio et al found role for PKCδ in α-granule secretion under flow. (Gilio, Harper et al. 2010)

[¥]Pula et al found no role for PKCδ in dense -granule secretion (Pula, Schuh et al. 2006)

Table 1.2 Summary of the roles of the novel isoforms PKCδ and PKCε in platelet activation. Summary of published data using platelets from PKCδ-/- and PKCε-/- mice to determine the role of the individual isoforms in platelet function.

Function	PKCθ						
	(Soriani, Moran et al. 2006)	(Hall, Harper et al. 2008)	(Harper and Poole 2009)	(Gilio, Harper et al. 2010)	(Nagy, Bhavaraju et al. 2009)		(Cohen, Braiman et al. 2009)
		GPVI	GPVI	GPVI	GPVI	PAR	PAR
Aggregation	-	No role	Negative	-	Positive	Positive	Positive (WB)
Dense granule secretion.	-	No role	Negative	-	Positive	Positive	-
Alpha granule secretion	-	Negative	-	Negative (under flow)	Positive	Positive	Positive (WB)
Inside-out signalling (αIIbβ3 activation)	No role	Negative	-	-	Positive	Positive	-
Outside-in signalling (Spreading on fibrinogen)	Positive	Positive	-	-	-		-
Spreading on collagen	No role	No role	-	-	-		-
Filopodia development	Positive (fibrinogen)	Positive	-	-	-		-
Thromboxane A2 formation	-	-	-	-	Positive	Positive	-
Intracellular Ca^{2+}	-	-	-	Negative	-	-	-
Coagulation (PS exposure on collagen under flow)	-	-	-	Negative	-		-
Thrombus formation on collagen under flow *in vitro*	-	Negative	-	Negative	-		-
Thrombus formation in vivo (fecal occlusion)	-	-	-		Positive		-
Thrombus formation in vivo (Tail Bleeding)	No bleeding phenotype	-	-		-		Positive

WB – Whole Blood

Table 1.3 Summary of the roles PKCθ isoforms in platelet activation. Summary of published data using PKCθ-/- mice enabling a comparison of previously published results.

1.9 AIMS

PKC is thought to be a key regulator in several cellular processes and is considered to be a central player in many agonist induced signalling pathways involved in platelet activation, although in several cases our understanding is incomplete and complicated by contradictory reports. As members of the PKC superfamily have been found to be associated with several diseases in a variety of cell types (Gustafson 2003), PKC is an attractive target for a wide-range of therapies, and whether or not PKC is a suitable target for anti-thrombotic and anti-platelet therapies is of considerable interest. However, a complete understanding of PKC isoform function is required before PKC is used as a potential therapeutic target.

The overall aim of this thesis is to further investigate the role of the different isoforms of PKC in platelet activation and haemostasis using both human and mouse platelets, with a particular interest in the roles for the novel isoforms.

The aims of the work presented in this thesis include:

1. To quantify the expression levels per platelet of each of the major PKC isoforms, PKCα, β, δ, θ and ε in human and mouse platelets.
2. To determine and compare the role of the different PKC isoforms in human platelet activation, aggregation and granule secretion downstream of the GPVI, CLEC-2 and PAR receptors using both broad spectrum and isoform-specific pharmacological inhibitors.
3. To determine the role of the PKC superfamily, and the individual PKC isoforms in ADP-induced platelet activation as this is relatively undefined, using both pharmacological inhibitors and transgenic mouse models.

4. To determine whether a robust role for the novel isoform PKCθ exists in the regulation of platelet activation and haemostasis using mice deficient in PKCθ.
5. To determine whether any redundancy exists between the novel isoforms PKCθ and PKCε in the regulation of mouse platelet activation and haemostasis using mice deficient in both novel isoforms.

CHAPTER 2.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General materials

Sodium chloride, potassium chloride, magnesium chloride, citric acid, tris (hydroxymethyl)-aminomethane (tris base), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetra acetic acid (EGTA), glycerol, nitrocellulose membranes and microscope slides were purchased from VWR (Lutterworth, UK). Bromophenol blue, glucose, B-mercaptoethanol, dimethylsulphoxide (DMSO), fatty acid free BSA, sodium dodecylsulphate (SDS) were purchased from Sigma (Poole, UK). Nonidet P-40 (NP-40) alternative was purchased from Calbiochem (Nottingham, UK). 4-12% Bis-Tris NuPAGE gels were purchased from Invitrogen (Paisley, UK). Coverslips were purchased from Menzel-Glaser (Braunschweig, Germany).

2.1.2 Blood anticoagulants

Sodium citrate and Heparin was purchased from Sigma (Poole, UK). P-PACK (D-Phe-Pro-Arg-chloromethylketone, HCl), was purchased from Merck Biosciences Ltd (Nottingham, UK).

2.1.3 Platelet agonists

The agonists used in this thesis are listed in Table 2.1.

2.1.4 Inhibitors and antagonists

Inhibitors used are listed in Table 2.2.

2.1.5 Antibodies

A summary of all the antibodies and the dilutions used is summarised in Table 2.3.

2.1.6 Fluorescent probes

FURA-2 AM was a product of Invitrogen/Molecular Probes, Eugene, OR, USA.

2.1.7 Solutions

The solutions used in this thesis are listed in Table 2.4.

AGONIST	TARGET	CONCENTRATIONS USED	SOURCE
ADP	$P2Y_1$ $P2Y_{12}$	3, 100µM	Sigma, Poole, UK
Collagen (HORM)	GPVI α2β1	3, 5, 10, 100µg/ml	Nycomed (München, Germany)
CRP (YGKO$(GPO)_{10}$GKOG)	GPVI	0.3, 1µg/ml	Dr RW Farndale (Cambridge UK)
Fibrinogen	$\alpha IIb\beta_3$	100µg/ml	Enzyme Research Labs, Swansea, UK
PAR-1 peptide (SFLLRN)	PAR-1	1-100µM	Sigma, Poole, UK
Phorbol myristate acetate (PMA)	PKC	1-100nM	Sigma, Poole, UK
Rhodocytin	CLEC-2	30-100nM	Gift from Dr JA Eble (University Frankfurt, Germany)
Thrombin	PAR-1 PAR-3 PAR-4	0.03, 0.1, 0.2U/ml	Sigma, Poole, UK
U46619	TxA_2	10µM	Sigma, Poole, UK

Table 2.1 Platelet Agonists.

Inhibitor/Antagonist	Target	used	Source
Gö6976	Classical PKC isoforms, Syk	0.1-100µM	Calbiochem/MERCK Biosciences (UK)
Gö6983	PKCα, PKCβ and PKCδ	0.1-100µM	Sigma, Poole, UK
PKCβ inhibitor (3(-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione)	PKCβ	0.3-100µM	Calbiochem/MERCK Biosciences (Nottingham, UK)
PKCθ inhibitor	PKCθ	0.3-100µM	Gift from Boehringer Ingelheim Pharmaceuticals
Ro31-8220 (Bisindolylmaleimide IX)	PKC	0.1-100µM	Sigma, Poole, UK
Ro31-8425 (Bisindolylmaleimide X)	PKC	0.1-100µM	Sigma, Poole, UK
Rottlerin	PKCδ	0.3-100µM	Calbiochem/MERCK Biosciences (Nottingham, UK)
AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride)	Serine proteases	2mM	Sigma, Poole, UK
Aprotinin,	Serine proteases	10µg/ml	Sigma, Poole, UK
Apyrase	ATP, ADP	0.05U/ml	Sigma, Poole, UK
ARC/cangrelor	$P2Y_{12}$	10µM	Gift from Astrazeneca
Indomethacin	Cyclooxygenase	10µM	Sigma, Poole, UK
Integrilin (Eptitibatide)	$\alpha IIb\beta_3$	9µM	University Hospital, Birmingham Pharmacy
Leupeptin	Ser, thr, cys proteases	10µg/ml	Sigma, Poole, UK
MRS-2179	$P2Y_1$	100µM	Sigma, Poole, UK
Pepstatin A	Asp proteases	1µg/ml	Sigma, Poole, UK
Prostacyclin (PGI_2)	PGI_2 receptor (prevents platelet activation)	1µg/ml	Sigma, Poole, UK
Sodium fluoride	**Ser/Thr, acidic phosphatases**	2mM	Sigma, Poole, UK
Sodium orthovanadate	**Tyr and alkaline phosphatases**	5mM	Sigma, Poole, UK

Table 2.2. Inhibitors and antagonists

* WB – WESTERN BLOT FC – FLOW CYTOMETRY (FACS ANALYSIS).

ANTIBODY	HOST	USE*	SOURCE
PRIMARY			
PKCα	Rabbit	WB 1/500	Cell Signalling Technology (Beverly, MA, USA)
PKCβ	Mouse	WB 1/500	BD Biosciences
PKCδ	Mouse	WB 1/1000	BD Biosciences
PKCθ	Mouse	WB 1/1000	BD Biosciences
PKCε	Mouse	WB 1/1000	BD Biosciences
αIIb (mouse)	Rat	FC 1/100	Emfret Analytics
GPVI – FITC conjugate (mouse)	Rat	FC 1/100	Emfret Analytics
GPIb – FITC conjugate (mouse)	Rat	FC 1/100	Emfret Analytics
α2β1 – FITC conjugate (mouse)	Rat	FC 1/100	Emfret Analytics
P-selectin – FITC conjugate (mouse)	Rat	FC 1/100	BD Pharmingen
Actin	Goat	WB 1/5000	Santa Cruz Biotechnology, Inc. (CA, USA),
phospho(Ser)PKC-substrate ¥	Rabbit	WB 1/500	Cell Signalling Technology (Beverly, MA, USA)
CLEC-2 (human)	Goat	3-10µg/ml	R+D Systems (Abingdon, UK)
CLEC -2 (mouse)	Rat	3-10µg/ml	Gift from Dr. C. Reis e Sousa
SECONDARY			
Mouse IgG HRP – conjugate	Rabbit	WB 1/5000	Dako
Rabbit IgG HRP – conjugate	Swine	WB 1/5000	Dako
Goat IgG HRP-conjugate	Rabbit	WB 1/5000	Dako

¥ phosphor(Ser)PKC-substrate antibody recognises $(Lys/Arg)_{1-3}$ – X – (pSer) – Y – $(Lys/Arg)_{1-3}$ amino acid sequence where X is any amino acid and Y is any hydrophobic amino acid.

Table 2.3 Antibodies used.

SOLUTION	COMPONENTS
0.5% Agarose gel	0.5% agarose in 1xTAE
2 x NP40 lysis buffer	300mM NaCl, 20nM Tris base, 2mM EGTA, 2mM EDTA, 1mM PMSF, 10μg/ml aprotinin, 10μg/ml leupeptin, 0.7μg/ml pepstatin A, 2mM sodium orthovanadate, 1mM sodium fluoride, 4% NP-40, pH 7.3
2x Laemmli SDS PAGE loading buffer	4% SDS, 10% β-mercaptoethanol, 20% glycerol, 50mM Tris base, trace of bromophenol blue, pH 6.8
Acid Citrate Dextrose	120mM sodium citrate, 80mM citric acid and 110mM glucose
ATP Standard	Prepared as per manufacturer's instructions
Chronolume Luciferin/ Luciferase reagent.	Prepared as per manufacturer's instructions, just add deionised autoclaved water
DNA loading dye	0.25% bromophenol blue, 40% sucrose.
Modified Tyrodes-HEPEs Solution	138mM NaCl, 2.7mM KCl, 1mM MgCl2, 3mM NaH_2PO_4, 5mM glucose, 10mM HEPES, pH7.3
PBS	10mM sodium phosphate, 137mM NaCl (adjusted to pH 7.4)
SDS running buffer	1% SDS, 0.25M Tris, 1.92M Glycine, pH8.3
SDS transfer buffer	0.5% SDS running buffer, 10% Methanol.
TAE (50 x)	2M Tris, 50mM EDTA, 5.71% (1M) Glacial acetic acid
TBS	2.48mM Tris, 137mM NaCl (adjusted to pH 7.4).
TBS-T	0.1% TWEEN-20 dissolved in 1 x Tris buffered saline (TBS).
Western Blot Stripping Solution	2% SDS, 100mM β-mercaptoethanol, 62.5mM Tris-HCl pH 6.8.

Table 2.4. Solutions used.

2.1.8 Mice

PKC$\theta^{-/-}$ and PKC$\varepsilon^{-/-}$ deficient mice were bred as heterozygotes on a B6 background in the University of Birmingham from mice engineered by Jackson labs or Castrillo et al respectively (Sun, Arendt et al. 2000; Castrillo, Pennington et al. 2001). All results were compared to wild type (WT) litter-matched controls.

PKC$\theta^{-/-}/\varepsilon^{-/-}$ mice were bred from PKC$\theta^{-/-}/\varepsilon^{+/-}$ parents on a B6 background in the University of Birmingham, PKC$\theta^{-/-}/\varepsilon^{+/-}$ mice were bred from PKC$\theta^{+/-}/\varepsilon^{+/-}$ parents which were originally bred from PKC$\theta^{-/-}/\varepsilon^{+/+}$ and PKC$\theta^{+/+}/\varepsilon^{+/-}$ mice (as PKC$\theta^{+/+}/\varepsilon^{-/-}$ males are sterile). All results are compared to age-matched wild type background C57/BL6 and litter-matched PKC$\theta^{-/-}/\varepsilon^{+/+}$ controls.

Other WT C57BL/6 mice were bred at University of Birmingham.

Housing and husbandry was in accordance with Home Office regulations under the Animals (Scientific Procedures) Act 1986. Animals were bred under an approved Home Office Licence (Ref: PPL 30/2721).

2.1.9 Primers and genotyping

Genotyping was performed using PCR analysis of DNA extracted from tissue samples by phenol:chloroform DNA extraction, samples were then separated. Primers specific for the wild type (WT) and knock out (KO) alleles enabled identification of the mouse genotype. Primers and the genotyping PCR protocols are outlined in Table 2.5 and Figure 2.1.

ISOFORM	PRIMERS	DETECTION	PCR PROGRAM
PKCθ	WT – TTGGTTCTCTTGAACTCTGC KO - ACTGCATCTGCGTGTTCGAA Common - TAAGAGTAATCTTCCAGAGC	WT – 426bp band KO – 600bp band	1. 95°C - 3 mins 2. 95°C - 40 secs 3. 62°C - 40 secs 4. 72°C - 1 min (repeat steps 2-4 for 36 cycles) 5. 72°C - 2 mins 6. 4°C – hold
PKCε	PKC-E-D2 – CCAATGCTCAGGCAGCAAGTC PKC-E-D3 - CATGGTAGTGTTCAATGGCCTTC NEO-D1 - TCTCCTGTCATCTCACCTTGC	WT – 500bp band KO – 700bp band	1. 94°C - 3 min 2. 94°C - 30 sec 3. 51°C - 1 min 4. 72°C - 1 min (repeat steps 2-4 for 35 cycles) 5. 72°C - 2 min 6. 10°C - hold

WT – Wild type allele. KO – knock-out/null allele

Table 2.5. Primers and genotyping protocols

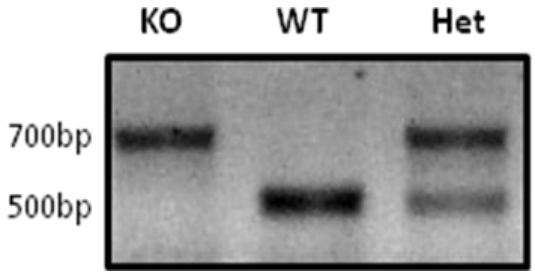


Figure 2.1. **Example PKC-null mice genotyping results.** DNA samples were run on agarose gels and visualised using ethidium bromide and ultraviolet light. Results shown here are for PKCε genotyping. 500bp single band represents homozygous for the wild type (WT $^{+/+}$) allele, 700bp single band represents homozygous for knockout allele (KO $^{-/-}$), samples with both bands are heterozygous (Het $^{+/-}$).

2.2 METHODS – Platelet Preparation

2.2.1 Human platelet preparation

Studies on human platelets were carried out with ethical approval from the Oxford Research Ethic Council (Ref: 08/H0605/123). Blood was drawn from aspirin-free, healthy, consenting volunteers.

2.2.1.1 Platelet rich plasma preparation

Whole blood was drawn from a healthy consenting volunteer into a syringe containing one-tenth of total volume sodium citrate. For plasma containing extracellular calcium; blood was collected into 60μM PPACK. Blood was centrifuged at 200g for 20 minutes at room temperature to obtain platelet-rich plasma (PRP) which was then kept at room temp. The remaining blood was centrifuged at 1000g for 10 minutes at room temperature to obtain platelet-poor plasma. Platelets were counted using a Coulter Particle Counter and size analyser to ensure a platelet count of at least 10^8 platelets/ml.

2.2.1.2 Washed platelet preparation

Whole blood was drawn from a healthy consenting volunteer into syringe containing one-tenth of total volume of acid citrate dextrose (ACD), additional ACD was then added to the blood then centrifuged at 200g for 20 minutes at room temperature to obtain PRP. 10μg prostacyclin (PGI_2) was added to the PRP and then centrifuged at 1000g for 10 minutes at room temperature to pellet the platelets, platelets were resuspended in ACD containing modified Tyrodes-HEPEs buffer, 10μg PGI_2 added and spun for a second time. Supernatant is removed and discarded and platelets were resuspended in modified

Tyrodes-HEPEs buffer. Cells were counted using a Coulter Particle Counter and size analyser and were adjusted to the required concentration.

2.2.1.3 ADP-sensitive washed platelet preparation

For ADP-sensitive washed platelets; blood was taken into one-sixth total volume of ACD. Blood was then centrifuged at 200g for 20 minutes at room temperature to obtain PRP. 10μg PGI_2 was added to the PRP and then centrifuged at 1000g for 10 minutes at room temperature to pellet the platelets. Platelets were then resuspended in modified Tyrodes-HEPEs buffer containing 0.05U/ml apyrase. Cells were counted and size analysed and were adjusted to the required concentration. The platelets were left for 1 hour at 37°C.

2.2.2 Mouse platelet preparation

Animals were bred and blood removed under an approved Home Office Licence (Ref: PPL 30/2721). Blood was drawn by cardiac puncture or from the vena cavae of terminally CO_2-narcosed mice, anaesthetised with gaseous isofluorane.

2.2.2.1 Platelet rich plasma preparation

For mouse PRP, blood was taken into 100μl sodium citrate and centrifuged at 200g for 6 minutes to obtain PRP. The needle was then removed to prevent platelet shearing and the blood was added to 200μl modified Tyrodes-HEPEs buffer pH 7.3 in a 1.5ml Eppendorf. Blood was spun at 2000 rpm for 5 minutes using a microfuge. The top 70% of blood was removed into a new 1.5ml Eppendorf and centrifuged at 200g for 6 minutes at room temperature. PRP was removed and 200ul modified Tyrodes-HEPEs buffer was added to the remaining cells to extract more PRP and centrifuged again at 200g for a

further 6 minutes. Cells were counted using a Coulter Particle Counter and size analyser and were adjusted to a concentration of 2 x 10^8 diluted with modified Tyrodes-HEPEs buffer.

2.2.2.2 Washed platelet preparation

For washed platelets, blood was drawn into 100µM ACD. The needle was then removed to prevent platelet shearing and the blood was added to 200µl modified Tyrodes-HEPEs buffer pH 7.3 in a 1.5ml Eppendorf and prepared as detailed for PRP above. Once PRP was obtained 10µg PGI_2 was added to the PRP and then centrifuged at 1000g for 6 minutes room temperature to pellet the platelets. Platelets were resuspended in 200ul modified Tyrodes-HEPEs buffer pH 7.3. Cells were counted and adjusted to a concentration of 2 x 10^8 diluted with modified Tyrodes-HEPEs buffer.

2.3 METHODS – Functional Studies

2.3.1 Aggregometry and ATP release

Light transmission aggregometry enables the ability of various platelet agonists to induce *in vitro* aggregation to be measured. The platelet sample is stirred in a cuvette between a light course and a photocell. As platelets aggregate the sample absorbs less light increasing light transmission which is detected by the aggregometer. Aggregation was measured using light transmission under stirring (1200 rpm) conditions at 37°C. The optical density of the platelet suspension was measured against a blank reading from

either platelet poor plasma (PPP) or modified Tyrodes-HEPEs buffer when using PRP and washed platelets respectively and recorded in real time on a chart recorder.

ATP is stored in the dense granules and measurement of its release from platelets is an indicator of the extent of platelet dense granule secretion following agonist stimulation. Dense granule release was monitored using the Chrono-Lume luciferin/luciferase reagent to detect adenosine triphosphate (ATP) release. Light emission from the luciferase reaction, catalysed by secreted ATP, was recorded in real time on a chart recorder.

Aggregation and dense granule secretion of human and mouse platelets were monitored simultaneously using a Chrono-log Corporation Lumi-dual aggregometer. Platelets were preincubated at 37°C for 1 minute prior to incubation in the presence or absence of the desired inhibitor for a further 2 minutes (1 minute without stirring, 1 minute with stirring) before stimulation with agonist. Luciferin/luciferase was added 2 minutes prior to addition of agonist. Each sample was then allowed to aggregate/secrete for at least 2.5 minutes. Finally an ATP standard was added in order to quantify secretion.

2.3.2 Alpha granule secretion and expression analysis by Flow Cytometry

One-colour analysis of α-granule secretion (measured by detecting P-selectin expression at the cell surface) and surface receptor protein expression was carried out using flow cytometry. Flow cytometry enables these proteins of interest to be counted by passing a suspension of cells through beams of light and fluorescence detectors enabling detection of protein expression.

Platelets were diluted to 2 x 10^6/ml and 50µl was stimulated with or without agonist for 10 minutes at 37°C under non stirring conditions. The sample was then incubated with

5µl of α-P-selectin, FITC-conjugated antibody (for α-granule secretion) or 5µl of a FITC-conjugated antibody raised against the surface receptor of interest in the dark at room temperature for 20 minutes. A further 200-450µl of modified Tyrodes-HEPEs buffer was added and samples were analyzed by FACs within 30 minutes.

Flow cytometry analysis (using Becton Dickinson FACsCalibur) was performed on each sample, a total of 10,000 events per sample were collected and the platelet population was identified by forward and side scatter profile and protein expression identified using fluorescence detection of the antibodies. Data was analysed using CellQuest software.

2.3.3 Measurement of intracellular calcium

Washed human platelets were prepared as previously described and then incubated with 3µM of the fluorescent probe Fura-2-AM for 1 hour at 37°C in the dark. FURA-2-AM is membrane permeable, once across the cell membrane, cellular esterases remove the acetoxymethyl esters generating FURA-2 which acts as a calcium indicator by binding intracellular calcium. The samples were then incubated in the presence or absence of inhibitors for 2 minutes prior to agonist stimulation (1 min with stirring). Agonist was added to platelet suspension at 37°C under continuous stirring (1200 rpm). Fluorescence changes were monitored using a fluorimeter (340nM excitation and 510nM emission). Changes in intracellular calcium concentration were calculated using the Grynkiewicz equation (Grynkiewicz, Poenie et al. 1985). 2mM $CaCl_2$ is added to set basal levels, 0.1% Triton x-100 is added to obtain fluorescence at maximal Ca^{2+} and 8mM EGTA is added to obtain fluorescence signal at near to zero levels of free Ca^{2+}.

2.3.4 Platelet spreading

To determine the ability of platelets to undergo αIIbβ3 outside-in signalling, platelet spreading (filopodia and lamellipodia generation) on fibrinogen coated coverslips was established. Coverslips were incubated with a suspension of fibrinogen (100μg/ml) or collagen (100μg/ml) for 1 hr at room temperature before washing with phosphate-buffered saline (PBS) and blocking with denatured fatty acid free BSA (5mg/ml) for 1 hr. Platelets at $2x10^7$/ml were added to the coverslips and left to incubate for 45 minutes at 37°C. Non adherent platelets were removed, the coverslip washed once with PBS, before fixing using formalin and left for 10 minutes before being washed three times in PBS and mounted. Adherent platelets were then imaged using DIC optics with a Zeiss 63x oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200M microscope. A Hamamatsu Orca 285 camera and Slidebook 4.0 software (Intelligent Imaging Innovations Inc, Denver, USA) was used for image capture and subsequent analysis.

To calculate the surface area of platelets, images were manually outlined and quantitated by determining the number of pixels within each outline using a Java plug-in, Image J software package. Adhesion data in each experiment was obtained by counting the number of platelets on 5 images of each coverslip that were chosen at random with each image encompassing an area of $15400\mu M^2$. The number of filopodia or lamellipodia per platelet were also counted, and platelets scored as having none, few (1-2), some (3,4,5), or many (6 or more) filopodia or lamellipodia and the relative frequency determined. Statistical analysis was performed as described below.

2.3.5 *In vitro* flow analysis

To determine aggregate formation on collagen under shear, mouse whole blood was drawn in to sodium heparin (5 IU/ml) and PPACK (40 μM). Glass capillary tubes (1 x 0.1 mm; Camlab, Cambridge, UK) were coated with 100 μg/ml type I collagen for 1 hr at room temperature under slow rotation. The capillaries were washed and blocked with PBS containing 5 mg/ml BSA for 1 hr at room temperature before being mounted on the stage of an inverted microscope (DM IRB; Leica, Milton Keynes, UK).

Anti-coagulated whole blood was preincubated with 2μM $DiOC_6$ for 10 minutes at 37°C to fluorescently label the cells and enable their visualisation. The blood was then perfused through the chamber for 4 min at a wall shear rate of 1000 s^{-1}, followed by washing for 3 min at the same shear rate with modified Tyrodes-HEPEs buffer before imaged using phase-contrast microscopy, Zeiss Axiovert 200M microscope equipped with a Hamamatsu Orca 285 digital camera (Hamamatsu Photonics UK Ltd, Herts, UK). Image analysis was performed off-line using a Java plug-in, ImageJ software. Platelet adhesion results are expressed as the percentage of surface area covered by platelets. Contents of capillaries were subsequently lysed and levels of adherent platelets assessed by blotting and probing for actin (see section 2.2.10).

2.3.6 Tail bleeding

Tail bleeding experiments were performed on 20-35g male and female WT, $PKC\theta^{-/-}$, $PKC\varepsilon^{-/-}$ and $PKC\theta^{-/-}/\varepsilon^{-/-}$ mice. Mice were anesthetized with isofluorane and subsequently injected with the analgesic buprenorphine intraperitoneally. The terminal 3mm of tail was removed using a sharp razor blade and blood lost was collected. Mice were allowed to bleed until they lost either 15% blood volume (calculated prior to the experiment

based on the animal weight and assuming blood volume of 70ml/kg) or for 20min. Data are presented as ratio between amount of blood loss (mg)/mouse weight (g) or as a rate of bleeding (mg/min).

2.4 METHODS – Protein Biochemistry

2.4.1 Western blot analysis

Western blot analysis is widely used to detect specific proteins in a given sample, for example a cell lysate. Here the proteins within the cell lysates are first separated using SDS-PAGE gel electrophoresis, which separates denatured proteins by the length of the polypeptide. The proteins are then transferred, in this case to a nitrocellulose membrane where they are then detected using target protein specific antibodies.

Washed platelets (5×10^8/ml) were incubated with the desired inhibitors and stimulated with the desired agonists under non-stirring conditions and left for 5-10minutes at 37°C. Stimulations were stopped by addition of an equal volume of ice cold 2x NP-40 lysis buffer. From this, a whole cell lysate sample was taken into 2x Laemmli loading buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 50mM Tris base, trace of bromophenol blue, pH 6.8) and incubated at 90°C for 5 minutes. Samples were separated by SDS-PAGE using varying percentage Tris- gels then transferred to nitrocellulose membranes.

Membranes were then blocked at room temperature for 1 hr in either 5% BSA or 4% non-fat milk. Membranes were then incubated with primary antibody (usually in 1:1000 dilution in BSA or milk/TBS-T) and washed for 5 minutes 3 times in TBS-T followed by incubation with the secondary HRP-linked antibody (usually in 1:1000 dilution in BSA or milk/TBS-T) and washed using TBS-T for 5 minutes 5 times. Membranes were then

visualised using enhanced chemiluminescence. To strip the membrane of antibodies the membrane was incubated with stripping buffer for 30 min at 50°C. They were then washed thoroughly in TBS-T, blocked and reprobed as described above. Phosphoserine PKC substrate proteins were detected by incubation overnight at 4°C with a monoclonal anti-phosphoserine PKC substrate antibody. This antibody detects endogenous levels of proteins when phosphorylated at a Ser residue within the PKC substrate recognition sequence; $(Lys/Arg)_{1-3}$ – X – (pSer) – Y – $(Lys/Arg)_{1-3}$, where X is any amino acid and Y is any hydrophobic amino acid.

2.4.2 Quantification of PKC isoforms in human and mouse platelets.

The PKC isoforms were quantified using washed platelet samples from five human donors and 3 WT mice. The number of copies of each isoform per platelet was determined using antibodies raised against the individual human isoforms, and the relative expression of levels of each PKC isoform was determined using a quantitative Western blotting method as previously used (Tomlinson, Kurosaki et al. 1999; Tomlinson, Kane et al. 2004; Protty, Watkins et al. 2009). Reference samples of GST-tagged forms of each human PKC isoform (purchased from Enzo Life Sciences) were subjected to SDS PAGE analysis and Western blotting and the bands were quantified using ECL in combination with the Biorad GelDoc system which enables quantitative Western blotting. Expression levels of each of the isoforms was calculated by comparing the level of PKC isoforms present in platelet lysate samples to those of the reference samples. The amount of PKC isoform per platelet was then calculated as detailed in Figure 2.2 and is expressed as the number of molecules per platelet and as a concentration.

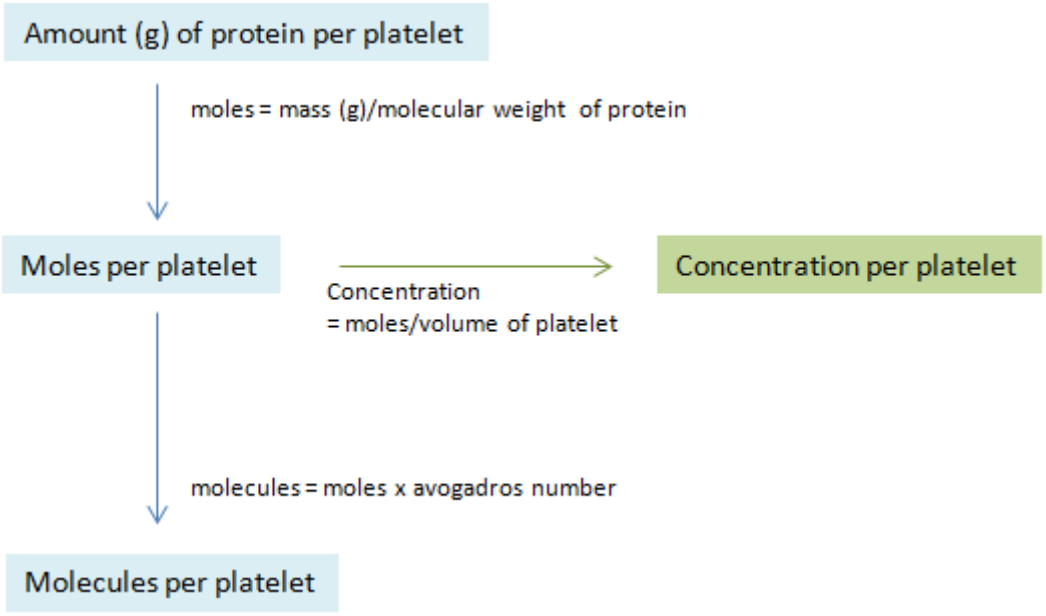


Figure 2.2. Quantification of the number of molecules per platelet. Calculation for the conversion of amount (g) of protein present per platelet to number of molecules and concentration per platelet.

2.5 METHODS – Data Analysis

2.5.1 Statistical analysis

Statistical analyses were carried on data using GraphPad prism software. When comparing two sets of data, an unpaired, 2-tailed Student's t test statistical analysis was used. If more than two means were present, significance was determined by one way ANOVA. $P \leq 0.05$ was considered statistically significant. Unless stated otherwise, values are expressed as mean ±SEM.

CHAPTER 3.

QUANTIFICATION OF THE ISOFORMS OF PROTEIN KINASE C IN HUMAN AND MOUSE PLATELETS.

3.1 INTRODUCTION

The PKC superfamily and its individual isoforms are critical mediators in platelet agonist induced signalling pathways, positively and negatively regulating several processes involved in platelet activation. At least ten separate isoforms of PKC have been identified which are divided into three subtypes, the classical (or conventional), novel and atypical isoforms, dependent on the basis of their structure and regulation or activation. Of these isoforms, both human and mouse platelets express the classical isoforms PKCα and PKCβ, and the novel isoforms PKCδ and PKCθ. Mouse platelets also express the additional novel isoform PKCε, whereas its presence in human platelets is controversial. There is also evidence for the expression of other isoforms, PKC η and ζ (Hall, Harper et al. 2008; Konopatskaya, Gilio et al. 2009; Nagy, Bhavaraju et al. 2009) (Murugappan, Tuluc et al. 2004; Buensuceso, Obergfell et al. 2005; Pears, Thornber et al. 2008; Bynagari, Nagy et al. 2009; Cohen, Braiman et al. 2009). In addition to the use of PKC broad spectrum and isoform-specific pharmacological inhibitors, recent understanding for the role of the major PKC isoforms expressed in platelets has progressed due to the use of transgenic mice models, allowing the study of mouse platelets that are deficient in a particular isoform.

Interestingly despite the apparent individual target substrates and non-redundant functions for each of the PKC isoforms identified so far in the regulation of platelet activation, the different isoforms of PKC show high sequence homology within their catalytic domains, suggesting different mechanisms of regulation which could also be affected by levels of expression. The relative expression level of the isoforms in both human and mouse platelets is unknown. Quantification of these levels may provide insight into the relative importance and significance of the different isoforms of PKC in the regulation of platelet activation, and would also allow a direct comparison between the two species to determine if the results obtained using null mice are directly applicable to human platelets.

3.1.1 AIMS:

The data and results presented in this chapter focus on the detection and quantification of the five major PKC isoforms expressed in human and WT mouse platelets; PKCα, β, δ, θ and PKCε, using a quantitative western blotting protocol (as detailed in the Methods section 2.4.2).

3.2 RESULTS

3.2.1 Conservation of antibody recognition sequences or regions in mouse PKC isoforms.

To enable the quantification of the PKC isoforms in human and mouse platelets, GST-tagged recombinant full length human proteins of each of the individual human isoforms were used. `Isoform-specific' antisera are commercially available and have been well

characterised (see for e.g. (Hall, Harper et al. 2008; Pears, Thornber et al. 2008; Konopatskaya, Gilio et al. 2009)). In order to use these antibodies for quantification it was necessary to show that they did not show cross reactivity with other isoforms. Therefore all five isoforms were analysed by western blot using each of the antisera as shown in Figure 3.1. Detection of the GST tag using an anti-GST antibody was used to confirm the amount and presence of each isoform loaded. Each antibody was only able to recognise and detect the isoform it was raised against and did not show any cross reactivity with any of the other isoforms (Figure 3.1).

The use of the human versions of each isoform as standards in the quantification of the mouse isoforms is a possible limitation of this study. Mouse recombinant protein standards are not available commercially and cloning of the mouse variants proved unsuccessful. Therefore, the conservation of the antibody recognition sequence (for the antibodies used) between the human and mouse isoforms was analysed to ensure high conservation (Table 3.1). Sequence alignment of the human and mouse protein variants enabled the identification of conserved regions. All isoforms show a total sequence homology of at least 95% between human and mouse variants. Further comparison of the antibody recognition sequences for the isoform-specific antisera revealed that the conservation between the human and mouse isoform variants was at least 97% (Table 3.1). This makes it likely that the antisera show similar reactivity to the isoforms from the two species, but the possibility that they do not must be kept in mind especially as four of the five antibodies used were monoclonal (see discussion).

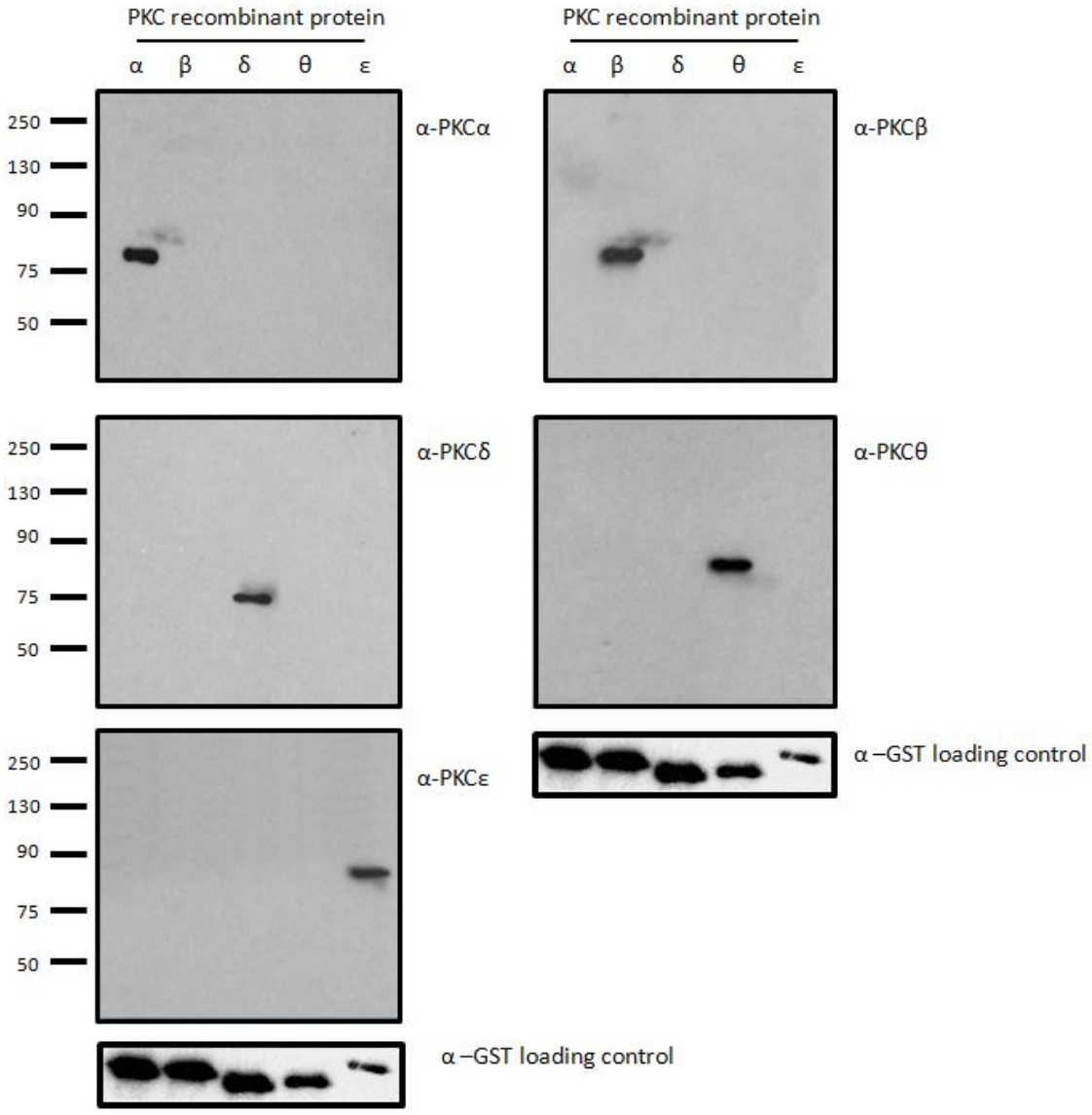


Figure 3.1 PKC isoform-specific antibodies show no cross reactivity for the other PKC isoforms. 5ng GST tagged- human recombinant proteins for each of the PKC isoforms, PKCα, β, δ, ε and θ were resolved by SDS-PAGE electrophoresis and western blotting performed. Isoform-specific antibodies raised against a particular isoform were used. GST was used as a loading control. The results are representative of 3 experiments.

ISOFORM	REGION ANTIBODY RAISED AGAINST	ANTIBODY TYPE.	SPECIES ANTIBODY RAISED AGAINST	CONSERVATION OF SAME REGION IN MOUSE.
PKCα	Whole protein	Polyclonal	Human	99.7%
PKCβ	a.a 126-324	Monoclonal	Human	97%
PKCδ	a.a 114-289	Monoclonal	Human	97.7%
PKCθ	a.a 21-217	Monoclonal	Human	98.5%
PKCε	a.a 1-175	Monoclonal	Human	99.4%

Table 3.1 Conservation of the antibody recognition regions of the human and mouse variants of the major PKC isoforms. Sequence alignment of the human and mouse variants of each PKC isoform and the conservation within each specific sequence determined. (a.a = amino acid residues)

3.2.2 Presence of the PKC isoforms in human and murine platelets.

The presence of the PKC isoforms, PKCα, β, δ, θ and ε in both human and WT mouse platelets was confirmed using these antibodies (Figure 3.2). In support of previously published data human platelets were found to express PKCα, β, δ and θ while PKCε was undetectable (Figure 3.2). All five isoforms were found to be expressed in mouse platelets (Figure 3.2).

3.2.3 Quantification of PKC isoforms in human platelets.

Having verified the antibodies chosen and the presence of the isoforms in platelets, the levels of each of the PKC isoforms in human and mouse platelets was quantified using a similar approach to one previously used to quantify Tec tyrosine kinases in lymphocytes (Tomlinson, Kurosaki et al. 1999; Tomlinson, Kane et al. 2004; Protty, Watkins et al. 2009). This involves a western blotting protocol comparing the signal for an isoform expressed in a platelet sample to a dilution series of purified protein standard of known concentration (Figure 3.3A). The Biorad GelDoc system allowed the linear range in the western blot signal to be identified for each of the isoforms (example shown in Figure 3.3A). The concentration of the platelet samples was altered so that it fell in the linear range for each isoform. Platelets from five donors were analysed on 3 occasions for each isoform. This enabled the number of protein molecules per platelet (Figure 3.3B) and intracellular concentration (Figure 3.3C) to be calculated as detailed in the methods section 2.4.2. In human platelets the classical isoforms PKCα and PKCβ and the novel isoform PKCδ were calculated to be expressed at similar levels with approximately 11,000-12,000 copies per platelet, corresponding to an intracellular concentration of

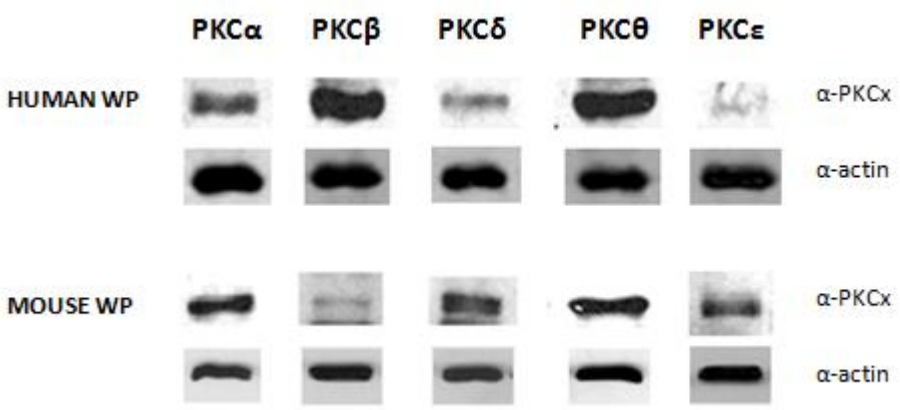
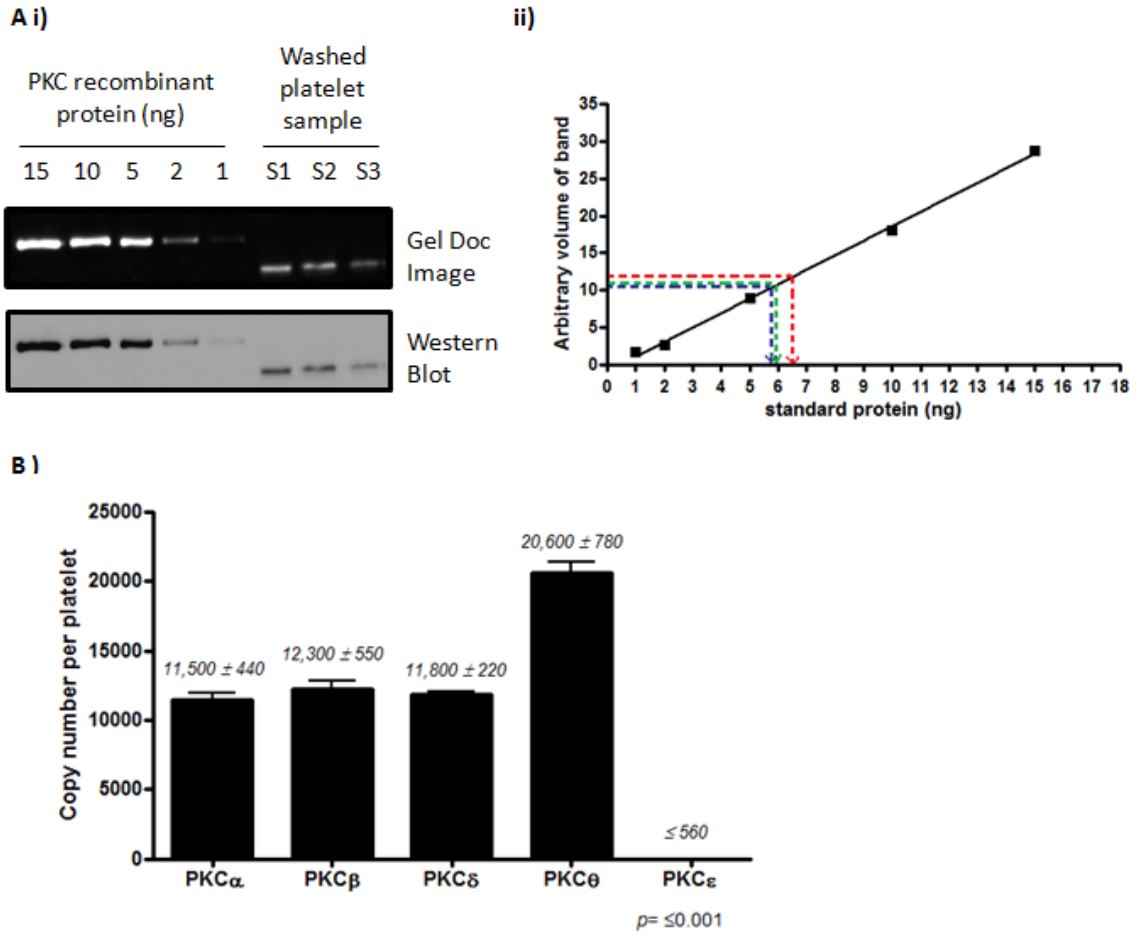


Figure 3.2 Expression and detection of the major PKC isoforms in human and mouse platelets. Human and mouse washed platelets (WP) were lysed and samples separated by SDS-PAGE electrophoresis. The expression of PKCα, β, δ, ε and θ was determined by western blot using antibodies raised against each isoform. Actin was used as a loading control. The results are representative of three experiments.



C)

Isoform	Molecules per platelet	Concentration (μM)
α	11,500 ± 440	2.6
β	12,300 ± 550	2.8
δ	11,800 ± 220	2.7
θ	20,600 ± 780	4.6
ε	Less than 550	Less than 0.12

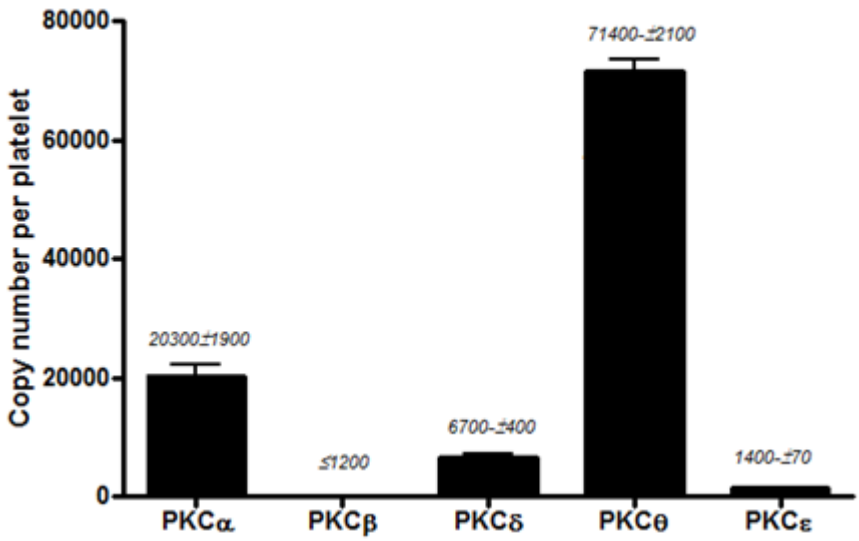
Figure 3.3 Quantification of the major PKC isoforms in human platelets. A) Whole cell lysates (20μl at $2.5x10^8$/ml from human washed platelets (S1, 2, 3) were resolved alongside recombinant protein standards, PKCα as an example is shown, using SDS PAGE and then i) subjected to western blotting using isoform-specific antisera. Quantitative western blotting was performed using ECL and the BioRad Gel Doc system. ii) Isoform expression levels in human platelets were compared to the recombinant protein standards via their relative intensities (coloured lines represent different samples) and converted to B) copy number per cell and C) intracellular concentration. The data represents platelets from five human donors, data for each isoform was repeated at least 3 times. Data are presented as mean ± SEM.

~2.6-2.8μM. In comparison, the novel isoform, PKCθ, was expressed at a 1.75-fold greater level, with approximately 20,000 copies per platelet and an estimated intracellular concentration of 4.6μM. PKCε fell below the level of detection. These observations are interesting as the classical isoforms of PKC, especially PKCα, are thought to play the major roles in the regulation of platelet activation and yet are not the most highly expressed isoforms.

3.2.4 Quantification of PKC isoforms in mouse platelets.

A similar approach was used to quantify the expression of the PKC isoforms in mouse platelets, lysates from 3 WT mice were used, with each analysed on 3 occasions (Figure 3.4). Quantification of the level of the major PKC isoforms in mouse platelets identified a different pattern to that found in human platelets. As with human platelets, PKCθ is the most highly expressed but is present at a much higher level with approximately 70,000 copies equating to an intracellular concentration of 16μM. PKCα is also expressed at a slightly higher level than in human platelets with an intracellular concentration of 4.6μM, corresponding to 20,000 copies per cell. Interestingly both PKCβ and PKCδ are expressed at much lower levels in mouse than in human with only 7,000 copies of PKCδ per platelet (1.5μM) and fewer than 1,200 copies of PKCβ (0.27μM). Interestingly the novel isoform PKCε, which has previously been considered to be a major isoform in mouse platelets following robust detection via western blot, is only expressed at low levels in comparison to the other isoforms (PKCα, δ, θ), with only 1500 copies per platelet and an intracellular concentration of approximately 0.3μM.

A)



B)

Isoform	Molecules per platelet	Concentration (μM)
α	20,300 ± 1900	4.6
β	Less than 1200	Less than 0.27
δ	6,700 ± 400	1.5
θ	71,400 ± 2100	16
ε	1,400 ± 70	0.31

Figure 3.4 Quantification of the major PKC isoforms in mouse platelets. PKC isoforms levels in whole cell lysates from mouse washed platelets were compared to recombinant human protein standards using a quantitative western blotting system as previously detailed in the figure legend for figure .3. Data was then converted to A and B) copy number per cell and intracellular concentration. The data represents platelets from ≥ 3 mice, data for each isoform was repeated at least 3 times. Data are presented as mean ± SEM.

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3.3 DISCUSSION

Expression of several PKC isoforms has been observed in human (PKCα, β, δ and θ) and mouse platelets (PKCα, β, δ, θ and ε) and is confirmed here. Interestingly despite their distinct functional (and in many cases non-redundant) roles in the regulation of the processes involved in platelet activation and thrombus formation indicative of different isoform-specific substrate targets, the different isoforms of PKC show high sequence homology within their catalytic domains (Shattil and Brass 1987; King and Rittenhouse 1989; Ryu, Kim et al. 1990; Toullec, Pianetti et al. 1991; Walker and Watson 1993; Wilkinson, Parker et al. 1993; Yoshioka, Shirakawa et al. 2001; Quinton, Kim et al. 2002; Tabuchi, Yoshioka et al. 2003; Murugappan, Tuluc et al. 2004; Harper and Poole 2007; Strehl, Munnix et al. 2007; Hall, Harper et al. 2008; Pears, Thornber et al. 2008; Harper and Poole 2009; Konopatskaya, Gilio et al. 2009; Nagy, Bhavaraju et al. 2009; Gilio, Harper et al. 2010; Harper, Molkentin et al. 2010; Harper and Poole 2010; Harper and Poole 2010). This therefore suggests that different mechanisms of regulation must exist for each of the isoforms. It is already known that there are structural differences in the regulatory domains between the different classes of the PKC isoforms which enable the classical isoforms to be regulated by Ca^{2+} and DAG, whilst the novel isoforms are Ca^{2+} insensitive as a result of their modified C2-like domain (Newton 1997; Mellor and Parker 1998). However, these differences in structure do not explain the differences in function and target substrates within the different classes (i.e. between PKCα and β and between the novel isoforms PKCδ, θ and ε). To determine whether differences in the levels of isoform expression could explain differences in isoform involvement, the levels of expression of the different isoforms per platelet was quantified.

In support of previously published data (Pears, Thornber et al. 2008) (Thornber, unpublished data), robust expression of PKCα, β, δ, and θ was detected in both human and mouse platelets using isoform-specific antisera. Although PKCε cannot be detected in human platelets, it is expressed in mouse platelets. Species-specific protein expression when studying platelets is not unexpected, as the PAR1 receptor, the major thrombin receptor in human platelets, is not present in mouse platelets. These inter-species differences do however, highlight a potential problem with using mouse platelets to determine and understand the role of PKC and other proteins in human platelets.

Using a quantitative western blotting protocol, the classical isoforms PKCα and PKCβ and the novel isoform PKCδ were all found to be expressed at similar levels in human platelets (11,000-12,000 copies), in contrast PKCθ appears to be expressed at almost double these levels (20,000 copies). Interestingly very little variation in isoform expression levels was seen between the different donors, showing the expression of the different isoforms is consistent between individuals and not highly variable. Several other signalling proteins have also been quantified in human platelets. Tetraspanin CD9 is present at approximately 50,000 copies per platelet (Protty, Watkins et al. 2009), and the integrin αIIbβ3 is expressed at 80,000 copies (Wagner, Mascelli et al. 1996). These levels are significantly higher than those of the PKC isoforms, although they are in the same order of magnitude. Interestingly, the PKC isoforms in human platelets are expressed at higher levels than both the collagen receptor GPVI, and the thrombin receptor PAR1, which are thought to be present in platelets at approximately 4000-6000 copies and 1200 copies per platelet respectively (Best, Senis et al. 2003; Dupont, Fontana et al. 2003), thereby suggesting the PKC isoforms are expressed in the same linear range as several other signalling proteins expressed in platelets.

The pattern of PKC isoform expression was found to be considerably different in mouse platelets, although this does assume that the antibodies recognise the proteins with equal efficiency in the two systems. This is likely due to the high conservation of the epitopes, but it would be preferable to repeat the analysis using mouse recombinant PKC isoforms as standards or at least to confirm the results using different isoform-specific antibodies. As with human platelets, PKCθ is the most highly expressed isoform, but it is expressed at much higher levels with a concentration of approximately 16μM per platelet, 3.5 times higher than that in human platelets. PKCα is also expressed at a higher concentration in mouse platelets at approximately 4.6μM per platelet compared to 2.6μM in human platelets. In contrast both PKCβ and PKCδ are expressed at much lower levels in mice; with concentrations of 0.27μM and 1.5μM per platelet compared to 2.8μM and 2.7μM respectively. PKCε was also only found to be expressed at low levels with an intracellular concentration of around 0.31μM. Such low levels of expression for PKCβ and PKCε in mouse platelets is interesting as both isoforms have a clearly defined role in the regulation of mouse platelet activation, in particular PKCβ which has been shown to play a relatively major role in the positive regulation of thrombus formation on collagen using platelets from PKCβ null mice (Gilio, Harper et al. 2010). Interestingly PKCβ which is expressed at levels similar to PKCα in human platelets is present at less than $1/10^{th}$ of the amount of PKCα in mouse platelets. In contrast PKCθ is highly expressed, yet does not appear to have a clearly defined major role in the regulation of platelet activation, several roles have been identified by several groups but many do not appear to be robust (see Chapter 6 for further discussion). How can we be sure that with such varying levels of protein expression, the functions of the individual PKC isoforms are the same between species?

These results presented in this chapter highlight different levels of expression for the different isoforms of PKC and different patterns of expression between species. There does not however, appear to be an obvious correlation between expression levels and the extent of isoform function. For example, previously published data and studies in future chapters using human platelets and isoform-specific inhibitors have identified major positive phenotypes for PKCα and PKCβ in platelet activation downstream of several agonists compared to those of the novel isoforms PKCδ and PKCθ which show less severe phenotypes (Gilio, Harper et al. 2010). However, in human platelets PKCα and β appear to be expressed at the same level as PKCδ, and at levels which are considerably lower than the level of PKCθ expression. Studies using mice deficient in single PKC isoforms have also highlighted major positive roles, that are thought to be non redundant, for the classical isoforms in mouse platelet activation and more minor roles for the novel isoforms of PKC (Murugappan, Tuluc et al. 2004; Soriani, Moran et al. 2006; Yacoub, Theoret et al. 2006; Hall, Harper et al. 2008; Pears, Thornber et al. 2008; Cohen, Braiman et al. 2009; Harper and Poole 2009; Konopatskaya, Gilio et al. 2009; Nagy, Bhavaraju et al. 2009; Gilio, Harper et al. 2010). However, it is interesting that PKCβ is expressed at such low levels than the other isoforms despite its robust functional phenotype. Additionally expression of PKCα in mouse platelets is considerably lower than that of PKCθ, which has not been reported to play as major a functional role as either of the classical isoforms.

As there appears to be no clear correlation between expression and function, the results presented in this chapter suggests that other factors must be important for the regulation and functionality of the different PKC isoforms that enables isoform-specificity, such as differences in activation and subcellular localisation patterns that

determines the enzymes ability to interact with specific cofactors, interacting proteins and binding partners, which are not dependent on isoform expression levels (Cohen, Braiman et al. 2011).

It is known that phosphorylation of PKC is a requirement for kinase activity and it is thought that the different isoforms of PKC undergo different phosphorylation patterns and hence activation events. The novel isoforms, unlike the classical isoforms, appear to undergo additional isoform-specific tyrosine phosphorylation of the catalytic or regulatory domains following activation by particular agonists. Following agonist induced platelet activation, PKCδ has been shown to be phosphorylated at two tyrosine residues (Tyr311 and Tyr565) (Hall, Jones et al. 2007; Murugappan, Chari et al. 2009), PKCθ undergoes phosphorylation at Tyr90 (Soriani, Moran et al. 2006) and PKCε is constitutively phosphorylated (Pears, Thornber et al. 2008). It is likely that it is these differences in activation and regulation that enable differences in cellular localisation, such as targeting to the plasma and nuclear membranes and subcellular compartments from the cytosol which makes the isoforms accessible to their specific substrates in an agonist dependent manner, PKCδ for example has already been shown to specifically target VASP which is required for the regulation of filopodia formation (Pula, Schuh et al. 2006).

The data presented in this chapter identifies varying levels of expression and intracellular concentrations of the 5 major PKC isoforms expressed in human and mouse platelets. Interestingly quantification of the PKC isoforms in both human and mouse platelets identified that there are significant differences in the expression levels and pattern of expression of the PKC isoforms between the two species. This provides another example of inter-species differences and taking this into consideration caution should be

observed when using mouse models as tools to understand PKC isoforms (and potentially other proteins) and their roles in human platelets. These differences could have implications for the use of mouse models in the understanding of human platelet function and in the development of any PKC targeting therapies.

CHAPTER 4.

THE ROLE OF THE PKC ISOFORMS IN HUMAN PLATELET ACTIVATION – A PHARMACOLOGICAL APPROACH

4.1 INTRODUCTION

Platelet activation requires a complex balance of positive and negative signalling pathways, several of which are regulated by PKC isoforms. Pharmacological studies, have shown that several key processes involved in platelet activation including integrin activation and granule secretion are positively regulated by PKC downstream of several platelet agonists, as they are reduced or ablated following treatment with broad spectrum PKC inhibitors (Shattil and Brass 1987; Toullec, Pianetti et al. 1991; Walker and Watson 1993; Hers, Donath et al. 1998; Paul, Jin et al. 1999; Quinton, Kim et al. 2002). Broad spectrum inhibitors have also identified negative regulatory roles for PKC in processes involved in platelet activation, including receptor desensitisation and Ca^{2+} extrusion (Rink and Sage 1990; Cavallini and Alexandre 1994; Hardy, Conley et al. 2005) (Strehl, Munnix et al. 2007). These findings raise the possibility of isoform-specific roles of PKC in regulating platelet activation.

Platelets are anucleate and studies on human platelets are therefore limited to the use of pharmacological inhibitors. In this chapter the effects of isoform non-selective and selective inhibitors of the PKC superfamily on human platelet function was investigated in order to determine the roles of PKC isoforms in regulating platelet activation. This work has focussed on the major platelet activating receptors, GPVI (using both collagen

and collagen-related peptide, CRP) and CLEC-2 (using rhodocytin) in view of the similar pathways through which they stimulate platelet activation. Both signalling via tyrosine kinase linked pathways, although significant differences between these pathways exist, for example rhodocytin induced responses have a greater dependence on secretion compared to GPVI and because the role of some of the PKC isoforms in GPVI signalling is controversial. These results were then contrasted with a strong agonist (thrombin) that signals through two G protein coupled PAR receptors, PAR-1 and PAR-4. There is increasing evidence for differences in the mode of regulation via PKC downstream of different platelet activating receptors, (see section '1.8.2 PKC and Platelet Activation' onwards in Chapter 1- Introduction) and therefore whether differences exist at the level of PKC involvement was investigated.

The inhibitors chosen for investigation are as follows: the broad spectrum PKC isoform inhibitors, Ro31-8220 and Ro31-8425, were used to inhibit all PKC activity as both have been shown to inhibit both aggregation and dense granule secretion downstream of several platelet agonists (Walker and Watson 1993; Quinton, Kim et al. 2002; Strehl, Munnix et al. 2007; Gilio, Harper et al. 2010). The modified bisindolylmaleimide, Gö6983 primarily targets the classical PKC isoforms but unfortunately can also inhibit PKCδ at higher inhibitor concentrations (Gschwendt, Dieterich et al. 1996). This inhibitor is however included here as the frequently used classical isoform inhibitor Gö6976, has recently been shown to inhibit the tyrosine kinase Syk in human platelets (Getz, Mayanglambam et al. 2011). As Syk is a key mediator in tyrosine kinase linked signalling pathways, such as GPVI and CLEC-2 signalling, Gö6976 is not included here. Isoform-specific inhibitors that were available and have been used previously in platelet studies include, a PKCβ inhibitor, (3(-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-

pyrrole-2,5-dione) that selectively inhibits the β isoform over the others (Tanaka, Sagawa et al. 2004), and was used in the studies by Gilio et al (Gilio, Harper et al. 2010), and a PKCθ inhibitor, compound 20 (potency PKCθ>δ>α/β) donated by Boehringer Pharmaceuticals (Cywin, Dahmann et al. 2007). This PKCθ inhibitor was used by Zanin-Zhorov et al (2010) in the study of T cell function and a similar inhibitor was also used by Gilio et al (2010) although the exact inhibitor used is unclear (Zanin-Zhorov, Ding et al. 2010). We also included Rottlerin, a phloroglucinol derivative which has frequently been used as a PKCδ inhibitor (Crosby and Poole 2003; Murugappan, Tuluc et al. 2004; Pula, Schuh et al. 2006; Gilio, Harper et al. 2010) although this has been reported to have non-PKC targets (Cohen 1999; Davies, Reddy et al. 2000; Bain, Plater et al. 2007; Soltoff 2007). A list of the inhibitors used and their IC_{50} values for individual PKC isoforms are summarised in Table 4.1 and a summary of the roles previously identified for the different isoforms of PKC using the above inhibitors is summarised in Table 4.2.

4.1.1 AIMS

In this chapter the relative effects of both broad spectrum and isoform-selective inhibitors of the PKC superfamily on human washed platelet function was studied in order to determine the roles for individual PKC isoforms in the regulation of human platelet activation downstream of the major platelet agonist receptors, GPVI, PAR and CLEC-2. Aggregation and dense granule secretion were monitored following stimulation with maximal and sub-maximal concentrations of agonist in the presence and absence of varying concentrations of a range of PKC inhibitors.

	IC_{50} value (μM)				Reference
	PKCα	PKCβ	PKCδ	PKCθ	
Ro31-8220	0.005	0.024	-	-	(Wilkinson, Parker et al. 1993)
Ro31-8245	0.008	0.014	0.013	-	(Wilkinson, Parker et al. 1993)
Gö6983	0.007	0.007	-	-	(Gschwendt, Dieterich et al. 1996)
PKCβ inhibitor	0.331	0.02	-	-	(Tanaka, Sagawa et al. 2004)
Rottlerin	30	42	3-6	-	(Gschwendt, Muller et al. 1994)
PKCθ inhibitor	PKCθ inhibitor shows at least 10 fold selectivity for PKCθ over PKCα, although the IC_{50} values for the other PKC isoforms are unknown			0.018	(Cywin, Dahmann et al. 2007)

Table 4.1. PKC inhibitor IC_{50} values for inhibition of the major PKC isoforms expressed in human platelets as determined in the studies referenced under idealised conditions. '-' is data not known.

Inhibitors	Role for PKC identified downstream of the following platelet receptors:				
	GPVI	CLEC-2	PAR	P2Y (ADP)	TP (TxA_2)
Ro31-8220	+ve	-	+ve	+ve	+ve
Ro31-8425	+ve	-	+ve	-	-
Go6983		-	-	-	-
Go6976	+ve	-	+ve	-	-
PKCβ inhibitor	+ve	-	-	-	-
Rottlerin	-ve	-	+ve	-	-
PKCθ inhibitor	-ve*	-	-	-	-

* as identified using a PKCθ inhibitor, which may not be the same as the one used here.

Table 4.2. Roles identified for the PKC superfamily in the regulation of platelet activation downstream of several receptor signalling pathways using pharmacological inhibitors, both broad spectrum and Isoform-specific inhibitors. References are as detailed above. Positive roles for PKC (or PKC isoform) identified as treatment with inhibitors caused a reduction on platelet aggregation or secretion, negative roles for PKC (or PKC isoform) identified as treatment with inhibitors caused a potentiation of platelet aggregation or secretion.

4.2 RESULTS

4.2.1 Efficacy of PKC inhibitors

The ability of the inhibitors to reduce PKC-dependent phosphorylation induced by the phorbol ester, PMA (a direct activator of PKC), in washed platelets was investigated (Figure 1). Proteins were separated by SDS-PAGE electrophoresis before western blotting using a phospho-specific PKC substrate Ab, which recognises PKC-dependent substrate phosphorylation (Pula, Schuh et al. 2006).

PMA induces an increase in phosphorylation of a number of proteins (the number of bands observed increased) in inhibitor untreated platelets in comparison to unstimulated controls. The broad spectrum inhibitors Ro31-8220 and Ro31-8425 produce concentration-dependent inhibition of phosphorylation of all proteins with thresholds at 0.1-0.3μM for Ro31-8220 and 0.3-1μM for Ro31-8425 with almost complete inhibition occurring by 10μM in both cases (Figure 4.1). The classical isoform inhibitor Gö6983 also induced inhibition of phosphorylation with a threshold of 0.1μM with almost complete inhibition by 10μM with the exception of a substrate at approximately 75kDa. The PKCβ-specific inhibitor showed significant inhibition of phosphorylation by 10μM and total inhibition at 30μM, perhaps suggesting loss of selectivity at such high concentrations. At 1μM inhibitor, a reduction to approximately 30% of phosphorylation is observed in the majority of substrate bands. The novel isoform inhibitors, rottlerin and the PKCθ inhibitor, appeared to have very little effect on phosphorylation on most of the substrates even at high concentrations (30μM) although significant inhibition of phosphorylation of higher molecular weight bands was observed at low concentrations of Rottlerin (0.3μM). The lack of a significant change in PKC

dependent substrate phosphorylation suggests relatively minor roles for these isoforms in PKC dependent activation in human platelets. Although it is possible that phosphorylation and activation of high molecular weight proteins are dependent on PKCδ. Interestingly, some bands show different patterns of inhibition following treatment with the different inhibitors, suggesting some PKC substrate phosphorylation patterns are Isoform-specific and therefore certain PKC substrates are only affected in the presence of the corresponding inhibitors. For example, the molecular weight band at approximately 90kDa is to some extent inhibited following treatment with all of the PKC inhibitors used, suggesting that this particular PKC substrate is not phosphorylated and therefore regulated by a specific isoform of PKC. In contrast the high molecular weight band at approximately 250kDa, is not inhibited in the presence of the PKCθ inhibitor but is inhibited in the presence of other inhibitors highlighting no role for PKCθ in the phosphorylation and regulation of this particular substrate. These results also appear to suggest that the classical isoforms of PKC represent the major component of platelet PKC activity in human platelets, whilst the novel PKC isoforms in comparison appear to have relatively minor roles.

4.2.2 The effect of the PKC inhibitors on PKC dependent platelet aggregation and dense granule secretion.

Western blot analysis of PKC dependent substrate phosphorylation appeared to show inhibitory effects on PKC substrate phosphorylation for most of the inhibitors used. In order to determine the role of the PKC isoforms in platelet activation, both aggregation and dense granule secretion were assessed following activation by PMA in the presence of increasing concentrations of each of the inhibitors (Figure 4.2 and Figure 4.3).

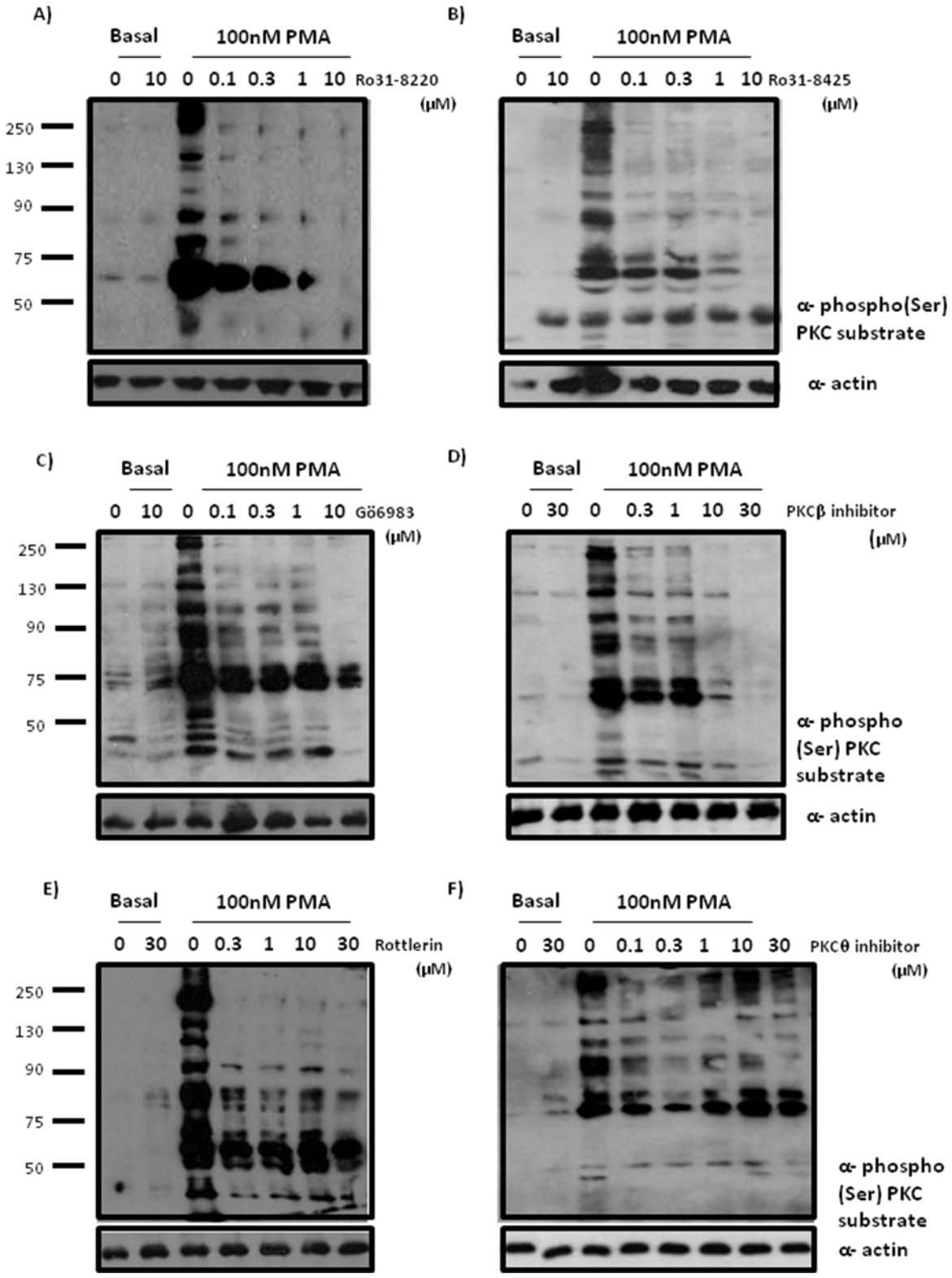

Figure 4.1 Effect of PKC Isoform-specific inhibitors on PKC mediated phosphorylation. Effect of broad spectrum inhibitors (A) Ro31-8220, (B) Ro31-8425, classical isoform inhibitors (C) Gö6983 and (D) PKCβ inhibitor and novel isoform inhibitors (E) Rottlerin and (F) PKCθ inhibitor on PKC substrate phosphorylation in human washed platelets. Whole cell lysates were resolved by SDS-PAGE and subject to western blot analysis. Phosphorylation by PKC in washed platelet lysates was detected using α-phospho(ser)-

PKC substrate antibody, in untreated/unstimulated platelets (Basal) and platelets following activation by PMA (100nM) in the presence or absence of increasing concentrations of the inhibitors, (ranging between 0, 0.1, 0.3, 1 ,10 and 30µM). Actin was used as a loading control. Representative blots shown, n=3.

Concentrations as low as 0.1 μM of both of the broad spectrum inhibitors Ro31-8220 and Ro31-8425 caused complete inhibition of aggregation and dense granule secretion to PMA (Figure 4.2). This concentration of Ro31-8425 is 100 fold lower than that used by Gilio et al (2010). Gö6983 also achieved complete inhibition of both responses at 0.1μM even though this concentration only induced partial loss of PKC substrate phosphorylation. The PKCβ (1μM) and PKCθ (1μM) inhibitors reduced aggregation and secretion by approximately 50% inhibition in response to PMA, whereas rottlerin, caused potentiation of aggregation and secretion at concentrations of 10μM and higher. These results support previously published data for an overall positive role for the PKC family in platelet activation, but also suggest differential roles for individual isoforms, including a potential negative role for PKCδ. The marked inhibition observed at low concentrations of Gö6983 indicates that PKCα and PKCβ could be the major positive mediators of aggregation and secretion to PMA, supported by PKCθ. In contrast PKCδ does not appear to play a positive role in PMA induced aggregation and secretion (Figure 4.2 and 4.3).

The effect of the different PKC inhibitors on human platelet activation in response to several receptor agonists, including CRP and Collagen, PAR receptor agonist thrombin, and the CLEC-2 receptor agonist rhodocytin, was then investigated. Ro31-8425 was used as a representative broad spectrum inhibitor, as this was the inhibitor previously used in similar experiments by Gilio et al (2010).

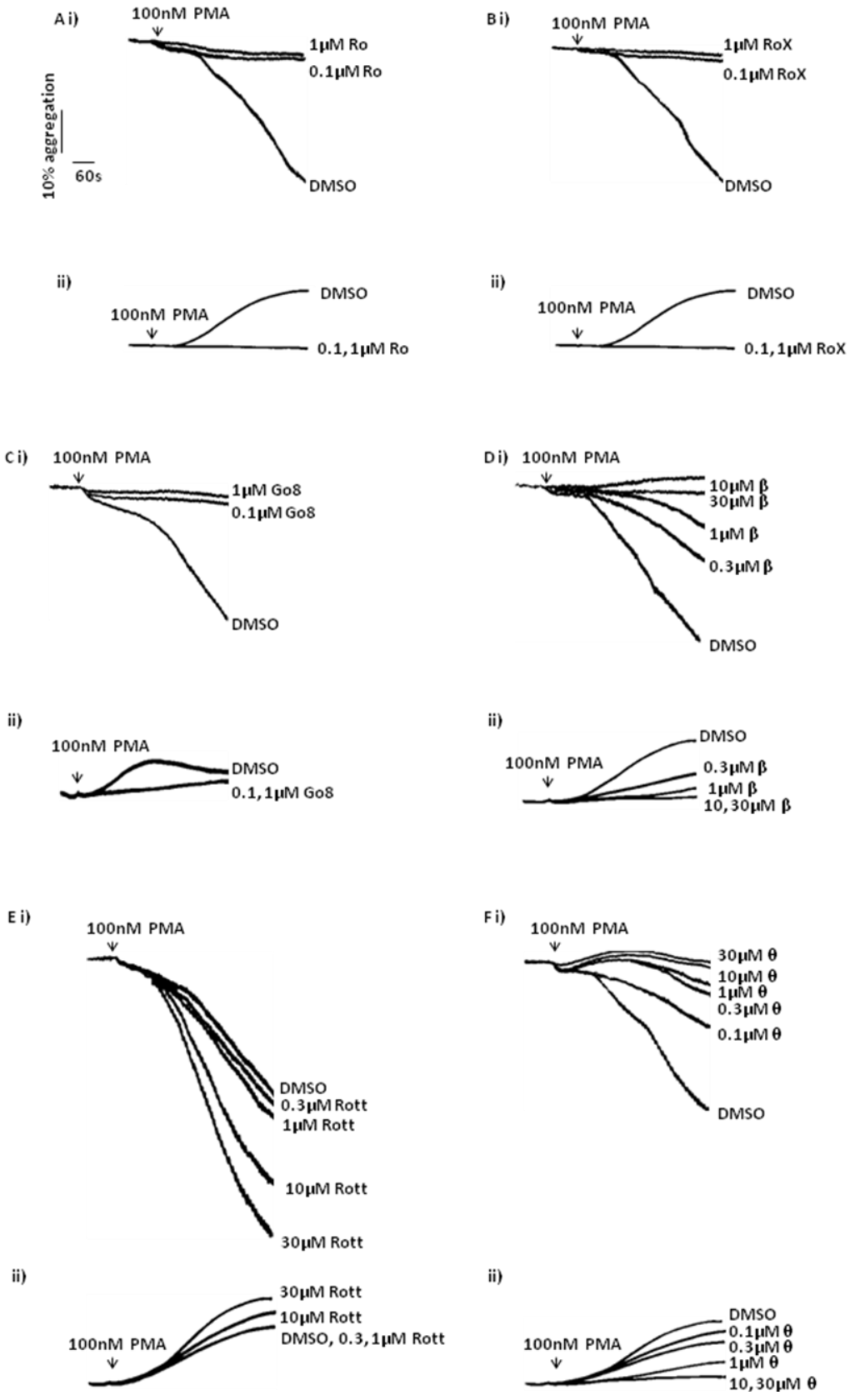

Figure 4.2. Inhibition of the classical and novel PKC isoforms differentially affects PKC dependent platelet activation. Washed human platelets were pre-treated with

increasing concentrations of the different PKC inhibitors, A) Ro31-8220 (Ro), B) Ro31-8425 (RoX), C) Gö6983 (Gö8), D) PKCβ inhibitor (β), E) Rottlerin (Rott) and F) PKCθ inhibitor (θ) and stimulated with PMA (100nM). Representative traces of the effect of inhibitors on i) aggregation (optical light transmission) and ii) dense granule secretion (luciferin-luciferase light emittance) are shown.

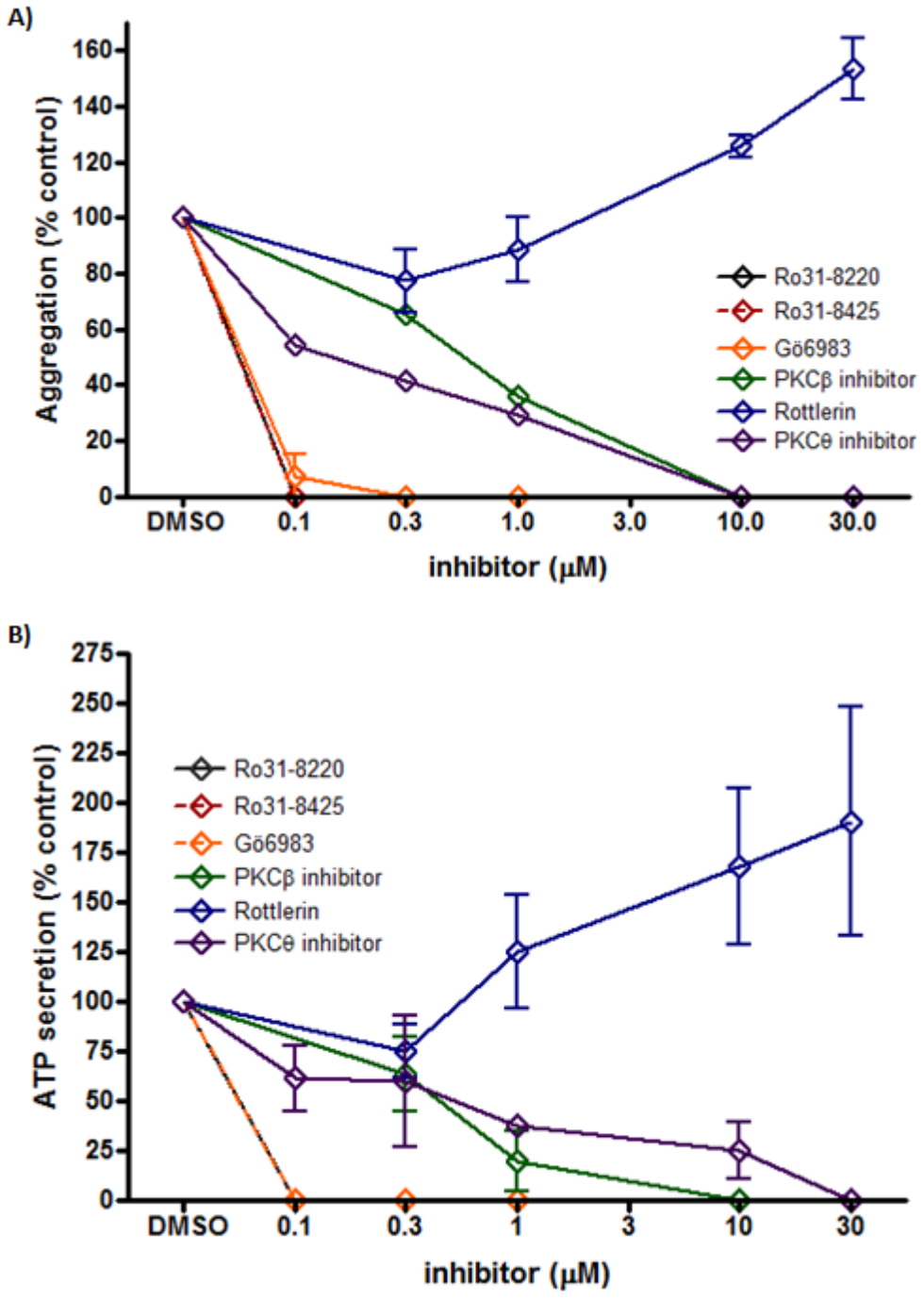

Figure 4.3 Inhibition of the classical and novel PKC isoforms differentially affects PKC dependent platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, Ro31-8220, Ro31-8425, Gö6983, PKCβ inhibitor and PKCθ inhibitor and stimulated with PMA (100nM). Dose response curves for each inhibitor are shown, A) aggregation and B) dense granule secretion. Lines for Ro31-8220, Ro31-8425 and Gö6983 show overlap as extent of inhibition was the same at the corresponding concentrations. Effects are expressed as percentages of control condition, mean ± SEM. ($n \geq 3$)

4.2.3. The effect of PKC inhibition on GPVI and Collagen-induced platelet activation.

4.2.3.1 The effect of the PKC inhibitors on GPVI induced PKC substrate phosphorylation

The effect of the different PKC inhibitors on PKC substrate phosphorylation downstream of GPVI activation was determined in platelets activated by a maximal and submaximal concentration of CRP (1 and 0.5µg/ml respectively). The two concentrations were used to ensure significant activation of PKC and to ensure activation was submaximal to enable inhibition or potentiation to be observed. However, similar patterns of phosphorylation were observed downstream of both CRP concentrations so for clarity only the results for 1µg/ml CRP are included here (Figure 4.4). A different pattern of phosphorylation, most notably decreased phosphorylation of higher molecular weight proteins, is seen following CRP stimulation in comparison to PMA stimulated samples highlighting agonist specific PKC phosphorylation of different substrates (Figure 4.5-4.10). Treatment with Ro31-8425 significantly inhibited substrate phosphorylation but only at concentrations of the inhibitor of 10µM and above, although some inhibition of phosphorylation (substrates at approximately 90 and 100kDa) does occur at lower concentrations (0.3-1µM). A similar pattern of inhibition was also observed following treatment with Gö6983. Treatment with the PKCβ inhibitor shows significant inhibition at 10µM and no inhibition of phosphorylation is apparent until high concentrations (30µM) of the PKCθ inhibitor. Interestingly, following stimulation by 1ug/ml CRP, some bands for example at approximately 55kDa and 70kDa appear potentiated in the presence of the PKCθ inhibitor. In contrast, treatment of platelets with increasing

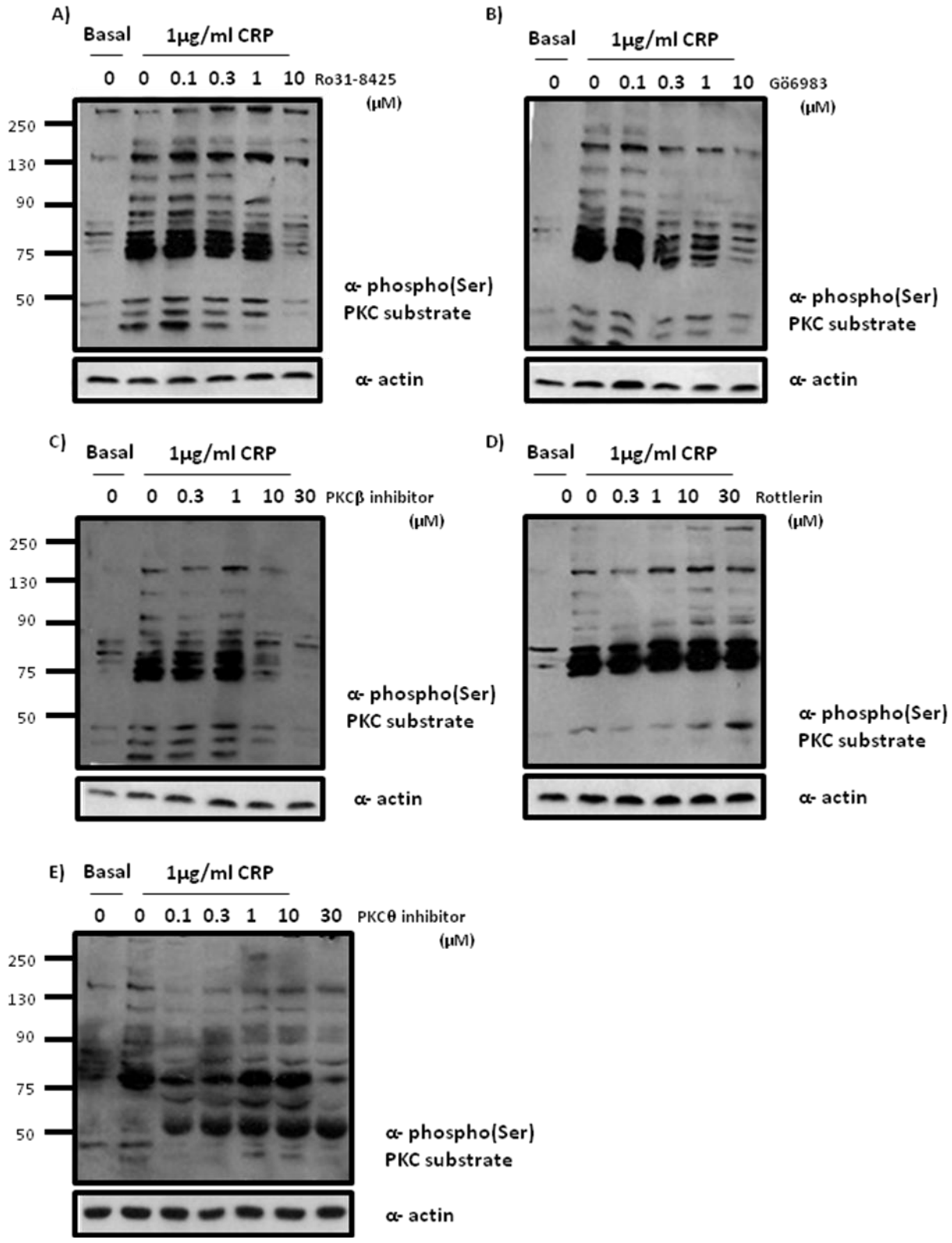

Figure 4.4 Effect of PKC Isoform-specific inhibitors on GPVI mediated PKC activation. Effect of the different PKC isoform inhibitors, the broad spectrum inhibitor (A) Ro31-8425, classical isoform inhibitors (B) Gö6983, and (C) PKCβ inhibitor and novel isoform inhibitors (D) Rottlerin and (E) PKCθ inhibitor on PKC substrate phosphorylation in human washed platelets. Whole cell lysates were resolved by SDS-PAGE and subject to

western blot analysis. Phosphorylation by PKC in washed platelet lysates was detected using α-phospho(Ser)-PKC substrate antibody, following activation by 1µg/ml CRP in the presence of increasing concentrations of the inhibitors, (ranging between 0.1, 0.3, 1 ,10 and 30µM). Actin was used as a loading control. Representative blots shown, n= 3.

concentrations of rottlerin appears to have no effect on PKC substrate phosphorylation suggesting very little role for PKCδ in the regulation of GPVI induced PKC activation.

4.2.3.2 The effect of the PKC inhibitors on GPVI and collagen-dependent platelet aggregation and dense granule secretion.

Following analysis of GPVI-induced PKC substrate phosphorylation, collagen- (GPVI and α2β1) and CRP (GPVI) –induced platelet aggregation and dense granule secretion were analysed using both intermediate (5μg/ml) and high (10μg/ml) concentrations of collagen (Figures 4.5 and 4.6, and 4.7 and 4.8 respectively) and 1μg/ml CRP (Figures 4.9 and 4.10) following treatment with increasing concentrations of the different inhibitors. Interestingly 50% inhibition of CRP induced platelet activation occurs at lower doses of all of the inhibitors used compared to inhibition of collagen induced responses most likely due to the extra positive reinforcement through α2β1 signalling that occurs downstream of collagen in addition to GPVI activation.

As with PMA induced platelet activation, both aggregation and dense granule secretion were inhibited in a concentration dependent manner by the broad spectrum inhibitor Ro31-8425 at both intermediate (5μg/ml) and high (10μg/ml) concentrations of collagen and CRP (1μg/ml) (figures 4.5-4.10). Complete or near complete inhibition of aggregation was achieved at 10μM of the inhibitor downstream of collagen whilst only 1μM was required to inhibit dense granule secretion and for total inhibition of both aggregation and secretion downstream of stimulation by 1μg/ml CRP. In contrast, sensitivity of both aggregation and dense granule secretion to low concentrations of inhibitor is not as great in the presence of Gö6983 where concentrations of 10μM elicits 80% but not total inhibition of aggregation and secretion downstream of both high and intermediate concentrations of collagen. As with Ro31-8425 inhibition by Gö6983 is

more sensitive, and total inhibition is achieved at lower concentrations of the inhibitor downstream of CRP in comparison to collagen. Similarly, 10μM of the PKCβ inhibitor was able to induce at most 50% inhibition of platelet activation downstream of collagen, yet this concentration was sufficient enough for near complete inhibition of CRP induced platelet aggregation and dense granule secretion. The observed inhibition supports the well characterised positive roles for the classical isoforms of PKC. The lack of total inhibition of collagen induced platelet activation in the presence of the inhibitors of the classical isoforms highlights that the novel isoforms of PKC may also act to positively regulate these processes.

In contrast to previously published results, using the same concentration range of inhibitor and agonist, inhibition rather than potentiation of both aggregation and dense granule secretion downstream of both GPVI agonists (5μg/ml collagen and 1μg/ml CRP) is seen in the presence of increasing concentrations of rottlerin and the PKCθ inhibitor. Following stimulation by high collagen (10μg/ml) only a minor inhibition of aggregation is seen until concentrations as high as 30μM. Dense granule secretion shows an increased sensitivity to both of the inhibitors, with approximately 50% inhibition observed at 10μM of either inhibitor. As high concentrations are required to achieve 50% inhibition, these results suggest a relatively minor positive role for both PKCδ and PKCθ in GPVI and collagen dependent platelet aggregation and dense granule secretion.

4.2.4. The effect of PKC inhibition on CLEC-2 induced platelet activation.

Studies have shown that the platelet CLEC-2 signals through a similar tyrosine kinase linked receptor signalling pathway to that used by GPVI, although several differences

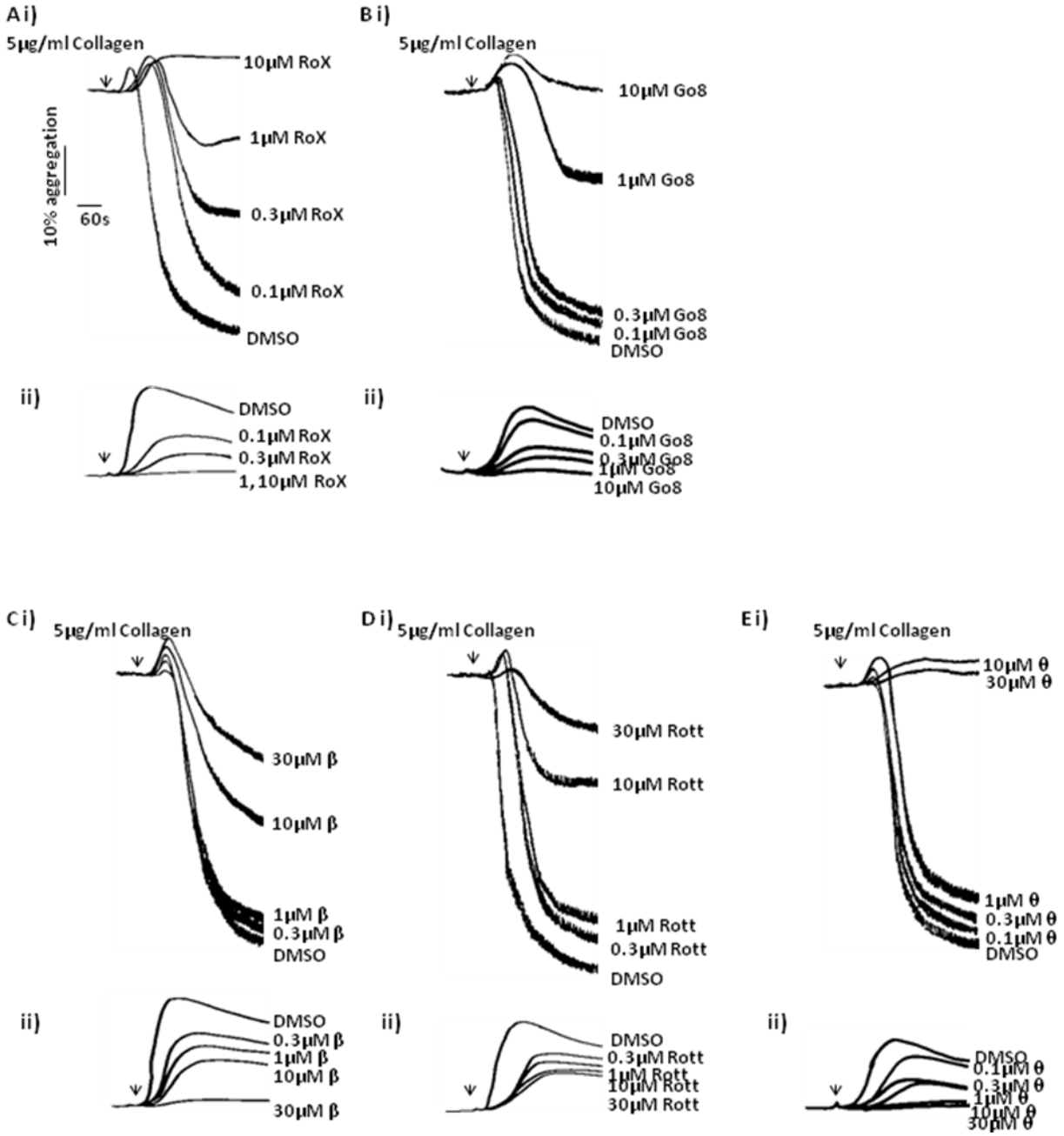

Figure 4.5. Inhibition of the classical and novel PKC isoforms differentially affects (low) collagen mediated platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, A) Ro31-8425 (RoX), B) Gö6983 (Gö8), C) PKCβ inhibitor (β) and D) Rottlerin (Rott) and E) PKCθ inhibitor (θ) and stimulated with collagen (5µg/ml). Representative traces of the effect of inhibitors on i) aggregation (optical light transmission) and ii) dense granule secretion (luciferin-luciferase light emittance) are shown.

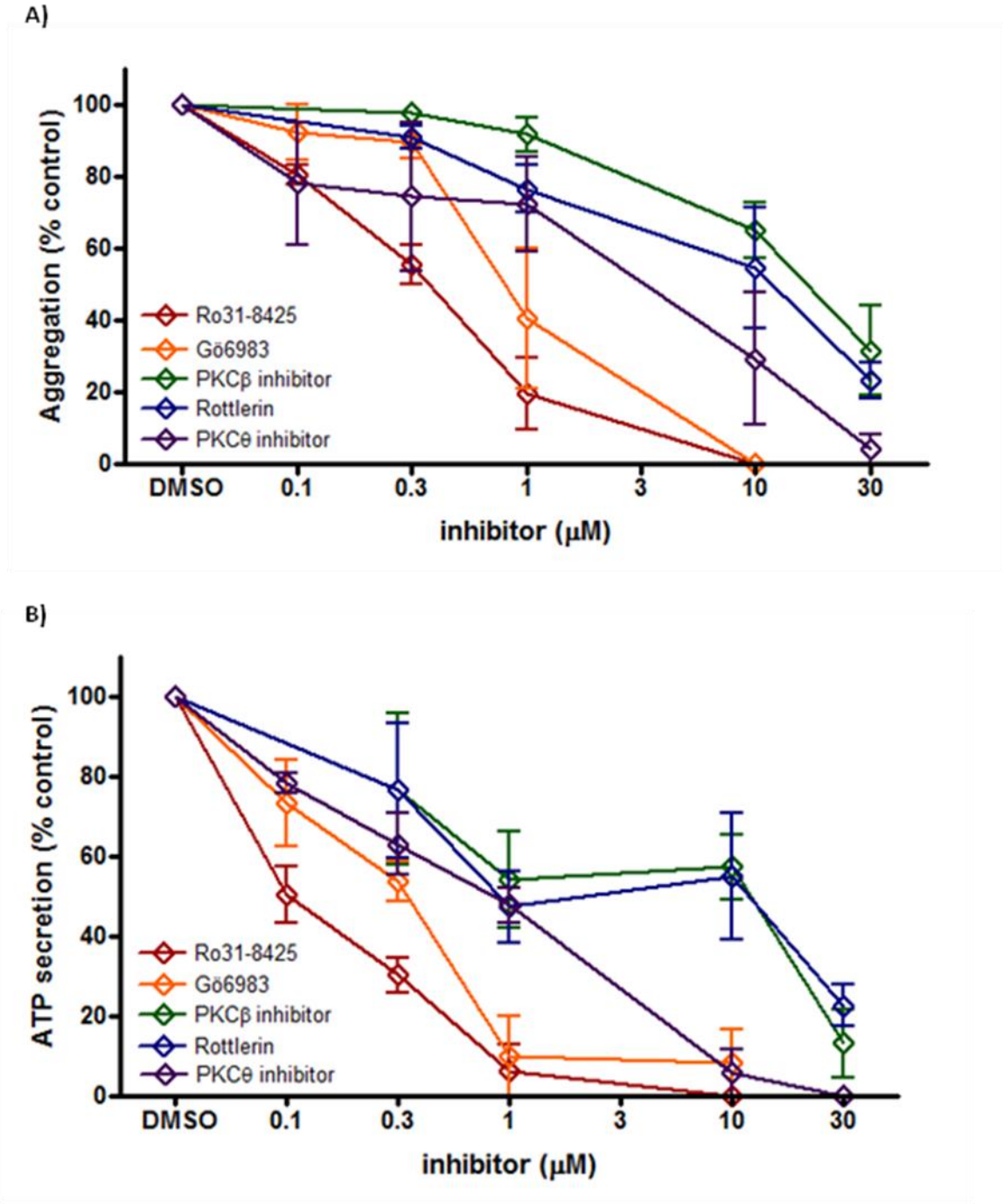

Figure 4.6 Inhibition of the classical and novel PKC isoforms differentially affects (low) collagen mediated platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, Ro31-8425, Gö6983, PKCβ inhibitor, Rottlerin and PKCθ inhibitor and stimulated with Collagen (5μg/ml). Dose response curves for each inhibitor are shown, A) aggregation and B) dense granule secretion. Effects are expressed as percentages of control condition, mean ± SEM. ($n \geq 3$)

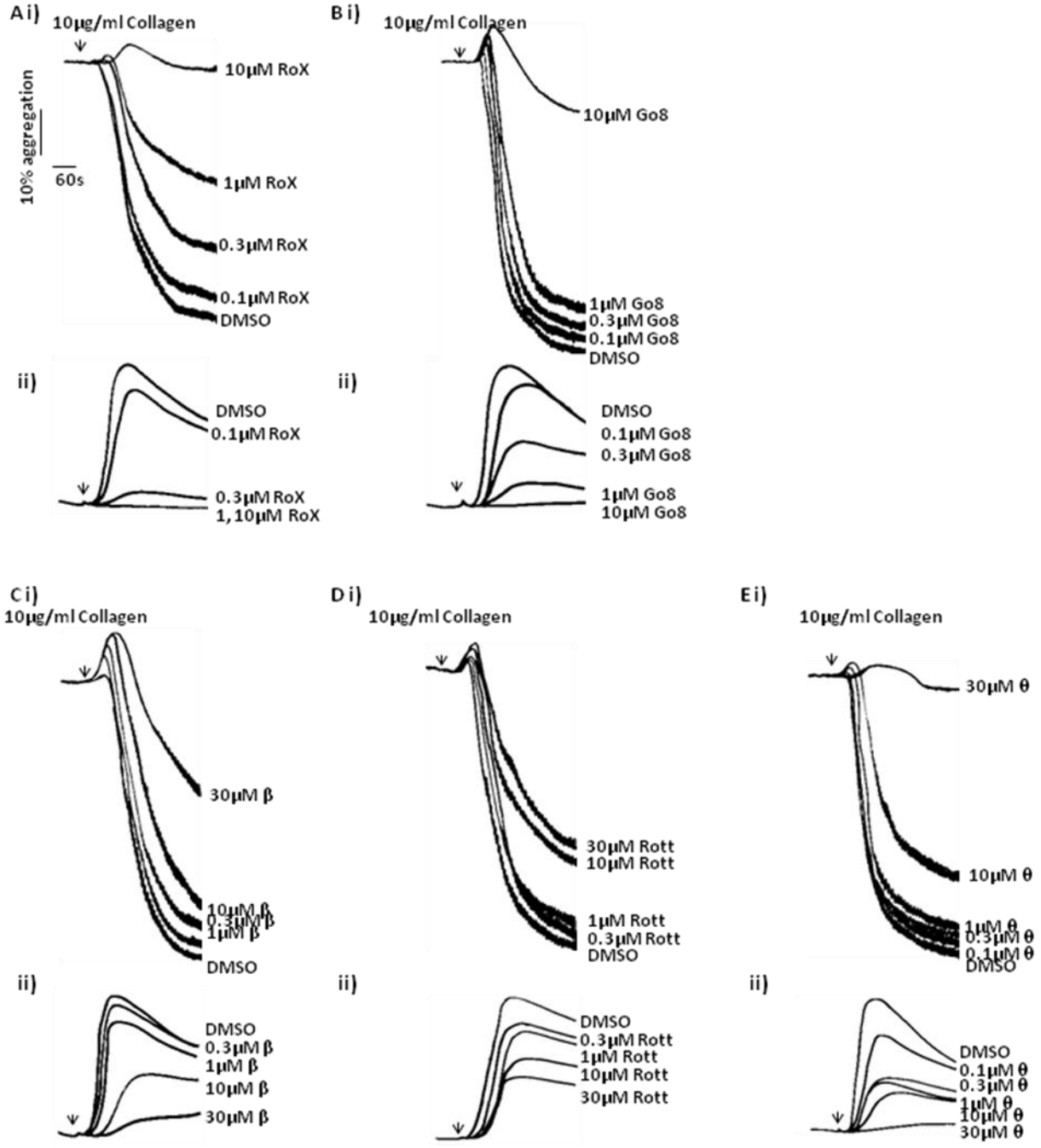

Figure 4.7. Inhibition of the classical and novel PKC isoforms differentially affects high collagen mediated platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, A) Ro31-8425 (RoX), B) Gö6983 (Gö8), C) PKCβ inhibitor (β) and D) Rottlerin (Rott) and E) PKCθ inhibitor (θ) and stimulated with collagen (10µg/ml). Representative traces of the effect of inhibitors on i) aggregation (optical light transmission) and ii) dense granule secretion (luciferin-luciferase light emittance) are shown.

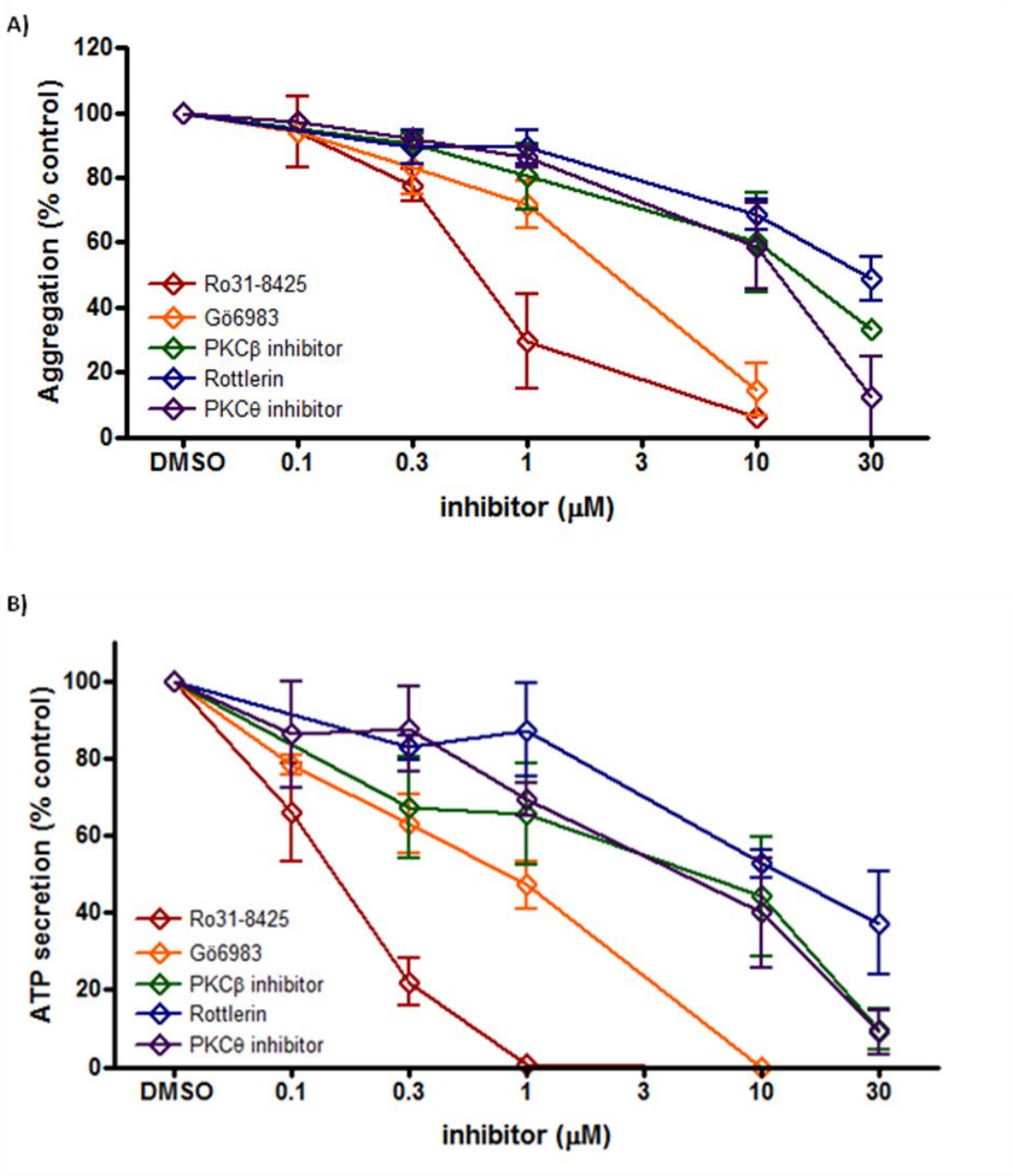

Figure 4.8 Inhibition of the classical and novel PKC isoforms differentially affects high collagen mediated platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, Ro31-8425, Gö6983, PKCβ inhibitor, Rottlerin and PKCθ inhibitor and stimulated with Collagen (10μg/ml). Dose response curves for each inhibitor are shown, A) aggregation and B) dense granule secretion. Effects are expressed as percentages of control condition, mean ± SEM. ($n \geq 3$)

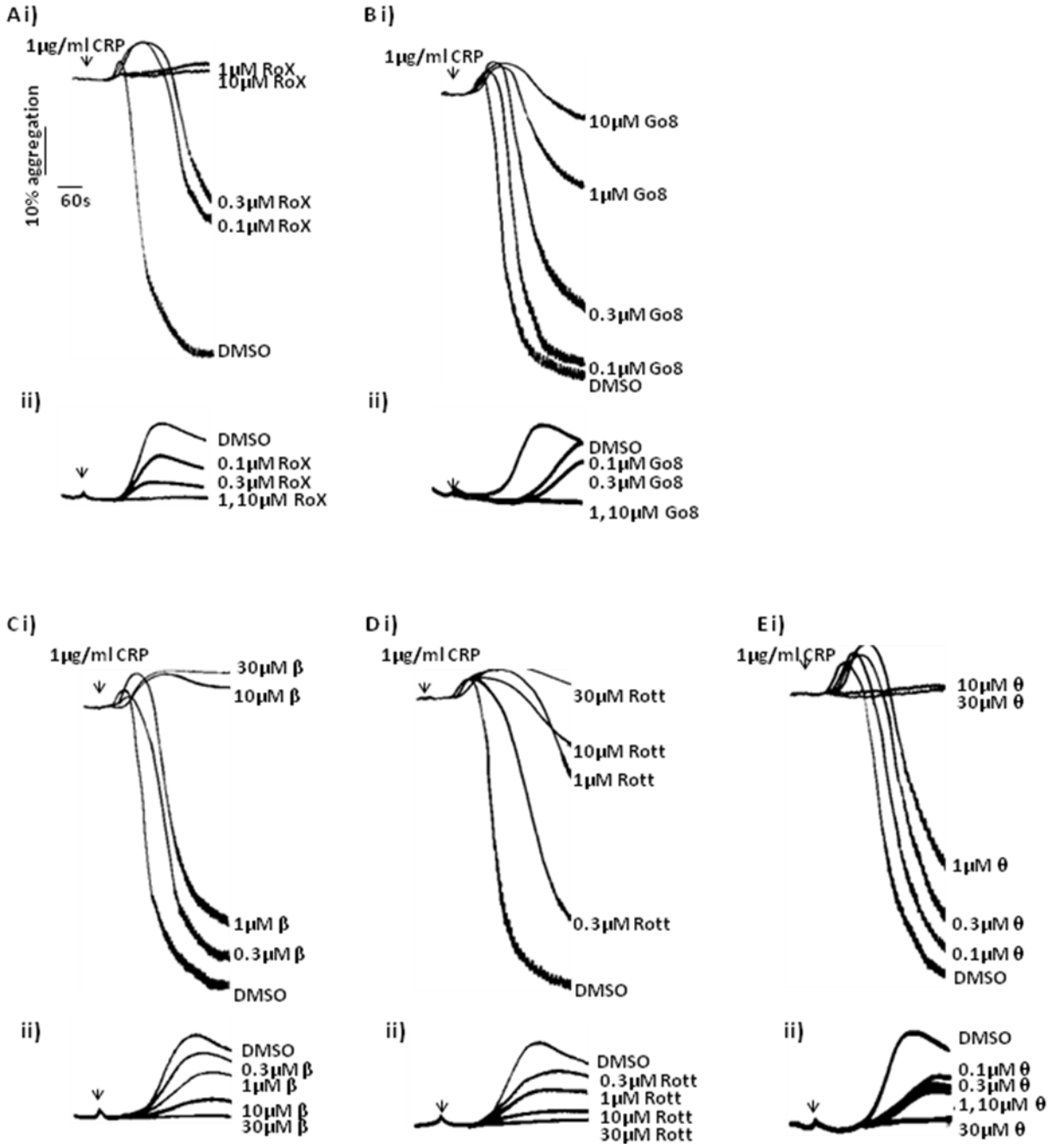

Figure 4.9. Inhibition of the classical and novel PKC isoforms differentially affects GPVI mediated platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, A) Ro31-8425 (RoX), B) Gö6983 (Gö8), C) PKCβ inhibitor (β) and D) Rottlerin (Rott) and E) PKCθ inhibitor (θ) and stimulated with CRP (1µg/ml). Representative traces of the effect of inhibitors on i) aggregation (optical light transmission) and ii) dense granule secretion (luciferin-luciferase light emittance) are shown.

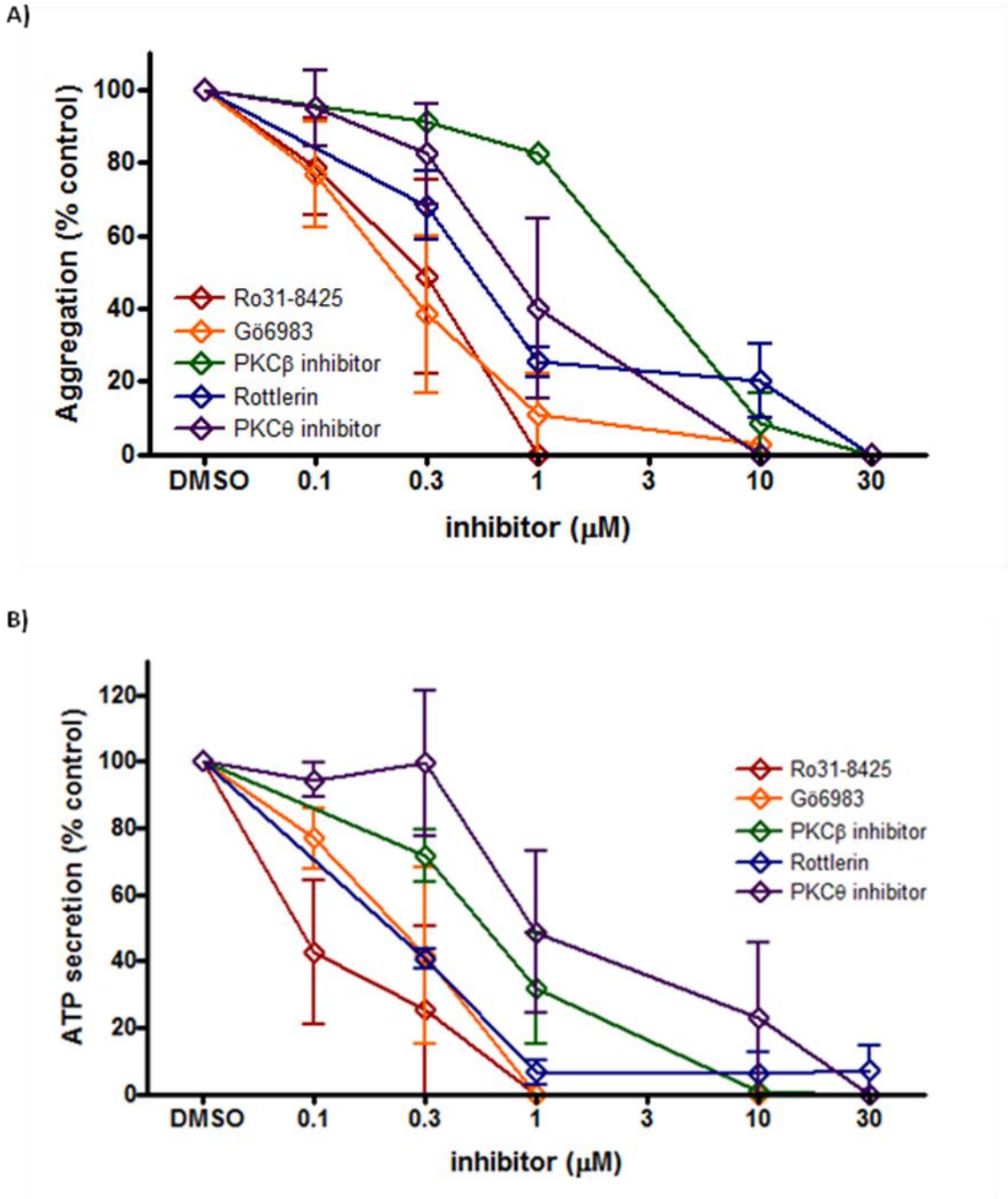

Figure 4.10 Inhibition of the classical and novel PKC isoforms differentially affects GPVI mediated platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, Ro31-8425, Gö6983, PKCβ inhibitor, Rottlerin and PKCθ inhibitor and stimulated with CRP (1μg/ml). Dose response curves for each inhibitor are shown, A) aggregation and B) dense granule secretion. Effects are expressed as percentages of control condition, mean ± SEM. ($n \geq 3$)

exist, highlighting two independent signalling pathways. Interestingly the role for PKC in the regulation of CLEC-2 signalling is relatively unknown. Like collagen and CRP, rhodocytin is a strong platelet agonist although unlike GPVI signalling, CLEC-2 signalling in human platelets has been shown to be very dependent on secretion.

4.2.4.1 The effect of the PKC inhibitors on CLEC-2 dependent platelet aggregation and dense granule secretion.

The effect of the different PKC inhibitors on CLEC-2 induced platelet aggregation and dense granule secretion was determined following activation by the snake toxin and CLEC-2 agonist rhodocytin (30nM) (figure 4.11 and 4.12). Similar to that observed downstream of GPVI signalling, a generally positive role for the PKC superfamily was identified in CLEC-2 induced platelet activation as both aggregation and dense granule secretion were, to some extent, inhibited in the presence of increasing concentrations of all of the PKC inhibitors used.

The broad spectrum inhibitor, Ro31-8425 elicited low dose inhibition of platelet aggregation and dense granule secretion, with approximately 50% inhibition occurring between 0.3-1μM inhibitor. Interestingly significant inhibition of rhodocytin-induced aggregation and dense granule secretion was also observed in the presence of low concentrations of the PKCθ inhibitor (0.1-0.3μM), concentrations that resulted in no alteration of GPVI-induced platelet activation. These observations indicate a major positive role for PKCθ in rhodocytin induced platelet aggregation and dense granule secretion in human platelets. As expected, treatment of platelets with Gö6983 showed a dose dependent inhibition of both aggregation and dense granule secretion but not total inhibition. The sensitivity to both the PKCβ inhibitor and the PKCδ inhibitor, rottlerin, was relatively low with high inhibitor concentrations required, the PKCβ inhibitor (30μM)

elicited 70-80% inhibition, whilst rottlerin (30μM) only inhibited aggregation and secretion by approximately 40%. These results suggest positive roles for PKC in CLEC-2 signalling with a major role for PKCθ a role for PKCα and relatively minor roles for PKCβ and PKCδ.

4.2.4.2 PKCθ inhibitor specificity and efficacy

Due to the striking pattern of inhibition observed in PKCθ inhibitor-treated platelets, the effect on this inhibitor on PKC activity in comparison to Ro31-8425 was determined by monitoring PKC substrate phosphorylation following platelet activation by rhodocytin (30nM) (Figure 4.13). Rhodocytin caused an increase in phosphorylation of a number of bands although the extent of phosphorylation was far weaker than that observed following stimulation by 1μg/ml CRP. As was expected, treatment of platelets with Ro31-8425 caused a significant reduction in PKC dependent phosphorylation of several substrates at low inhibitor concentrations (0.1μM) which were then severely reduced at high inhibitor concentrations (10μM). Following treatment with the PKCθ inhibitor, there appears to be relatively little effect on overall PKC substrate phosphorylation, a pattern which is similar to that observed following PMA activation. This suggests that the significant inhibition of rhodocytin induced aggregation and dense granule secretion in the presence of the PKCθ inhibitor is not a result of the PKCθ inhibitor non-specifically inhibiting total PKC activity or an upstream regulator of PKC activity consistent with a PKCθ Isoform-specific effect, although a PKC independent non-specific effect of the inhibitor cannot be ruled out. Interestingly, low dose inhibition of PKC substrates at 75 and 55kDa occurs at very low concentrations (0.1μM) of the PKCθ inhibitor, possibly

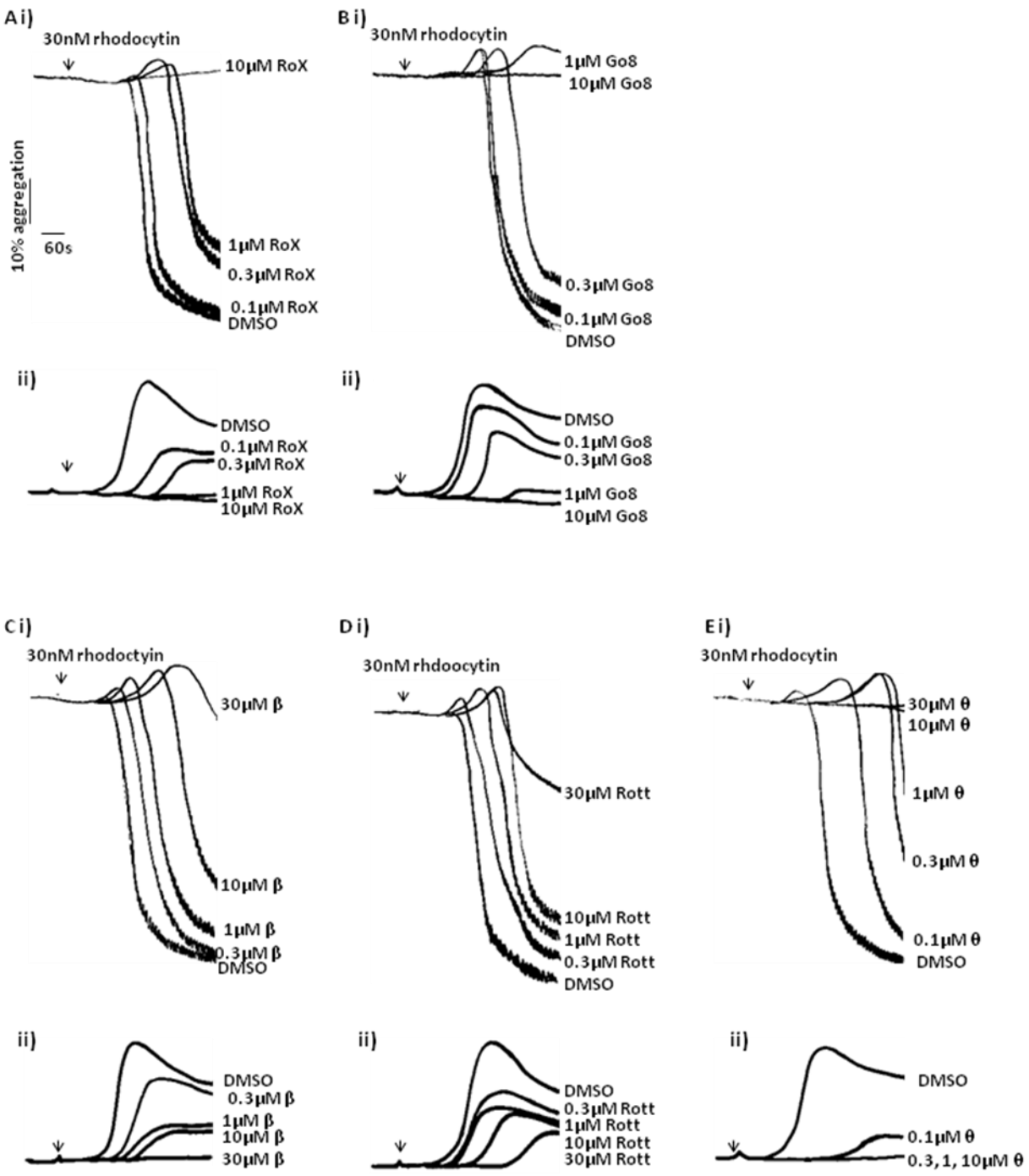

Figure 4.11. Inhibition of the PKC isoforms differentially affects CLEC-2 mediated platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, A) Ro31-8425 (RoX), B) Gö6983 (Gö8), C) PKCβ inhibitor (β) and D) Rottlerin (Rott) and E) PKCθ inhibitor (θ) and stimulated with rhodocytin (30nM). Representative traces of the effect of inhibitors on i) aggregation (optical light transmission) and ii) dense granule secretion (luciferin-luciferase light emittance) are shown ($n \geq 3$).

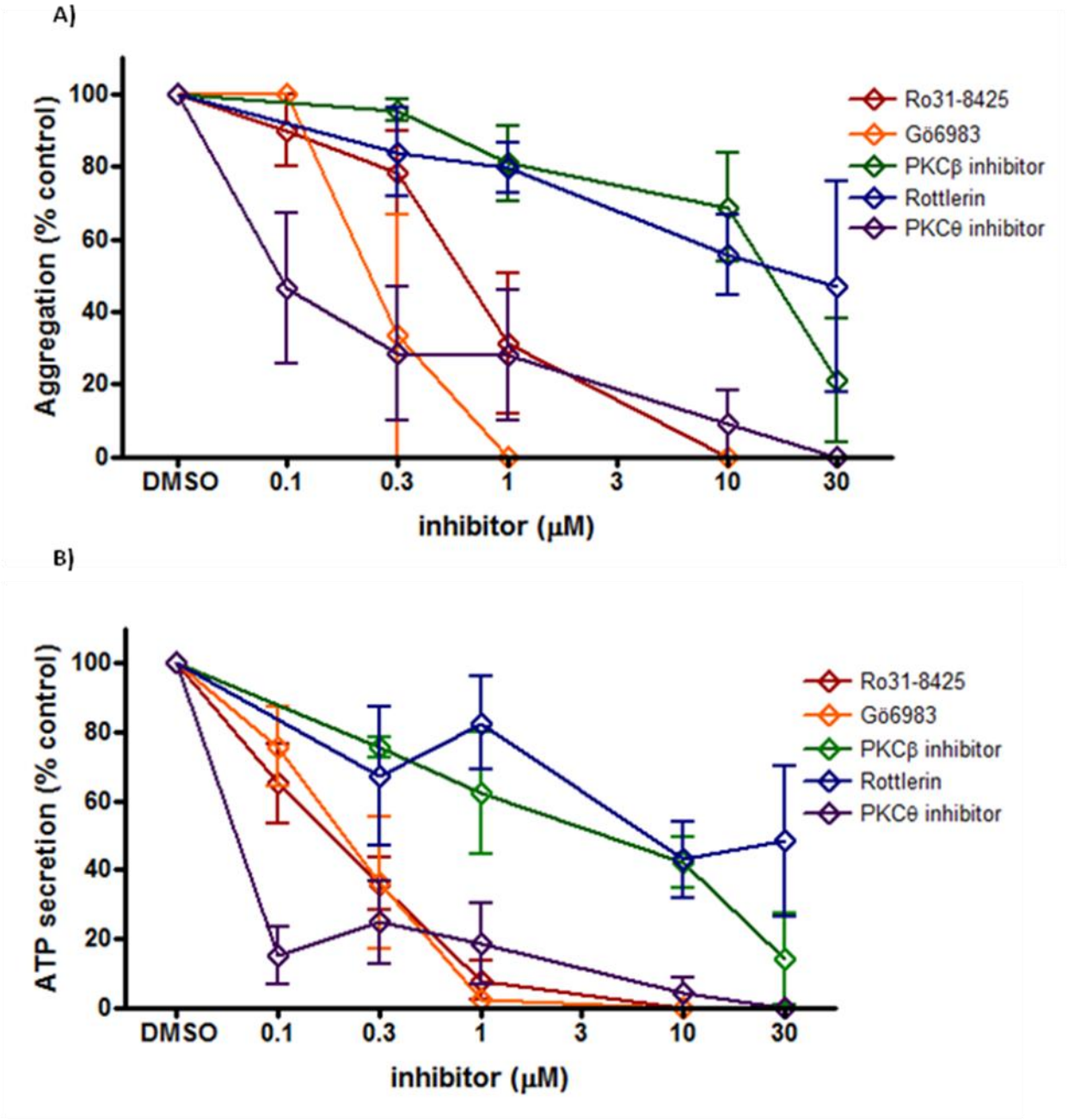

Figure 4.12 Inhibition of the classical and novel PKC isoforms differentially affects CLEC-2 mediated platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, Ro31-8425, Gö6983, PKCβ inhibitor, Rottlerin and PKCθ inhibitor and stimulated with Rhodocytin (30nM). Dose response curves for each inhibitor are shown, A) aggregation and B) dense granule secretion. Effects are expressed as percentages of control condition, mean ± SEM. ($n \geq 3$)

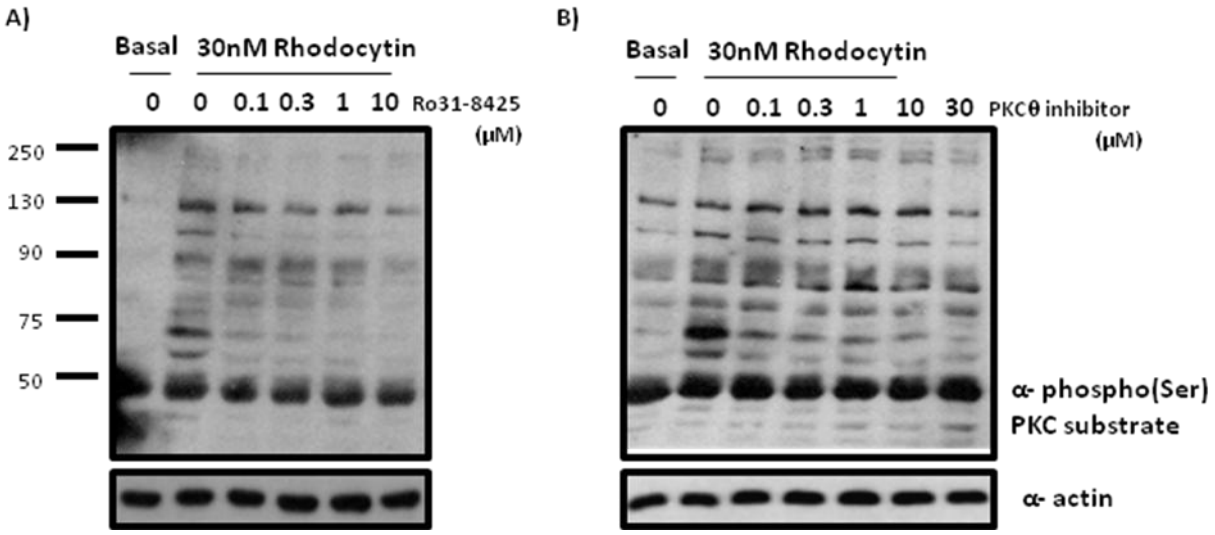

Figure 4.13 Effect of PKC Isoform-specific inhibitors on CLEC-2 mediated PKC activation. Effect of some of the different PKC isoform inhibitors, the broad spectrum inhibitor (A) Ro31-8425, and the novel isoform (B) PKCθ inhibitor on PKC substrate phosphorylation in human washed platelets. Whole cell lysates were resolved by SDS-PAGE and subject to western blot analysis. Phosphorylation by PKC in washed platelet lysates was detected using α-phospho(Ser)-PKC substrate antibody, following activation by Rhodocytin (30nM) in the presence of increasing concentrations of the inhibitors, (ranging between 0.1, 0.3, 1 ,10 and 30μM). Actin was used as a loading control. Representative blots shown, n= 3.

indicating these proteins could be direct targets of PKCθ that are involved in CLEC-2 induced platelet activation.

4.2.5. The effect of PKC inhibition on PAR induced platelet activation.

In contrast to GPVI and CLEC-2 receptors which signal through similar tyrosine kinase linked receptor signalling pathways, thrombin an endogenous and natural platelet ligand is another strong platelet agonist that activates platelets through a G protein coupled receptor signalling pathway via stimulation of the PAR1 and PAR4 receptors in human platelets.

4.2.5.1. The effect of the PKC inhibitors on PAR receptor induced PKC substrate phosphorylation

The effect of the different PKC inhibitors on PKC substrate phosphorylation was determined downstream of PAR receptor activation, following stimulation by intermediate and high concentrations of thrombin (0.05 and 0.1U/ml respectively) to ensure significant activation of PKC and to ensure activation was submaximal to enable inhibition or potentiation to be observed. Similar patterns of phosphorylation were observed downstream of both concentrations and so only the results for 0.1U/ml thrombin are included here (Figure 4.14). Thrombin induces strong phosphorylation of a number of proteins and PKC substrates in inhibitor untreated platelets compared to unstimulated controls. Unlike with GPVI and CLEC-2, although Ro31-8425 produced a dose dependent inhibition of this PKC substrate phosphorylation, significant inhibition of PKC activity was only seen at higher inhibitor concentrations (10μM) with majority of substrate phosphorylation abolished by 10μM A similar pattern of inhibition was achieved following treatment with increasing concentrations of Gö6983, although

platelet responses showed a reduced sensitivity to low inhibitor concentrations. High inhibitor concentrations of the PKCβ inhibitor (10 and 30μM) were also required to produce significant inhibition of PKC substrate phosphorylation and rottlerin appears to show no effect on the extent of thrombin (0.1U/ml) induced PKC substrate phosphorylation highlighting no role for PKCδ in PAR signalling. The PKCθ inhibitor also did not appear to have any effect on PKC substrate phosphorylation following stimulation by thrombin (0.1U/ml) until high inhibitor concentrations (30μM) were used. The use of high inhibitor concentrations perhaps points to a relatively minor role for PKC in the regulation of thrombin induced platelet activation.

4.2.5.2 The effect of the PKC inhibitors on PAR dependent platelet aggregation and dense granule secretion.

Following analysis of the effect of the inhibitors on thrombin induced PKC substrate phosphorylation, the effect of the inhibitors on thrombin (0.1U/ml) induced platelet aggregation and dense granule secretion was determined using human washed platelets (Figure 4.15 and 4.16). 0.1Uml thrombin induces sustained aggregation and dense granule secretion. All the inhibitors used displayed a dose dependent inhibition of both platelet aggregation and dense granule secretion highlighting positive roles for the PKC isoforms in PAR receptor induced platelet activation. As expected inhibition was the most sensitive following treatment with increasing concentrations of the broad spectrum inhibitor Ro31-8425. Significant inhibition of dense granule secretion was also observed with Gö6983 although interestingly inhibition of aggregation required higher concentrations of the inhibitor (orange line Figure 4.16), the PKCβ inhibitor also showed very little inhibition of platelet activation, it is possible that neither classical isoform

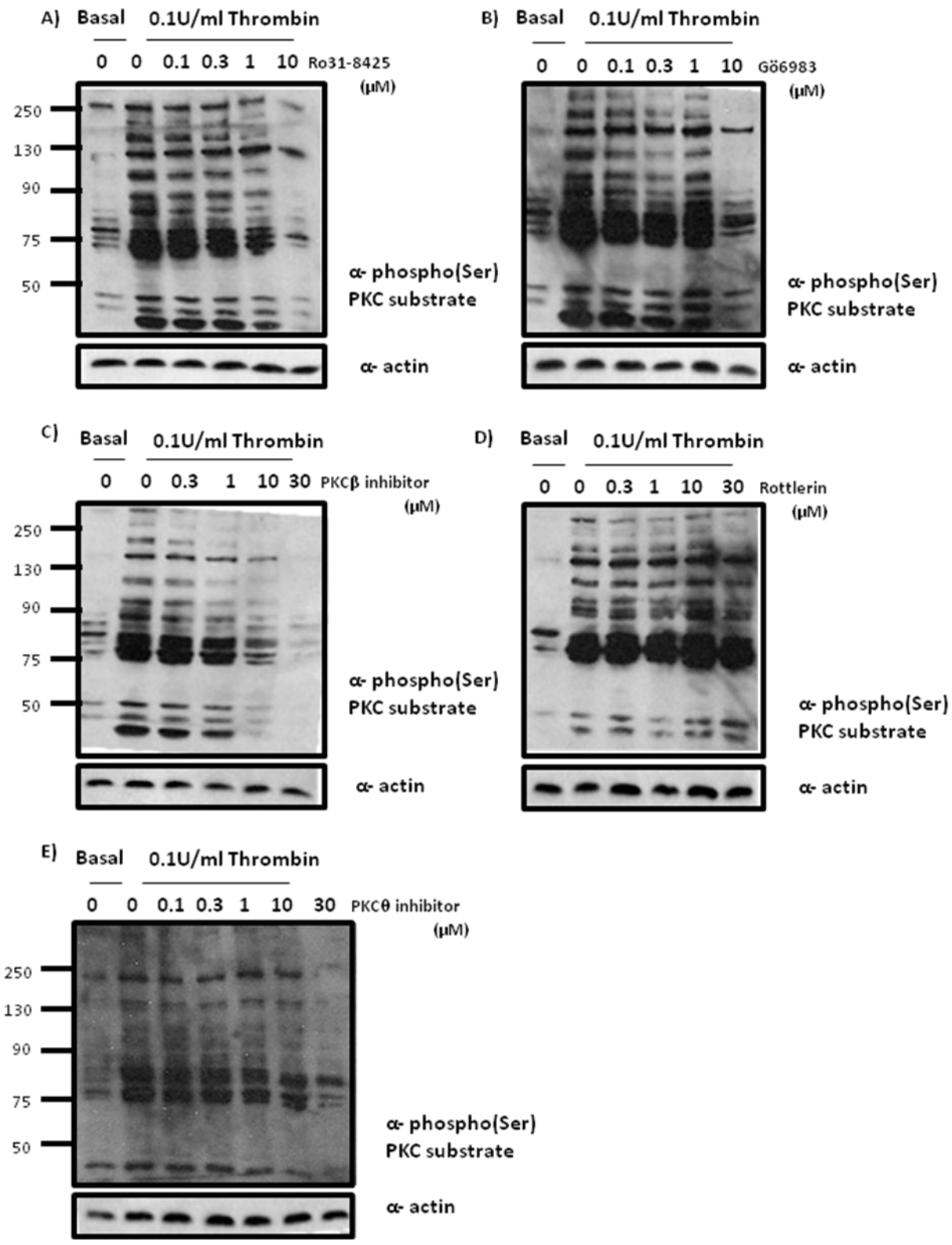

Figure 4.14 Effect of PKC Isoform-specific inhibitors on PAR mediated PKC activation. Effect of the different PKC isoform inhibitors, the broad spectrum inhibitor (A) Ro31-8425, classical isoform inhibitors (B) Gö6983 and (C) PKCβ inhibitor and novel isoform inhibitors (D) Rottlerin and (E) PKCθ inhibitor on PKC substrate phosphorylation in

human washed platelets. Whole cell lysates were resolved by SDS-PAGE and subject to western blot analysis. Phosphorylation by PKC in washed platelet lysates was detected using α-phospho(Ser)-PKC substrate antibody, following activation by Thrombin 0.1U/ml in the presence of increasing concentrations of the inhibitors, (ranging between 0.1, 0.3, 1 ,10 and 30μM). Actin was used as a loading control. Representative blots shown, n= 3.

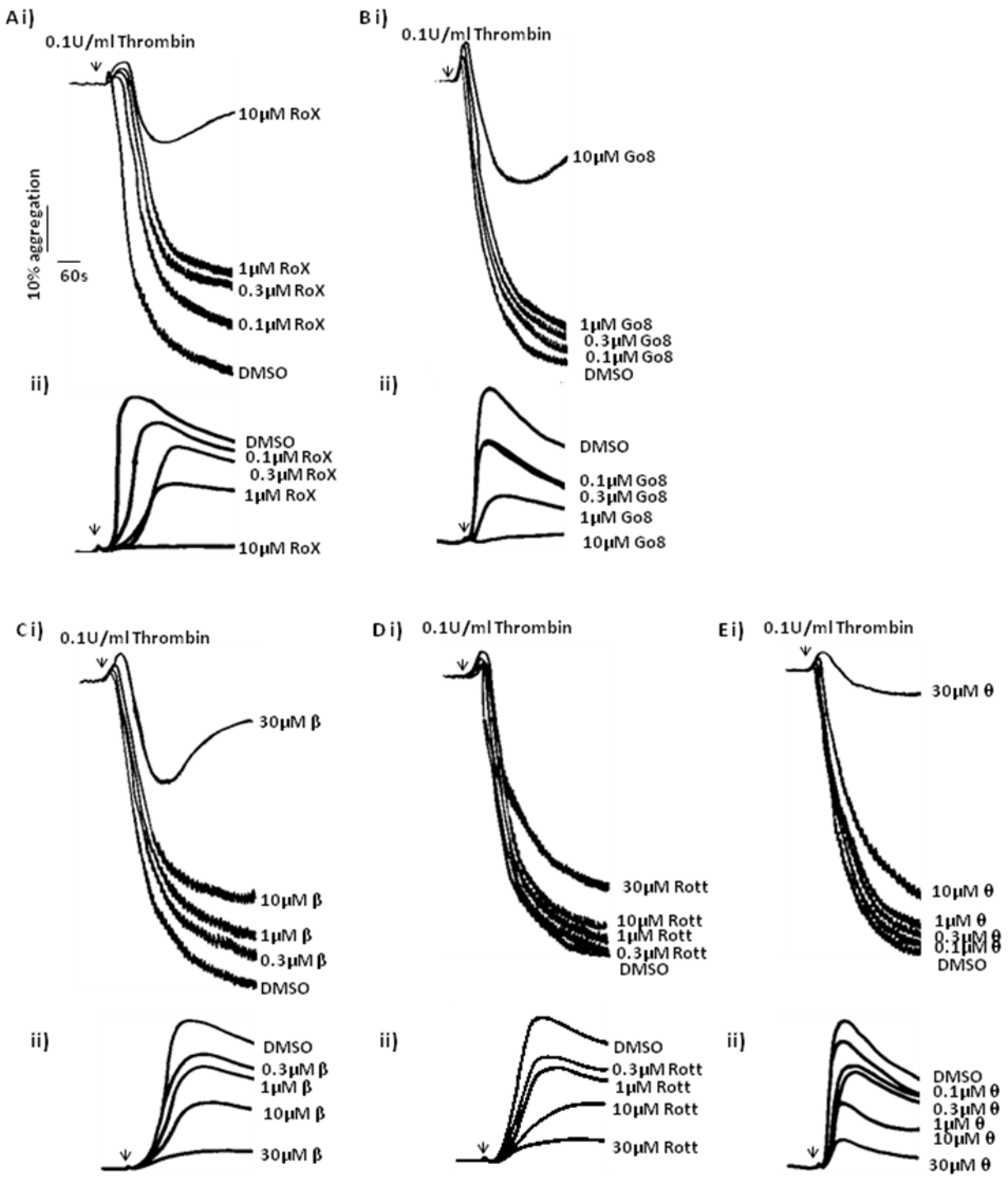

Figure 4.15. Inhibition of the classical and novel PKC isoforms differentially affects PAR mediated platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, A) Ro31-8425 (RoX), B) Gö6983 (Gö8), C) PKCβ inhibitor (β) and D) Rottlerin (Rott) and E) PKCθ inhibitor (θ) and stimulated with Thrombin (0.1U/ml). Representative traces of the effect of inhibitors on i) aggregation (optical light transmission) and ii) dense granule secretion (luciferin-luciferase light emittance) are shown n=3.

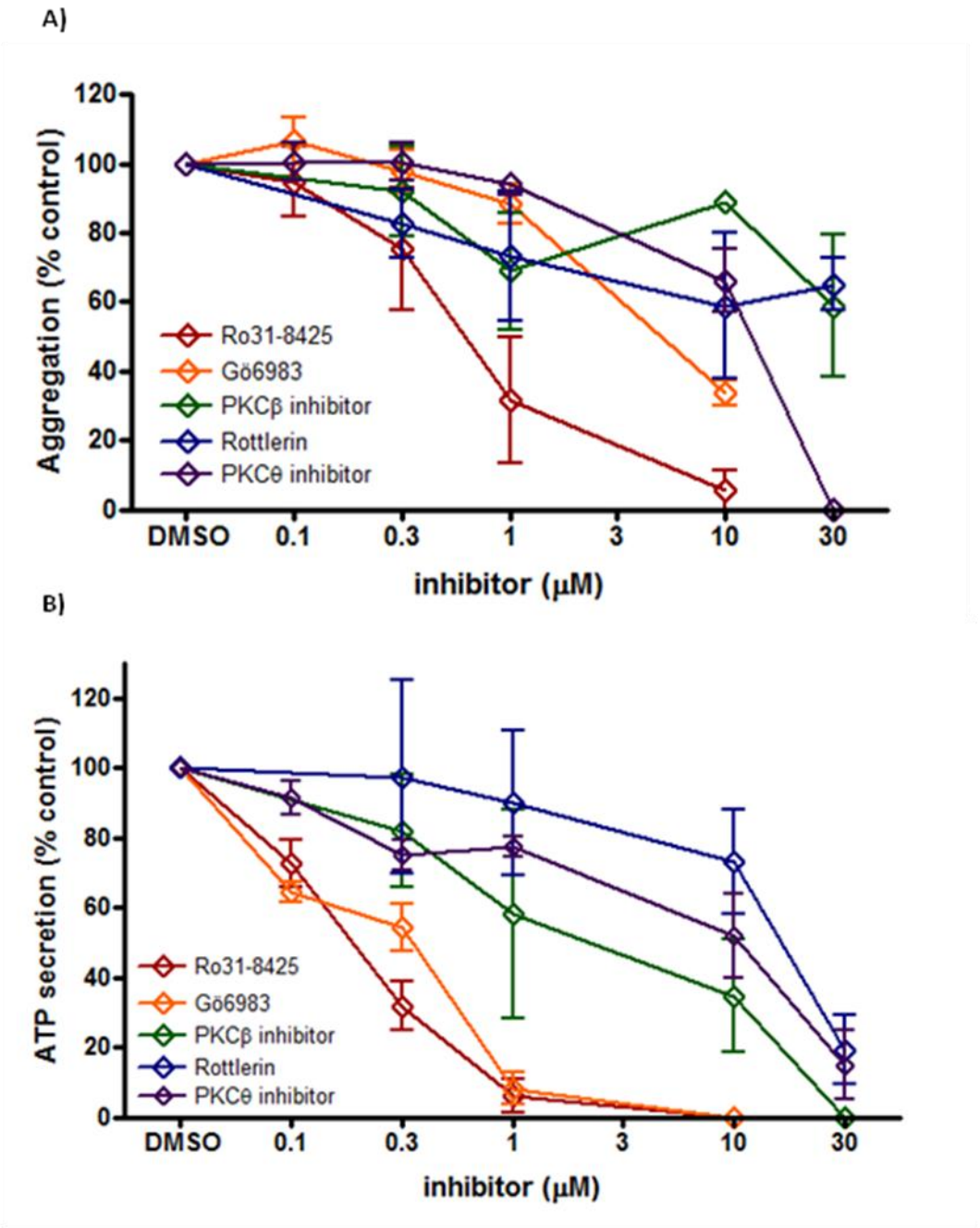

Figure 4.16 Inhibition of the classical and novel PKC isoforms differentially affects PAR mediated platelet activation. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, Ro31-8425, Gö6983, PKCβ inhibitor, Rottlerin and PKCθ inhibitor and stimulated with Thrombin (0.1U/ml). Dose response curves for each inhibitor are shown, A) aggregation and B) dense granule secretion. Effects are expressed as percentages of control condition, mean ± SEM. ($n \geq 3$)

plays a major role in the regulation of PAR induced aggregation, although it would appear in the absence of significant inhibition by PKCβ inhibitor, PKCα may play the key role in PAR receptor induced dense granule secretion. Interestingly no major role for either PKCδ or PKCθ was identified, as only slight inhibition of either aggregation or dense granule secretion is observed in the presence of intermediate concentrations of both rottlerin and the PKCθ inhibitor. Further supporting the possibility that the isoforms of PKC individually play relatively minor roles in the regulation of thrombin induced platelet activation, but combined play a significant overall positive role.

4.3 DISCUSSION

The family of PKC isoenzymes are well documented as key regulators of platelet activation with a general positive role for PKC identified downstream of several platelet agonists. Pharmacological studies using human platelets and genetic studies using 'knock-out' mouse models have identified positive and negative regulatory roles for the different PKC isoforms in several processes, required for platelet activation and thrombus formation, downstream of several platelet agonists indicating that this general positive role is too simplistic (Shattil and Brass 1987; King and Rittenhouse 1989; Ryu, Kim et al. 1990; Toullec, Pianetti et al. 1991; Walker and Watson 1993; Wilkinson, Parker et al. 1993; Yoshioka, Shirakawa et al. 2001; Quinton, Kim et al. 2002; Tabuchi, Yoshioka et al. 2003; Murugappan, Tuluc et al. 2004; Harper and Poole 2007; Strehl, Munnix et al. 2007; Hall, Harper et al. 2008; Pears, Thornber et al. 2008; Harper and Poole 2009; Konopatskaya, Gilio et al. 2009; Nagy, Bhavaraju et al. 2009; Gilio, Harper et al. 2010;

Harper, Molkentin et al. 2010; Harper and Poole 2010; Harper and Poole 2010). The classical isoforms PKCα and PKCβ have been suggested to be the major positive regulators of several processes involved in platelet activation whilst both positive and negative yet relatively minor roles for the novel isoforms PKCδ, PKCθ and PKCε have also been identified, highlighting distinct and specific roles for the individual isoforms of PKC in the complex regulation of platelet activation in mouse platelets (Heemskerk, Harper et al. 2011). However, although mouse models have become instrumental in the study of PKC isoforms, whether or not mouse models are representative models of humans is still debatable. Mouse platelets for example, express the novel isoform PKCε which has been shown to play a role in GPVI signalling and yet is not thought to be expressed in human platelets (Pears, Thornber et al. 2008). It is therefore feasible that the roles of the individual PKC isoforms may differ between species. As platelets are anucleate they cannot be genetically manipulated which limits the study of PKC in human platelets to the use of pharmacological inhibitors.

The work presented in this chapter set out to determine the roles for the individual isoforms of PKC downstream of the major platelet signalling receptors, GPVI, CLEC-2 and PAR receptors using pharmacological inhibitors. Both broad spectrum and Isoform-specific inhibitors, that are currently available were used, several of which had been used in a similar study by Gilio et al (2010) that focussed on GPVI signalling. These inhibitors included the pan-PKC inhibitors Ro31-8220 and Ro31-8425 (Wilkinson, Parker et al. 1993; Liu and Heckman 1998), Gö6983 which favours the classical isoforms (but can also inhibit PKCδ), a PKCβ inhibitor (Gschwendt, Dieterich et al. 1996; Tanaka, Sagawa et al. 2004), a PKCθ inhibitor, compound 20 donated by Boeringher Pharmaceuticals (Cywin, Dahmann et al. 2007) and Rottlerin, a PKCδ inhibitor (Gschwendt, Muller et al. 1994).

Full dose response concentration curves were investigated for several different agonists against phosphorylation and platelet functional responses, aggregation and dense granule secretion to enable direct comparison.

In general, all of the inhibitors used, show dose dependent inhibition of platelet aggregation and dense granule secretion downstream of the agonists for PKC (PMA), and the GPVI, CLEC-2 and PAR receptors. A summary of all the inhibitors downstream of the agonists used and the concentrations at which more than 50% inhibition of platelet activation was achieved is detailed in Table 4.2.

The Broad Spectrum PKC Inhibitors

The broad spectrum inhibitors elicit low concentration inhibition of platelet activation downstream of all of the agonists used, supporting previously published data for a key overall positive role for the PKC superfamily in the regulation of both GPVI and PAR receptor induced responses. The data presented here using the broad spectrum inhibitors also suggests a key positive role for the PKC superfamily in the regulation of CLEC-2 receptor mediated platelet activation identifying more similarities between the hemITAM CLEC-2 and ITAM GPVI tyrosine kinase linked signalling pathways and further supporting a central key role for PKC in the many platelet activation signalling pathways.

Gö6983

Similar observations that were made for Ro31-8425 were also made when treating platelets with Gö6983, suggesting that at the concentrations of Gö6983 used, PKCδ may also be being inhibited or that only the classical isoforms are involved. Interestingly higher concentrations of the inhibitor are required to inhibit platelet aggregation and dense granule secretion following stimulation by high collagen (10µg/ml) or thrombin

		Concentration of inhibitor that gives ~50% inhibition of platelet activation (μM)					
Agonist ↓	**Inhibitor →**	*Ro31-8220*	*Ro31-8245*	*Gö6983*	*PKCβ inhibitor*	*Rottlerin*	*PKCϑ inhibitor*
100nM PMA		0.1	0.1	0.1	0.3-1	10 *	0.3-1
5μg/ml Collagen		-	0.1-0.3	0.3-1	10-30	10-30	10-30
10μg/ml Collagen		-	0.3-1	1-10	10-30	10-30	10-30
1μg/ml CRP		-	0.1-0.3	0.3	1-10	0.3-1	1
30nM Rhodocytin		-	0.3-1	0.3	10-30	30+	0.1
0.1U/ml Thrombin		-	0.3	1	10-30	30+	30+

Table 4.2. Summary of the effect of the different PKC inhibitors on platelet activation downstream of several platelet agonists. Washed human platelets were pre-treated with increasing concentrations of the different PKC inhibitors, Ro31-8425, Gö6983, PKCβ inhibitor, Rottlerin and PKCθ inhibitor and stimulated with 100nM PMA, 5 or 10μg/ml Collagen, 1μg/ml CRP, 30nM rhodocytin and 0.1U/ml Thrombin. Approximate concentrations at which inhibition of platelet activation (as determined by monitoring aggregation and secretion) by 50% or more occurs for each PKC inhibitor following activation by each agonist tested are listed.

(0.1U/ml) suggesting that if Gö6983 is just targeting PKCα and PKCβ these agonist responses may not be as dependent on the classical isoforms in comparison to the other agonists tested.

PKCβ Inhibitor

In contrast, much higher inhibitor concentrations of the PKCβ inhibitor (10-30μM) are required for significant inhibition of platelet activation following stimulation by all the agonists used when compared to the concentrations required to inhibit PMA induced platelet activation (0.3-1μM). As CRP, intermediate collagen and rhodocytin induced responses are inhibited by low concentrations of Gö6983 but require high concentrations of PKCβ inhibitor for inhibition, this could imply that out of the classical PKC isoforms, PKCα plays the major role with support from PKCβ in the regulation of platelet activation downstream of tyrosine kinase linked signalling pathways, at agonist concentrations where aggregation is secretion dependent.

Rottlerin

Analysis of the novel isoform inhibitors and their effect on different agonist induced platelet activation identified a relatively minor role for PKCδ in the regulation of platelet signalling. Lower concentrations of rottlerin are required to elicit inhibition of CRP when compared to the other platelet agonists tested, suggesting PKCδ may play a role in the regulation of CRP (GPVI only) induced platelet activation, despite no apparent role downstream of collagen. This appears to suggest a relatively minor role for PKCδ, or redundancy with another isoform downstream of GPVI, CLEC-2 and PAR receptor signalling, but could also suggest that rottlerin is not a suitable PKCδ inhibitor. Analysis of agonist stimulated PKC dependent substrate phosphorylation in the presence of varying concentrations of rottlerin, identified very little effect on substrate

phosphorylation providing further support for current reservations as to the effectiveness of rottlerin as an inhibitor of PKC function (Davies, Reddy et al. 2000; Bain, Plater et al. 2007; Soltoff 2007). Interestingly, the negative roles for PKCδ identified in human platelet GPVI signalling using rottlerin by Gilio et al (2010), were not identified here. In contrast a minor inhibition of platelet activation downstream of both collagen and CRP was observed at high concentrations of rottlerin. These findings are in support of some data presented in the literature, but are also in conflict with others as a negative role for PKCδ in GPVI-mediated platelet aggregation and dense granule secretion has also been identified (Murugappan, Tuluc et al. 2004; Yacoub, Theoret et al. 2006; Chari, Getz et al. 2009; Gilio, Harper et al. 2010). These differences could reflect subtle differences in experimental design and conditions, and could highlight a relatively minor (and non-robust) role for PKCδ in the regulation of these processes, or could alternatively highlight limitations of inhibitor specificity and raises issues with the use of rottlerin in the study of PKCδ and the application of a pharmacological approach to study human platelet function.

PKCθ Inhibitor

Additionally, the negative role for PKCθ in human platelet GPVI signalling as found using a PKCθ inhibitor by Gilio et al (2010), was also not identified here. In contrast GPVI signalling, downstream of both collagen (supported by α2β1) and CRP was reduced in the presence of increasing concentrations of the PKCθ inhibitor. There is already controversy in the literature regarding the role of PKCθ in mouse platelet activation as both positive and negative roles for the isoform has been described, suggesting a non-robust minor phenotype that may be dependent on experimental conditions (Soriani, Moran et al. 2006; Hall, Harper et al. 2008; Cohen, Braiman et al. 2009; Harper and Poole

2009; Nagy, Bhavaraju et al. 2009; Harper and Poole 2010). These findings support that the different roles for PKCθ that have been identified are likely due to differences in experimental protocols and platelet preparations as a robust role for the isoform would be expected to be observed regardless of experimental conditions, suggesting PKCθ plays a relatively minor role in the regulation of platelet activation.

Despite displaying relatively minor inhibitory effects on GPVI and PAR agonist induced platelet aggregation and dense granule secretion, low concentrations of the PKCθ inhibitor elicited significant inhibition of both aggregation and secretion to rhodocytin highlighting a possible key role for PKCθ in the positive regulation of CLEC-2 signalling. In support of a role for PKCθ, analysis of PKC dependent substrate phosphorylation following stimulation by rhodocytin showed very little inhibition of overall PKC substrate phosphorylation, with the exception of a substrate at approximately 75kDa, indicating an Isoform-specific effect for PKCθ in CLEC-2 signalling. CLEC-2 signalling and subsequent platelet activation requires a major positive feedback effect from secretion, which most likely necessitates a role for all of the PKC isoforms in the regulation of this pathway. It is possible that if PKCθ functions to regulate dense granule secretion, inhibition of this would therefore significantly alter and reduce platelet aggregation.

Caution has to be taken when using pharmacological inhibitors, as non-specific inhibitor effects cannot necessarily be ruled out. For example in the majority of cases shown here, the Isoform-specific inhibitors, like with the broad spectrum inhibitors, show an inhibition of platelet activation but usually only at concentrations where there is a significant overall inhibition of PKC substrate phosphorylation. This could be an indication that the inhibition of platelet activation is not Isoform-specific, although a

major role for one particular isoform cannot be ruled out and therefore conclusions regarding specific isoform function should focus on lower inhibitor concentrations so as to ensure specificity. Otherwise roles for the particular isoforms cannot be determined easily using pharmacological inhibitors as their specificity cannot be assured. For a more detailed analysis of the role of the different isoforms of PKC in human platelet activation, more agonists, different inhibitors and wider ranges of inhibitor concentrations should be used in an attempt to ensure any conclusions drawn from inhibitor studies are as accurate as possible and are not based on non-specific inhibitor effects. Therefore although there are several concerns over the use of mouse models, these may currently offer the best model for the understanding of PKC isoform function.

Downstream of the majority of platelet agonists significant inhibition of platelet aggregation and dense granule secretion occurs following broad spectrum PKC inhibition (usually to low inhibitor concentrations) supporting previously published data for an overall positive role for the PKC superfamily in the processes involved in platelet activation. The data presented here also supports previously published data, indicating that the major regulators of granule secretion and aggregation are the classical isoforms, PKCα and PKCβ with PKCα playing the major role, as inhibition of both of the isoforms is significantly greater than the inhibition observed in just the presence of the PKCβ inhibitor. In comparison the novel isoforms appear to play relatively minor regulatory roles in the regulation of platelet activation although the PKCθ inhibitor had a major inhibitory effect on CLEC-2 induced responses, implying a possible major positive role for PKCθ in the regulation of CLEC-2 signalling.

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CHAPTER 5.

THE ROLE OF PROTEIN KINASE C IN ADP-INDUCED PLATELET ACTIVATION

5.1 INTRODUCTION

ADP, a weak platelet agonist, is released from platelet dense granules in response to all stimulatory platelet agonists and acts via a positive feedback mechanism to enhance and sustain platelet activation (Mills 1996; Jin, Quinton et al. 2002). ADP signals through $P2Y_1$ and $P2Y_{12}$ G protein-coupled receptors, which generate DAG and IP_3, and inhibit adenylyl cyclase and activate PI 3'-kinase (PI3K), respectively (Gachet, Hechler et al. 1997; Murugappa and Kunapuli 2006). These two second messenger pathways undergo marked synergy to mediate sustained aggregation, although on their own neither pathway is able to support full platelet aggregation. The $P2Y_1$ receptor undergoes marked desensitisation during preparation of washed platelets resulting in diminished or loss of response to ADP unless special procedures are used to prevent ADP accumulation. For this reason, ADP responses are commonly monitored in platelet rich plasma (PRP).

5.1.1 AIMS

The role for PKC and individual PKC isoforms in the regulation of ADP-induced platelet activation has not been extensively investigated in part because of the need for special conditions to retain ADP responsiveness in washed platelets and because many of the PKC inhibitors available undergo extensive protein binding in plasma. In this chapter, the

role of PKC in ADP-induced platelet activation was investigated using the pan-PKC inhibitor, Ro31-8220, isoform-specific PKC inhibitors and both human and mouse platelets utilising transgenic mice deficient in PKC isoforms.

5.2 RESULTS

5.2.1 Ro31-8220 shows reduced bioavailability in PRP compared to Washed Platelets

Many pharmacological reagents have reduced bioavailability in plasma as a result of plasma binding. The broad spectrum inhibitor Ro31-8220 is commonly used at a concentration of 10μM for full inhibition of PKC in washed platelets. However, the concentration required to completely inhibit PKC in PRP is not known. To address this, the effect of different concentrations of Ro31-8220 on aggregation and dense granule secretion in PRP and washed platelets was investigated downstream of the PAR-1 peptide TRAP (SFLLRN), as secretion is known to dependent on PKC (Figure 5.1). Ro31-8220 caused a dose-dependent inhibition of aggregation and dense granule secretion with increasing concentrations of the inhibitor (Figure 5.1Ai and ii). At 1μM in washed platelets, Ro31-8220 was able to achieve significant inhibition of aggregation and full inhibition of dense granule secretion to TRAP whereas a 100-fold higher concentration (100μM) was required to achieve complete inhibition of dense granule secretion in PRP, and this was still not sufficient to achieve the same level of inhibition of aggregation. These data therefore indicate that Ro31-8220 shows markedly reduced bioavailability in plasma which effectively lowers its concentration by more than two orders of magnitude. Ro31-8220 was therefore used in plasma at 100μM to achieve near maximal

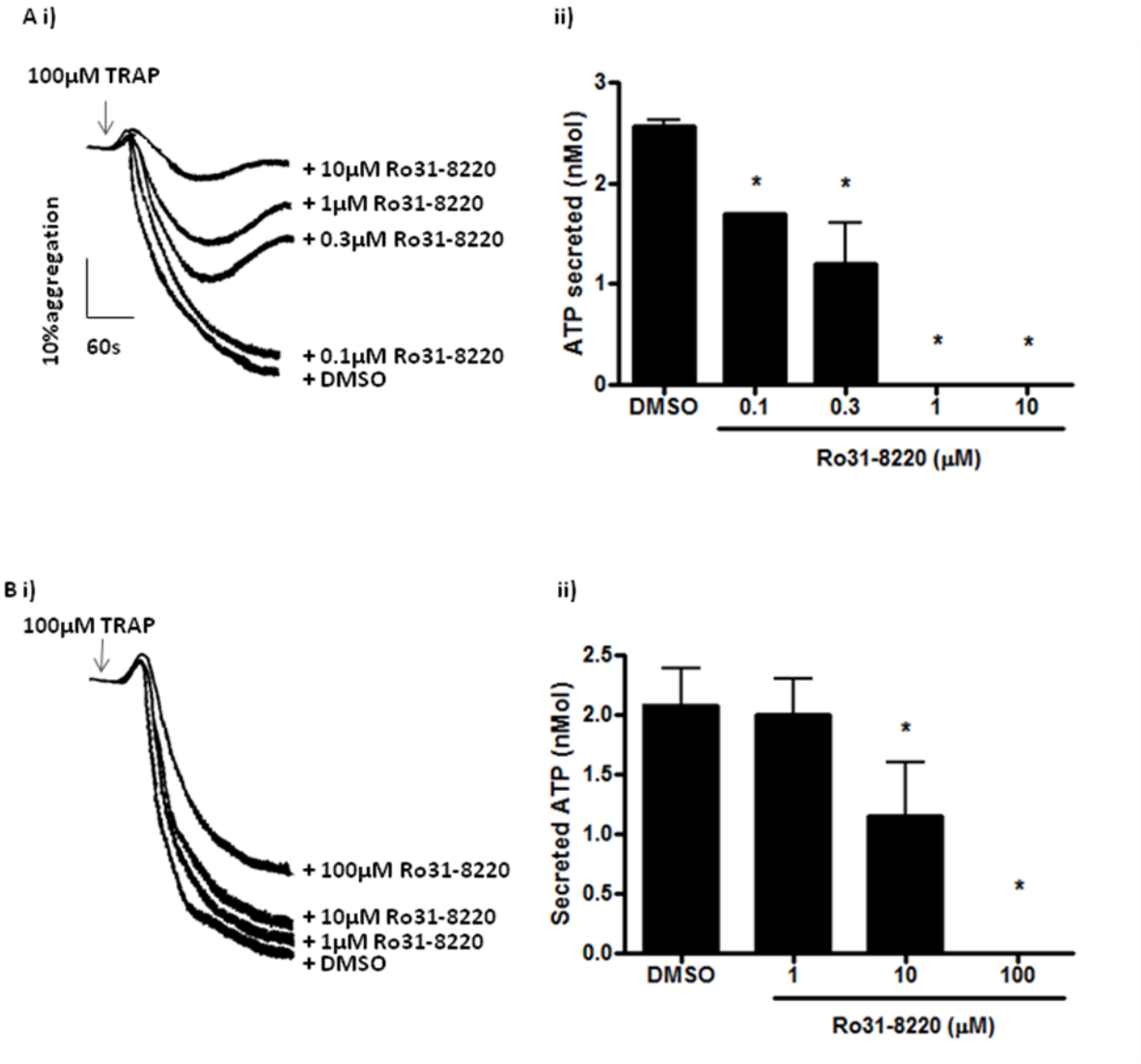


Figure 5.1. Effect of Ro31-8220 on aggregation and dense granule secretion of human washed platelets and citrated PRP in response to stimulation by PAR-1 peptide. Human washed platelets (A) and citrated PRP (B) were stimulated with 100µM TRAP (a PAR-1 peptide that stimulates the thrombin receptor and is known to be active in both washed platelets and PRP) following incubation with increasing concentrations of Ro31-8220. (i) Aggregation was measured by optical aggregometry. (ii) Dense granule secretion was measured by monitoring ATP secretion using luminometry. n=3 .* indicates $p<0.05$ in comparison to DMSO treated controls.

inhibition of PKC and 10μM was used for submaximal inhibition.

5.2.2 Submaximal inhibition of PKC potentiates ADP-induced dense granule secretion and aggregation in citrated PRP.

Citrated PRP, which buffers extracellular Ca^{2+}, is a standard method of PRP preparation and is frequently used for platelet testing in the clinic. Under these conditions a high concentration of ADP (100μM) is capable of producing sustained aggregation and dense granule secretion whilst at lower concentrations; ADP (3μM) stimulates transient aggregation but not secretion (Figure 5.2). Ro31-8220 caused a minor reduction in aggregation to 100μM (high) ADP at submaximal (10μM) and maximal (100μM) concentrations (Figure 5.2Ai). In contrast, at 100μM Ro31-8220 caused complete inhibition of dense granule secretion to 100μM ADP. Unexpectedly, however, at a tenfold lower concentration of Ro31-8220 (10μM) the rate of onset of secretion was dramatically increased although the overall level of secretion was reduced (Figure 5.2Aii). Moreover, this submaximal concentration of Ro31-8220 (10μM) also converted the transient aggregation response to a low concentration of ADP (3μM) to sustained aggregation and potentiated dense granule secretion (Figure 5.2Aiii and iv). This potentiation is not observed downstream of a submaximal concentration of the TRAP peptide (data not shown). These observations demonstrate that submaximal inhibition and hence partial blockade of PKC potentiates the rate of onset and magnitude of secretion to ADP, which at low concentrations (3μM ADP) leads to sustained aggregation. At a higher concentration of ADP the increase in the rate of onset of secretion associated with partial blockade of PKC is followed by a diminished overall response, presumably due to a balance between the inhibitory and stimulatory actions of PKC, as maximal PKC inhibition abolishes secretion.

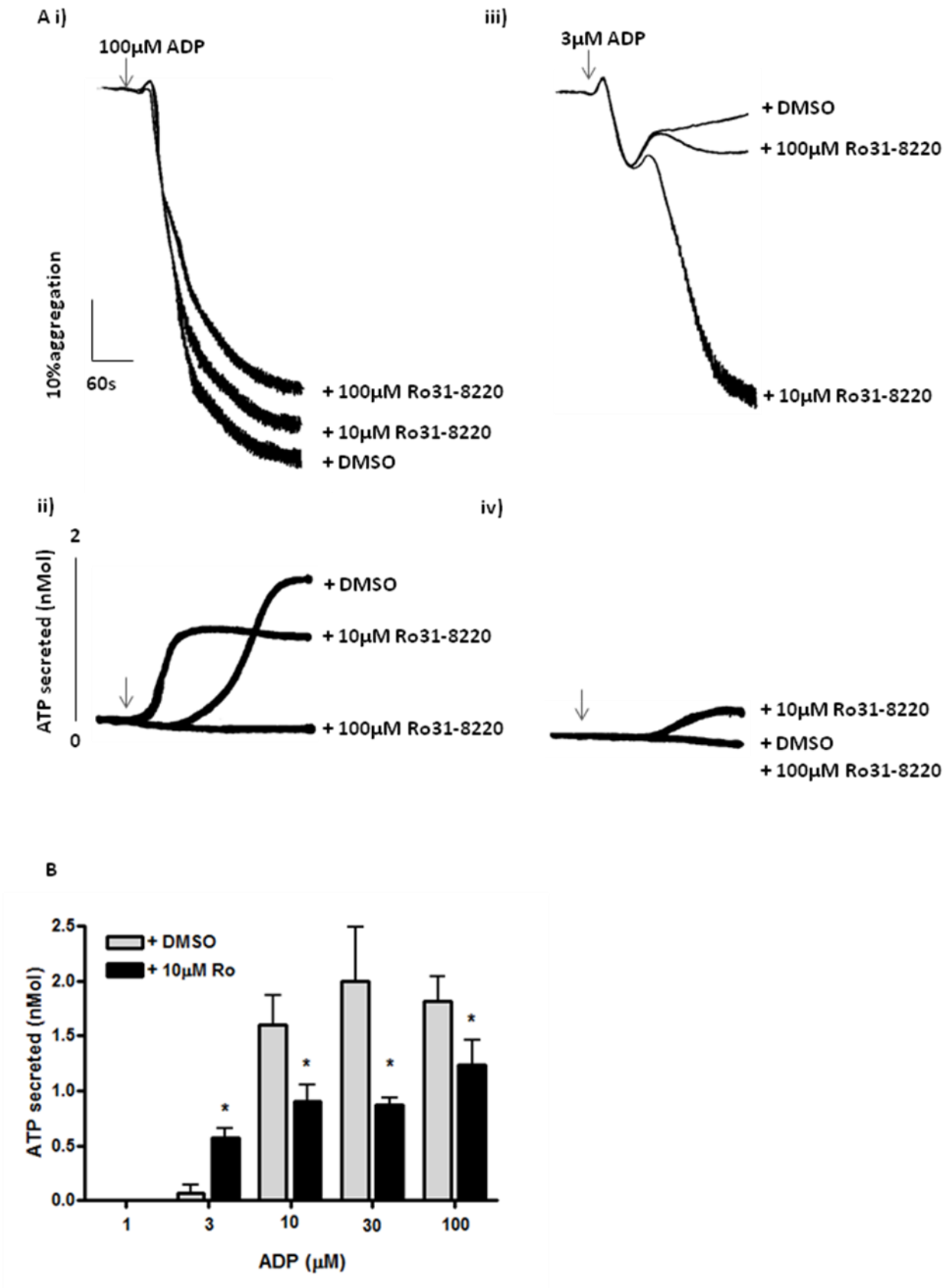


Figure 5.2. Effect of Ro31-8220 on aggregation and dense granule secretion of human citrated PRP in response to stimulation by ADP. Human citrated PRP was stimulated with either (A) maximal (100µM) ADP or (B) submaximal (3µM) ADP, aggregation and

dense granule secretion were monitored as described in legend to figure 1. A i) and iii) Aggregation monitored by optical aggregometry. Traces representative of n=3. ii) and iv) Representative traces of n=3 showing dense granule secretion, monitored by ATP release, to 3µM and 100µM ADP respectively in the absence or presence of submaximal and maximal Ro31-8220 (10 and 100µM). B) Dense granule secretion by PRP, monitored by ATP release to a range of ADP concentrations in the absence or presence of Ro31-8220 (10µM). Platelets were incubated with DMSO or Ro31-8220 (10µM) for 3 minutes prior to ADP stimulation. $n \geq 3$.* indicates $p<0.05$ in comparison to DMSO treated controls.

5.2.3 The role of thromboxane A_2 formation.

The ability of ADP to induce sustained aggregation and secretion in citrated plasma has been attributed to an increase in TxA_2 formation (Cattaneo, Gachet et al. 2002). In confirmation of this, significant inhibition of dense granule secretion following platelet activation by 100µM ADP is observed in the presence of the cyclooxygenase inhibitor indomethacin in citrated plasma (Figure 5.3A). To determine whether TxA_2 formation is necessary for the observed Ro31-220 induced potentiation, citrated PRP was treated with indomethacin in the presence of submaximal Ro31-8220 (10µM). This caused a partial restoration of aggregation and secretion. These data confirm a critical role for TxA_2 formation in mediating ADP-induced secretion, but also indicate that potentiation is not dependent on TxA_2 formation.

5.2.4 Outside-in signalling by integrin αIIbβ3 is essential for ADP-mediated platelet secretion.

In addition to TxA_2 production, outside-in signalling via the integrin αIIbβ3 also plays a critical role in mediating secretion by ADP (Jin, Quinton et al. 2002). Indirect evidence for this is the observation that the onset of ADP-induced secretion is concurrent with the second phase of aggregation (as shown in the control sample in Figure 5.2). In further support of this, Integrilin, an $\alpha IIb\beta_3$ inhibitor which completely inhibits aggregation to ADP (100µM), also reduced ATP secretion by approximately 90% (Figure 5.3B). Treatment of platelets with submaximal concentrations of Ro31-8220 (10µM) in the presence of Integrilin (9µM) did not recover dense granule secretion demonstrating an essential role for outside-in signalling through the integrin in ADP-induced secretion. Signalling via αIIbβ3 could contribute to the mechanism underlying the potentiation of dense granule secretion by submaximal inhibition of PKC.

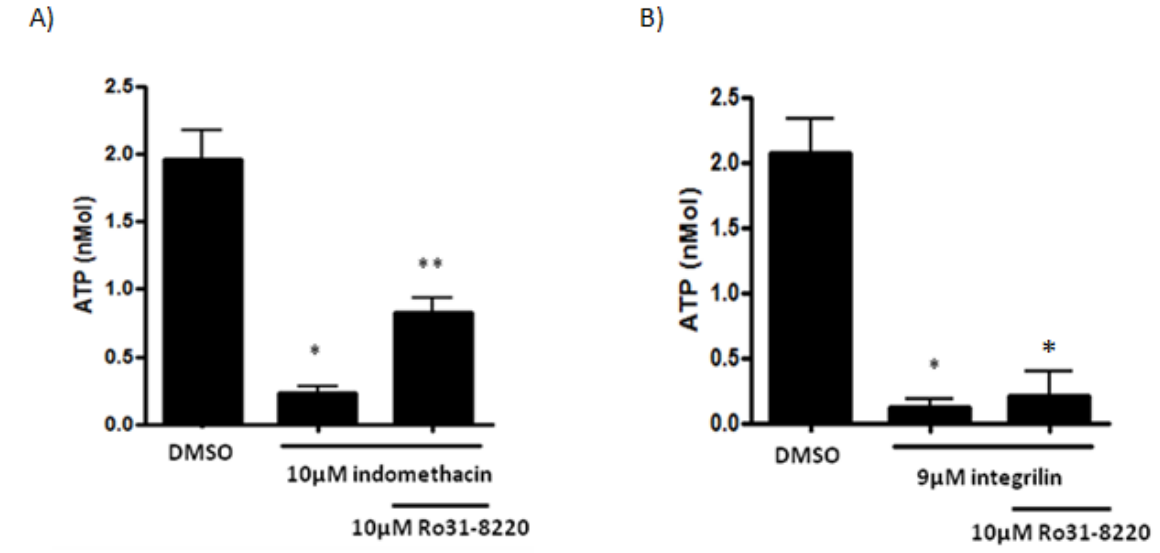


Figure 5.3. Effect of indomethacin and integrilin in the presence and absence of Ro31-8220 on aggregation and dense granule secretion of human citrated PRP in response to stimulation by ADP. Human citrated PRP was stimulated with maximal (100µM) ADP and dense granule secretion was monitored as described in legend to figure 1. A) Effect of Ro31-8220 and indomethacin on dense granule secretion of human PRP in response to ADP. PRP treated with or without (10µM) Ro31-8220 and/or (10µM) indomethacin. B) Effect of Ro31-8220 and integrilin on dense granule secretion of human PRP in response to ADP. PRP treated with or without (10µM) Ro31-8220 and/or (10µM) integrilin. Platelets were incubated with DMSO, Ro31-8220 (10µM), indomethacin (10µM) or integrilin (10µM) for 3 minutes prior to ADP stimulation. n≥3 .* indicates $p<0.05$ in comparison to DMSO treated controls. ** indicates $p<0.05$ in comparison to platelets treated with 10µM indomethacin and those treated with DMSO.

5.2.5 Potentiation is observed in the presence of extracellular Ca^{2+}.

Paradoxically it has been observed that ADP is able to stimulate sustained aggregation and marked dense granule secretion in citrated plasma which contains non-physiological, micromolar levels of Ca^{2+}, whereas in the presence of physiological, millimolar concentrations of the cation, it induces only transient aggregation and is unable to stimulate dense granule secretion. This difference has been shown to be associated with increased TxA_2 synthesis (Samuelsson, Goldyne et al. 1978) in citrated plasma suggesting extracellular Ca^{2+} inhibits ADP-induced TxA_2 formation (Cattaneo, Gachet et al. 2002) (Mustard, Perry et al. 1975; Packham, Bryant et al. 1989). The molecular basis of this paradox however is unknown.

In agreement with the above, in the presence of 1mM Ca^{2+} in PRP, ADP (100µM) induces reversible aggregation but not dense granule secretion (Figure 5.4). In the presence of submaximal concentrations of Ro31-8220 (10µM) both platelet aggregation and dense granule secretion to ADP (100µM) are potentiated, although it should be noted that the level of secretion was much lower than that observed in citrated plasma (Figure 5.2 and 5.4). In the presence of maximal PKC inhibition (100µM Ro31-8220), ADP-induced aggregation was decreased and no secretion was observed, similar to that already shown in citrated PRP. In contrast, sub-maximal concentrations of Ro31-8220 also potentiate ADP-induced secretion and aggregation in the presence of physiological concentrations of Ca^{2+}. This raises the possibility that the inability of ADP to stimulate sustained aggregation and secretion in Ca^{2+}-containing PRP is due to PKC exerting a constitutive feedback effect, and highlights the level of PKC activity as a key regulator of platelet activation by ADP in plasma containing low or physiologically normal Ca^{2+} levels.

5.2.6 The role of $P2Y_1$ and $P2Y_{12}$ in potentiation of ADP-induced platelet activation.

ADP activates both the $P2Y_1$ and $P2Y_{12}$ receptors and it is the synergy between the two that is thought to be necessary for ADP mediated platelet activation. To address which of the two ADP receptors are involved in the potentiation by submaximal PKC inhibition, human citrated PRP was treated with either the $P2Y_1$ inhibitor MRS-2179 (MRS) or the $P2Y_{12}$ inhibitor cangrelor/ARC-6699331MX (ARC) and stimulated by both high (100µM) or low (3µM) concentrations of ADP. Pre-treatment of platelets with the $P2Y_{12}$ inhibitor ARC blocked dense granule secretion induced by both low (3µM) and high (100µM) concentrations of ADP, completely inhibited aggregation to low concentrations of ADP and reduced aggregation to a diminished, transient response at near high ADP (100µM) (Figure 5.5). Significantly, neither response could be rescued in the presence of submaximal Ro31-8220 (10µM) (Figure 5.5). Interestingly inhibition of the $P2Y_1$ receptor, using MRS, had very little effect on aggregation and caused only minor inhibition of dense granule secretion following activation by high concentrations of ADP (100µM) (Figure 5.6). Treatment with Ro31-8220 (10µM) In the presence of MRS saw no difference in aggregation but, as with citrated plasma, potentiation of the rate of onset of dense granule secretion was observed. Following activation by lower concentrations of ADP (3µM), MRS (100µM) induced inhibition of the $P2Y_1$ receptor (Figure 5.6), inhibited shape change, aggregation and dense granule secretion. This inhibition however, could be rescued by the presence of submaximal Ro31-8220 and potentiation was observed (Figure 5.6). This recovery of platelet activation despite $P2Y_1$ inhibition therefore indicates that potentiation of aggregation and dense granule secretion is mediated by the $P2Y_{12}$ receptor or the synergy between $P2Y_1$ and $P2Y_{12}$.

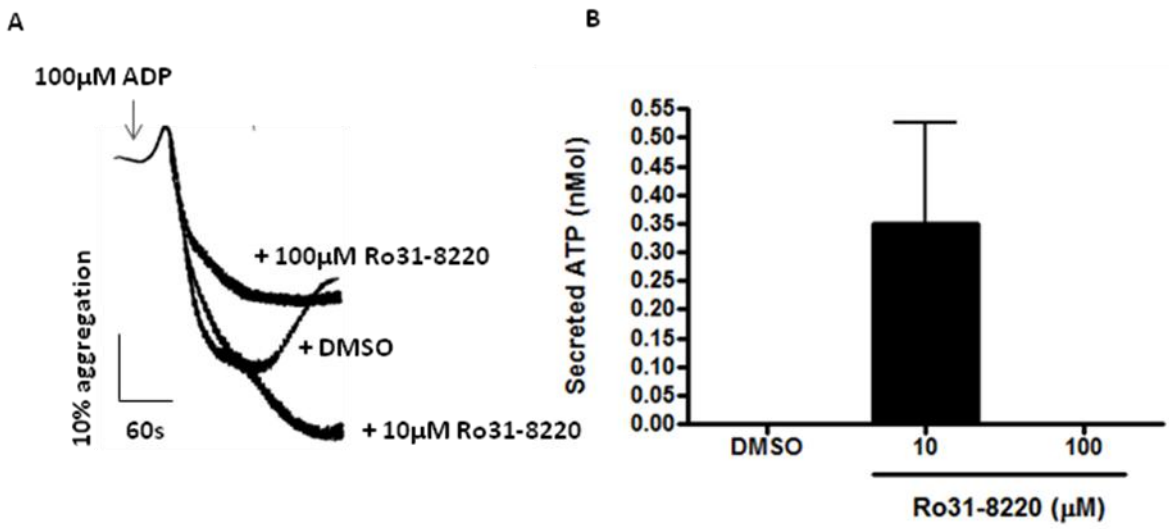


Figure 5.4. Effect of Ro31-8820 on aggregation and dense granule secretion of human PRP in the presence of extracellular calcium in response to stimulation by ADP. Human PRP was prepared using PPACK as an anticoagulant to maintain physiological levels of extracellular calcium and incubated in the presence or absence of 10 or 100µM Ro31-8220. (A) Aggregation and (B) dense granule secretion were monitored following stimulation by ADP (100µM). Traces representative of n=3.

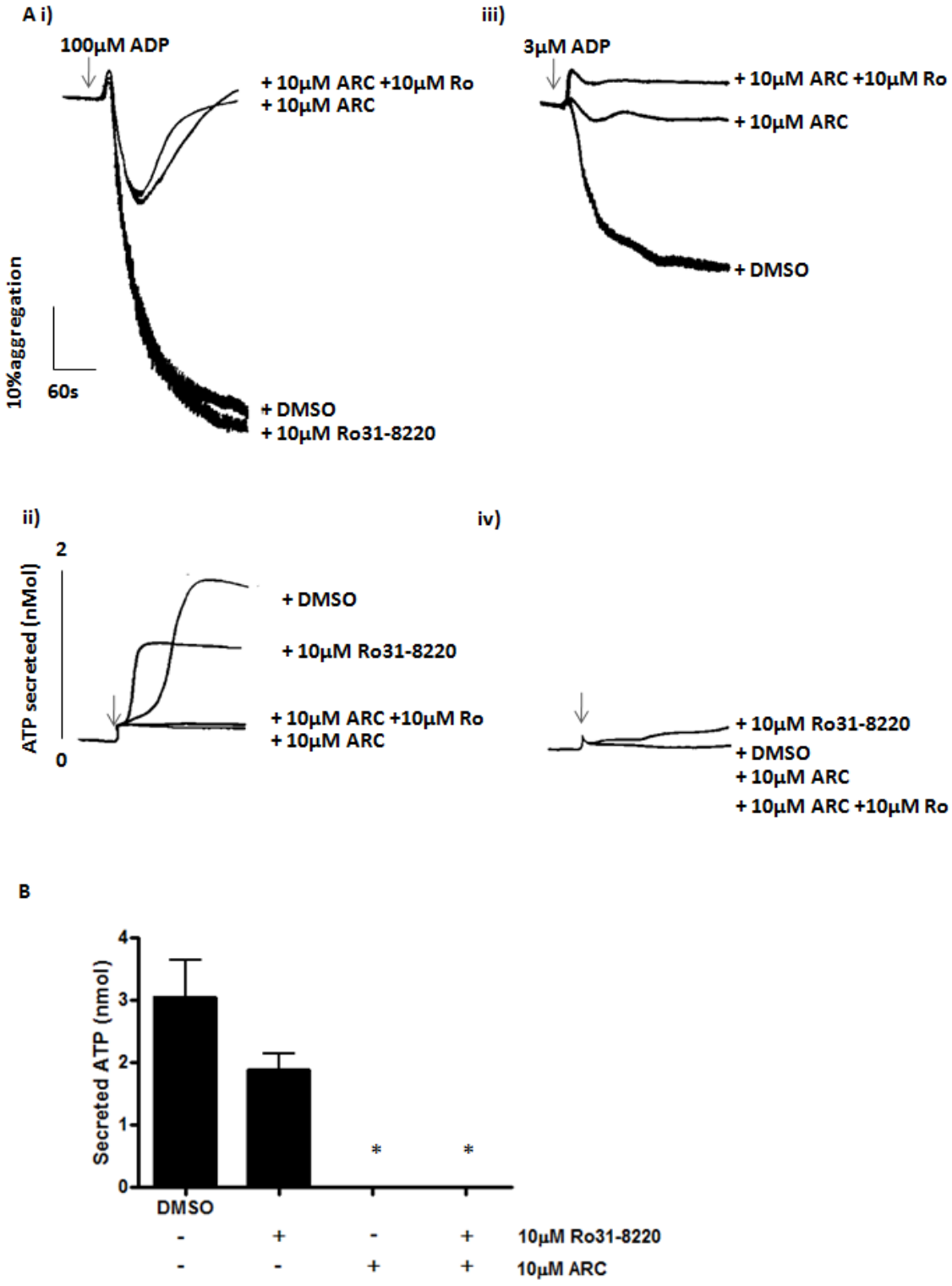


Figure 5.5. Effect of P2Y1 inhibition on aggregation and dense granule secretion of human citrated PRP in response to stimulation by ADP in the presence and absence of Ro31-8220. A. Human citrated PRP was stimulated with either (i, ii) maximal (100µM) or (iii, iv) submaximal (3µM) ADP, and aggregation and dense granule secretion were monitored as described in legend to figure 1. A i) and iii) Aggregation monitored by optical aggregometry. Traces representative of n=3. ii) and iv) Representative traces of n=3 showing dense granule secretion, monitored by ATP release, to 3µM and 100µM

ADP respectively in the absence or presence of ARC (10µM) and/or submaximal Ro31-8220 (10µM). B) Summary of dense granule secretion by PRP, monitored by ATP release, to ADP (100µM) in the absence or presence of ARC (10µM) and/or Ro31-8220 (10µM). Platelets were incubated with DMSO, Ro31-8220 (10µM) or ARC (10µM) for 3 minutes prior to ADP stimulation. $n \geq 3$.* indicates $p<0.05$ in comparison to DMSO treated controls

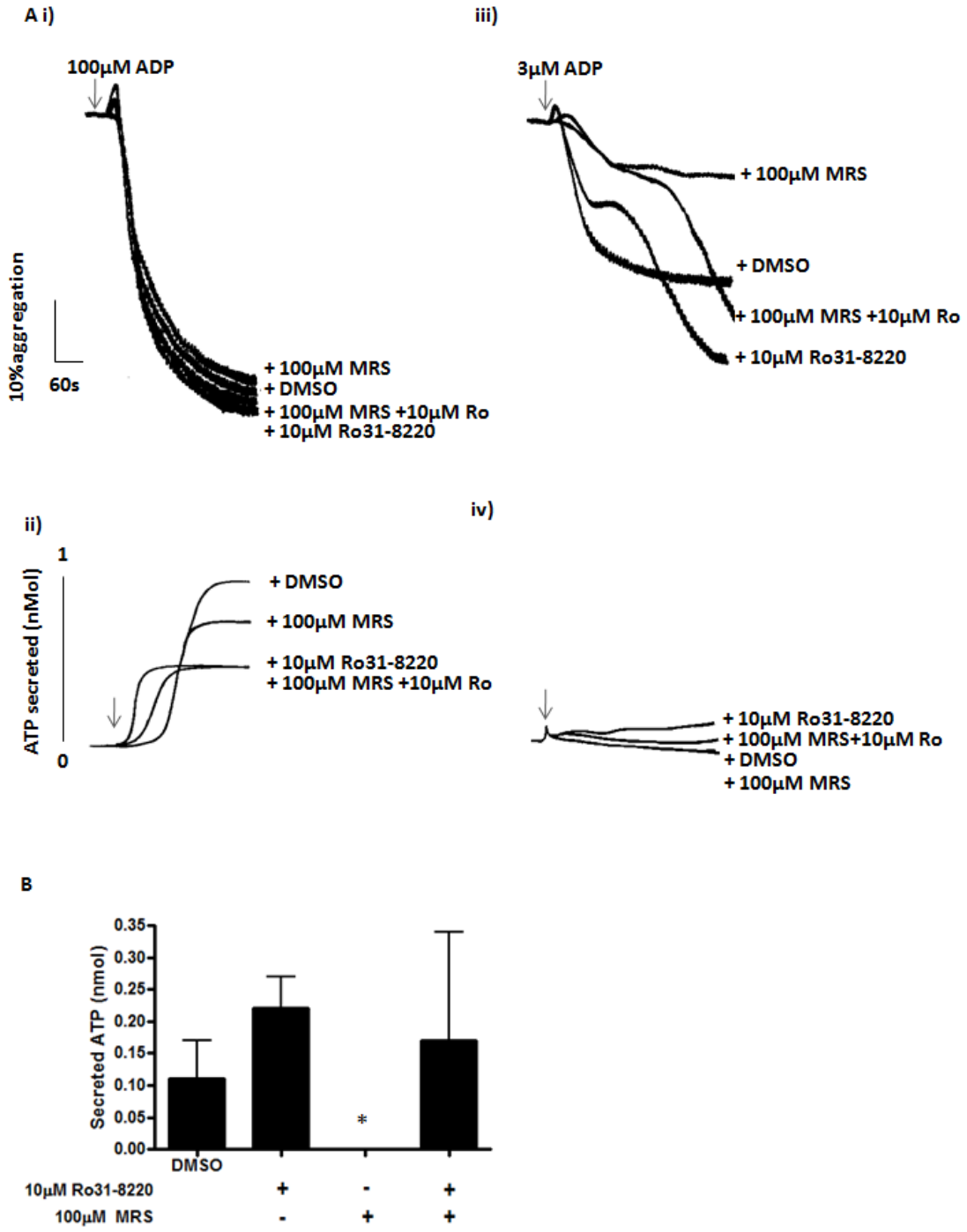


Figure 5.6. Effect of P2Y12 inhibition on aggregation and dense granule secretion of human citrated PRP in response to stimulation by ADP in the presence and absence of Ro31-8220. A. Human citrated PRP was stimulated with either (I, ii) maximal (100µM) or (iii, iv) submaximal (3µM) ADP, and aggregation and dense granule secretion were monitored as described in legend to figure 1. A i) and iii) Aggregation monitored by optical aggregometry. Traces representative of n=3. ii) and iv) Representative traces of n=3 showing dense granule secretion, monitored by ATP release, to 3µM and 100µM ADP respectively in the absence or presence of MRS (10µM) and/or submaximal Ro31-8220 (10µM). B) Summary of dense granule secretion by PRP, monitored by ATP release, to ADP (100µM) in the absence or presence of MRS (10µM) and/or Ro31-8220 (10µM).

Platelets were incubated with DMSO, Ro31-8220 (10µM) or MRS (10µM) for 3 minutes prior to ADP stimulation. n≥3

5.2.7 Submaximal inhibition of PKC potentiates aggregation and secretion in washed platelets.

One possible mechanism which could underlie the potentiation observed in the presence of partial inhibition of PKC is regulation of intracellular calcium. However, the protocol for the detection of changes in intracellular calcium levels requires the use of washed platelets instead of PRP, as fluorescence is used to detect and quantify the levels of calcium, and the presence of plasma creates background fluorescence. It was therefore necessary to determine whether potentiation was preserved in human washed platelets. As ADP reinforces activation through both the $P2Y_1$ and $P2Y_{12}$ receptors, special conditions are required to maintain ADP-mediated platelet activation in washed platelets, as the $P2Y_1$ receptor undergoes marked desensitisation upon exposure to ADP during the harsh preparation protocol. Washed platelets were therefore prepared in the presence of apyrase (0.05U/ml) which catalyses the hydrolysis of ADP and ATP released from the cells during platelet sample preparation.

To determine whether potentiation following partial blockade of PKC could be observed in washed platelets, a lower range of concentrations of Ro31-8220 were used because of the increased bioavailability (see Figure 5.1). ADP (100µM) stimulated weak, transient aggregation in washed platelets which was not accompanied by granule secretion (Figure 5.7A). In the presence of submaximal Ro31-8220 (now 0.3µM and 1µM), ADP (100µM) stimulated maximal, sustained aggregation and dense granule secretion, with both responses being inhibited in the presence of a maximally-effective concentration of the PKC inhibitor (10µM) (Figure 5.7A). These results indicate that potentiation can be observed in washed platelets and is therefore not dependent on the presence of plasma.

5.2.8 Ro31-8220 potentiates changes in intracellular Calcium downstream of ADP.

ADP-induced platelet activation is dependent on the mobilisation of intracellular Ca^{2+} from internal stores. It is known that the PKC superfamily has both inhibitory and stimulatory effects on Ca^{2+} mobilisation in platelets (Harper and Poole 2010) thereby providing a mechanism that could underlie Ro31-8220-mediated potentiation. To address this, washed platelets were loaded with the Ca^{2+} reporter dye FURA-2AM, which is converted to the calcium indicator Fura-2 following removal of the acetoxymethyl groups by cellular esterases (Grynkiewicz, Poenie et al. 1985). Treatment with Ro31-8220 caused a concentration dependent increase in intracellular Ca^{2+} levels induced by ADP in comparison to DMSO treated controls, with a threshold at 0.1µM Ro31-8220 and a peak effect at 1µM, which parallels the potentiation of aggregation and secretion (Figure 5.7B). Interestingly, the increase in Ca^{2+} was also sustained at a maximally-effective concentration of Ro31-8220 (10µM) where inhibition of both aggregation and dense granule secretion is observed (Figure 5.7A). This result indicates that Ro31-8220 dependent increases in intracellular calcium could provide a mechanism for the potentiation of aggregation and dense granule secretion observed following partial-inhibition of PKC. The presence of potentiated calcium levels at concentrations of Ro31-8220 that cause inhibition could indicate several additional positive roles for the PKC superfamily in the regulation and mechanisms of platelet aggregation and dense granule secretion.

5.2.9 Potentiation is PKC specific.

One concern with the findings so far, was that the effects observed in the presence of Ro31-8220 could be due to non-specific inhibition of an alternative inhibitor target,

rather than the well characterised inhibition of PKC. To ensure that Ro31-8220 was inhibiting PKC, PKC-dependent substrate phosphorylation was investigated, using an antibody raised against Phospho(Ser)-PKC substrates. PKC dependent substrate phosphorylation was monitored to see whether the pattern of phosphorylation changed in the presence of the inhibitor. As shown in Figure 5.8A, Ro31-8220 inhibited phosphorylation of several PKC substrates in platelets downstream of the phorbol ester PMA, a direct activator of PKC, (Liu and Heckman 1998) and following activation by ADP. Inhibition of PKC dependent substrate phosphorylation occurred with the same concentration response relationships as seen for aggregation and dense granule secretion on washed platelets (Figures 5.1 and 5.7). Weak inhibition of PKC dependent phosphorylation was observed between 0.1-0.3μM Ro31-8220 with full inhibition at 10μM. The blot shows some extent of inhibition to all the concentrations of Ro31-8220 that have been used most likely due to the combined inhibition of the multiple isoforms of PKC. These blots show that the potentiation of ADP-induced platelet activation by Ro31-8220 occurs over the same concentration range as those for inhibition of PKC dependent substrate phosphorylation (Figures 5.7 and 5.8A). This provides further support for the potentiation being a PKC dependent effect of the Ro31-8220 inhibitor.

In addition, the effect of low level activation of PKC using PMA on ADP-induced dense granule secretion was investigated. Seeing as partial-inhibition of PKC induces potentiation downstream of ADP, it was expected that the opposite should occur following low level activation of PKC; activation of PKC following treatment with in PMA should theoretically, result in the inhibition of ADP-induced platelet activation. Pre-treatment of PRP with increasing concentrations of PMA led to a decrease in dense granule secretion relative to control platelets following activation by ADP (Figure 5.8B).

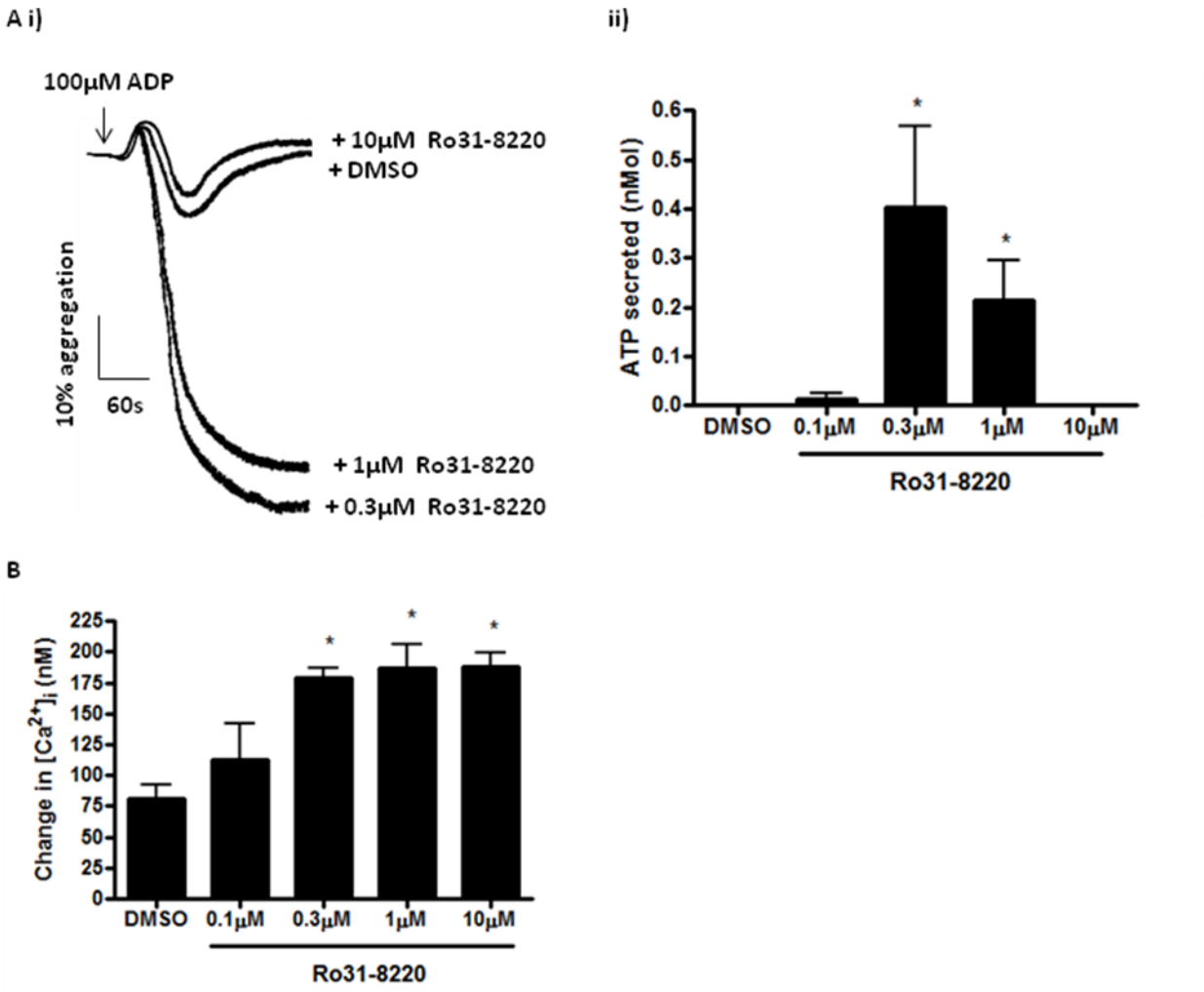


Figure 5.7. Mechanisms of potentiation by submaximal concentrations of Ro31-8220 in human washed platelets. (A) The effect of Ro31-8220 on aggregation and dense granule secretion in response to stimulation by ADP. Human ADP-sensitive washed platelets were stimulated with 100µM ADP in the presence and absence of varying concentrations of Ro31-8220 (0.1µM, 0.3µM, 1µM and 10µM) and (i) aggregation and (ii) dense granule secretion were monitored as previously described. (B) Effect of Ro31-8220 on intracellular calcium levels following ADP stimulation. Human washed platelets were loaded with FURA-2-AM and pre-treated with or without varying concentrations of Ro-31-8220 before stimulation with 100µM ADP. Fluorescence was measured before and after (1min) ADP addition and the increase in intracellular calcium concentration calculated using the Grynkiewicz equation. Platelets were incubated for 3 minutes with inhibitor(s) or DMSO prior to ADP stimulation. Representative traces shown. Data are presented as mean ± SEM, n≥3.* indicates $p<0.05$ in comparison to DMSO treated controls,

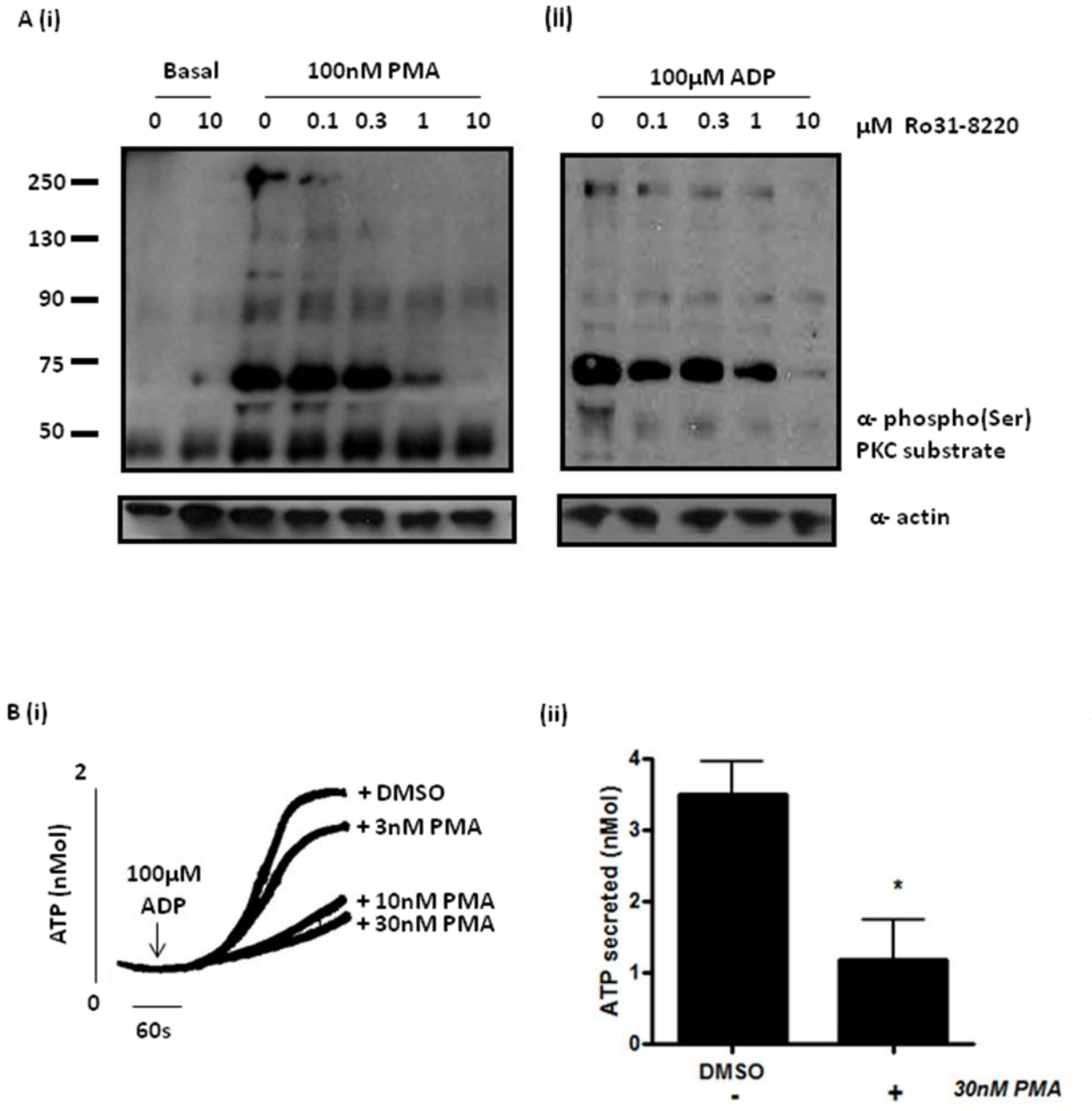


Figure 5.8. Effect of Ro31-8220 on PKC mediated phosphorylation and ADP-induced platelet aggregation. (A). Effect of Ro31-8220 on PKC substrate phosphorylation in human washed platelets. Whole cell lysates were resolved by SDS-PAGE and subject to western blot analysis. Phosphorylation of a PKC consensus sequence was detected using α-phospho(ser)-PKC substrate antibody, following activation by (i) PMA (100nM) and (ii) ADP (100μM) in the absence or presence of Ro31-8220 (0.1μM, 0.3μM, 1 μM and 10 μM). Actin was used as a loading control. (B) Effect of PKC activation by PMA on dense granule secretion from human citrated PRP in response to stimulation by ADP. Human citrated PRP was prepared and incubated with or without increasing concentrations of PMA (nM), and dense granule secretion was monitored by ATP release, following stimulation by 100μM ADP. (i) Representative trace. (ii) ATP release is presented as mean ± SEM, n≥3. Platelets were incubated with DMSO, PMA (30nM) or Ro31-8220 (10μM) for 3 minutes prior to ADP stimulation. n=3 .* indicates $p<0.05$ in comparison to DMSO treated controls.

These results demonstrate that low level activation of platelets by PMA mediates platelet inhibition in response to ADP, consistent with a model in which antagonism of constitutive signalling by the PKC superfamily underlies the potentiation of ADP-induced aggregation, further supporting the conclusion that potentiation is a PKC-specific effect.

Finally, in order to provide further support for the observed potentiation resulting from a PKC specific effect, a second pan-PKC inhibitor Ro31-8425 (Wilkinson, Parker et al. 1993; Gilio, Harper et al. 2010) was used. In both PRP and washed platelets the presence of submaximal Ro31-8425 (10μM and 0.3μM-1μM respectively), as with Ro31-8220, potentiated both aggregation and dense granule secretion to ADP (100μM), whereas both responses were inhibited in the presence of a maximally-effective concentration of the pan-PKC inhibitor (100μM and 10μM respectively) (Figure 5.9A and B). Furthermore, in washed platelets, ADP-stimulated Ca^{2+} mobilisation was potentiated over the same concentration of Ro31-8425 (0.3-10μM) (Figure 5.9C) and inhibition of PKC dependent substrate phosphorylation occurred with the same concentration response relationships as seen for aggregation and dense granule secretion on washed platelets (Figure 5.9D). These results are essentially the same as those with Ro31-8220 and support that the observed potentiation is a result of inhibition of PKC rather than a non-specific effect of the inhibitor.

5.2.10 A role for the novel PKCε isoform in the regulation of ADP-induced platelet activation in mice.

Satisfied that the results observed so far following treatment with Ro31-8220 were the result of a specific effect of the alteration of PKC activity, the focus was shifted to determine whether one of the many isoforms of PKC was specifically involved in this

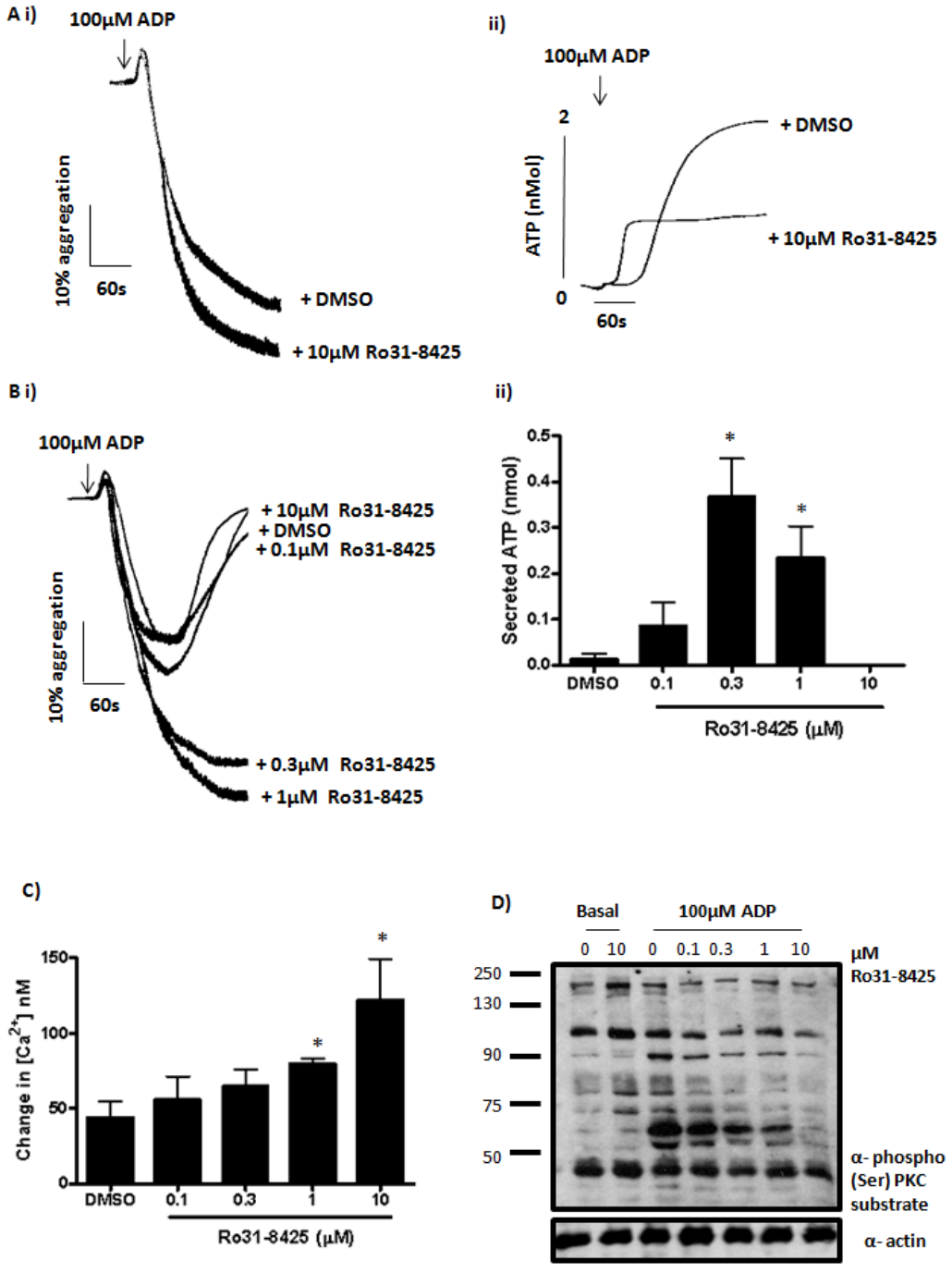


Figure 5.9. Effect of Ro31-8425 on platelet activation in human PRP and washed platelets in response to stimulation by ADP. (A) Human PRP and (B) human ADP-sensitive washed platelets were stimulated with 100µM ADP in the presence and absence of varying concentrations of Ro31-8425 (0.1-10µM) and (i) aggregation and (ii) dense granule secretion were monitored as previously described.(C) Effect of Ro31-8425 on intracellular calcium levels following ADP stimulation. Human washed platelets were loaded with FURA-2-AM, fluorescence measured and calcium levels calculated as

previously described in legend to Figure 5.7. (D) Effect of the Ro31-8425 on PKC substrate phosphorylation ADP stimulated human washed platelets. Whole cell lysates were resolved by SDS-PAGE and subject to western blot analysis. Phosphorylation by PKC was detected using α-phospho(ser)-PKC substrate antibody, following activation by ADP (100μM) in the absence or presence of Ro31-8425 (0.1 -10μM). Actin was used as a loading control. Platelets were incubated for 3 minutes with inhibitor(s) or DMSO prior to ADP stimulation. Representative traces shown, data are presented as mean ± SEM, n=3. *indicates $p \leq 0.05$ in comparison to controls.

phenomenon. The data so far indicates opposing regulatory roles for members of the PKC superfamily in platelet activation downstream of ADP, more specifically an inhibitory role that is sensitive to low concentrations of Ro31-8220 and hence low levels of PKC inhibition and an activatory role which is sensitive to high concentrations of the inhibitor and most likely maximal PKC inhibition. In the literature there is increasing evidence that suggests the novel isoforms of PKC have several negative roles in the different processes involved in platelet activation, whilst the classical isoforms appear to have positive roles (Tabuchi, Yoshioka et al. 2003; Murugappan, Tuluc et al. 2004; Pula, Schuh et al. 2006; Harper and Poole 2007; Hall, Harper et al. 2008; Pears, Thornber et al. 2008; Konopatskaya, Gilio et al. 2009; Nagy, Bhavaraju et al. 2009; Gilio, Harper et al. 2010; Harper, Molkentin et al. 2010; Harper and Poole 2010).

In order to determine whether one of the novel PKC isoforms was responsible for the negative role of PKC in ADP-induced platelet activation and hence the observed potentiation, mice that are deficient in a single isoform of PKC and that were available to us were tested. Initially, it was necessary to determine whether a similar potentiation of platelet activation could be observed in mouse platelets, downstream of ADP in the presence of submaximal PKC inhibition using wild-type (WT) mouse PRP. As was observed in human platelets, submaximal concentrations of Ro31-8220 and Ro31-8425 caused potentiation of dense granule secretion relative to untreated controls in WT mouse citrated PRP (Figure 5.10) although aggregation at high ADP remained unaffected. Subsequently ADP-induced responses in PRP from mice lacking either the novel PKC isoforms, PKCε or PKCθ was determined. Interestingly, although no difference in aggregation was observed in PRP harvested from PKCε$^{-/-}$ mice following activation by a

high concentration of ADP (100μM), dense granule secretion is potentiated in PKCε$^{-/-}$ mice in comparison to WT controls to a much greater extent than that seen following treatment with Ro31-8220 (Figure 5.10). This increased secretion is then reduced following treatment with submaximal Ro31-8220 (10μM) indicating a positive role for PKC in ADP-induced platelet activation in mouse platelets. However, the extent of dense granule secretion was not completely inhibited and can still be observed at a level greater than that achieved in WT platelets in the presence of Ro31-8220, therefore suggesting the involvement of an additional PKC isoform (Figure 5.10). Unlike in the PKCε$^{-/-}$ mice, no difference in ADP-induced secretion was observed in platelets from PKCθ$^{-/-}$ mice (data not shown). These results therefore demonstrate that the potentiation observed in mouse platelets is mediated, at least in part, through inhibition of the novel isoform PKCε. As PKCθ and PKCε null mice were the only mice available to us, platelet activation downstream of ADP was not monitored in mice deficient in any of the other isoforms of PKC.

5.2.11 A role for the classical isoform PKCβ in the regulation of ADP-induced platelet activation in human platelets.

The studies using mouse platelets highlighted a clear role for PKCε in the negative regulation of ADP-induced platelet activation. However, the presence of PKCε in human platelets is debatable, suggesting the involvement of another isoform in human platelets. Several inhibitors of the PKC superfamily, that are reported to be selective to various PKC isoforms, were tested. The inhibitors used include the classical isoform inhibitor Gö6983, a PKCβ inhibitor, a PKCθ inhibitor (Cywin, Dahmann et al. 2007) and the PKCδ inhibitor Rottlerin, (Figures 5.11, .512, 5.13).

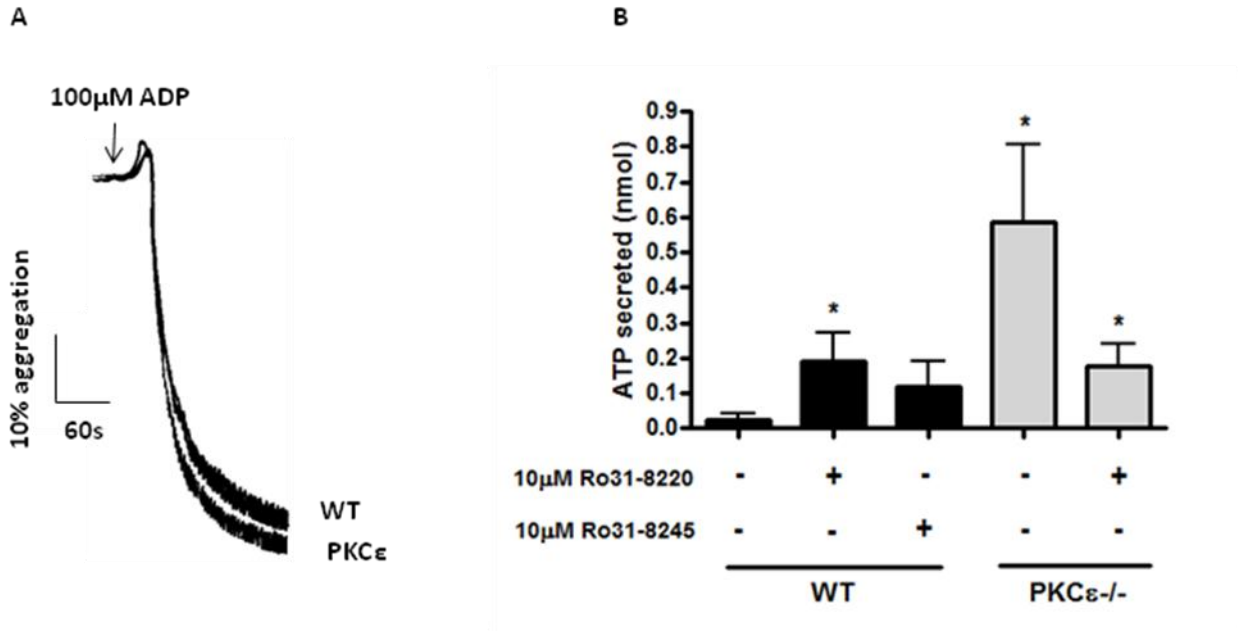


Figure 5.10. Potentiation of dense granule secretion by ADP in PKCε null mice. Mouse PRP from WT and PKCε deficient mice was prepared using citrate as an anticoagulant and incubated in the presence or absence of 10µM Ro31-8220 or 10µM Ro31-8245. Platelets were then stimulated with 100µM ADP. A) Aggregation of WT and PKCε null mice following activation by ADP was monitored by optical aggregometry. B) Dense granule secretion monitored by ATP release to 100µM ADP. Data are presented as mean ± SEM, n=3 *indicates p≤ 0.05 in comparison to WT controls.

Potentiation of aggregation and secretion to ADP (100μM) was observed in the presence of Gö6983 and the PKCβ inhibitor, in both human PRP and in washed platelets (Figures 5.11 and 5.12 A and B). A concentration dependent increase in intracellular Ca^{2+} by ADP was also observed in the presence of both of the inhibitors (0.3-10μM PKCβ inhibitor, Figure 5.11C (0.1-1μM Gö6983, Figure .12C) over the same concentration range as the potentiation of ADP-induced dense granule secretion and aggregation. Analysis of the extent of PKC dependent substrate phosphorylation in the presence of the two inhibitors following stimulation by 100μM ADP suggests inhibition of PKC activity in the presence of the concentrations of both inhibitors that induce potentiation of platelet activation (Figure 5.11D and 5.12D). In contrast neither Rottlerin (0.3-30μM) or the PKCθ inhibitor (0.1-30μM) potentiated these responses to ADP in washed platelets, (Figure 5.13) (no dense granule secretion observed, so data not shown). The involvement of PKCβ in human platelets led us to test whether the same was also true in mouse platelets, therefore the effect of Gö6983 and the PKCβ inhibitor on ADP-induced responses using WT mouse PRP was observed (Figure 5.14). As in human platelets the PKCβ inhibitor (10μM and 30μM) and the classical isoform inhibitor Gö6983 also potentiated ADP-induced responses in mouse PRP. Taken together, these results support a role for the classical isoform PKCβ in inhibiting activation by ADP in human platelets in addition to a role in combination with PKCε in mouse platelets.

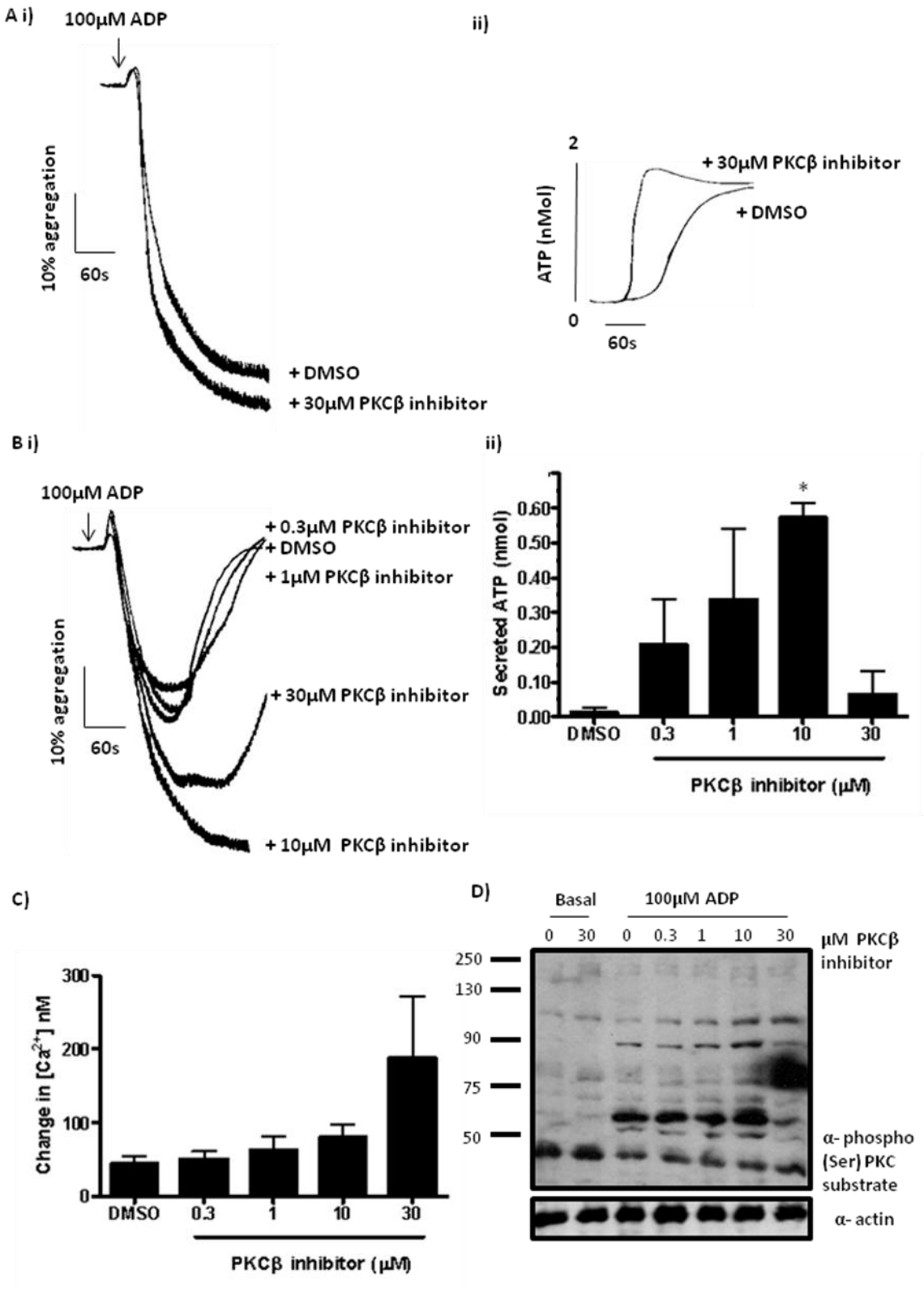


Figure 5.11. Effect of PKCβ inhibitor on platelet activation in human PRP and washed platelets in response to stimulation by ADP. (A) Human PRP and (B) Human ADP-sensitive washed platelets were stimulated with 100μM ADP in the presence and absence of varying concentrations of PKCβ inhibitor (0.3-30μM) and (i) aggregation and

(ii) dense granule secretion were monitored as previously described.(C) Effect of PKCβ inhibitor on intracellular calcium levels following ADP stimulation. Human washed platelets were loaded with FURA-2-AM, fluorescence measured and calcium levels calculated as previously described. (D) Effect of the PKCβ inhibitor on PKC substrate phosphorylation in ADP stimulated human washed platelets. Whole cell lysates were resolved by SDS-PAGE and subject to western blot analysis. Phosphorylation of a PKC consensus sequence was detected using α-phospho(ser)-PKC substrate antibody, following activation by ADP (100μM) in the presence of PKCβ inhibitor (0.3 -30μM). Actin was used as a loading control. Platelets were incubated for 3 minutes with inhibitor(s) or DMSO prior to ADP stimulation. Representative traces shown, data are presented as mean ± SEM, n=3. *indicates $p \leq 0.05$ in comparison to controls.

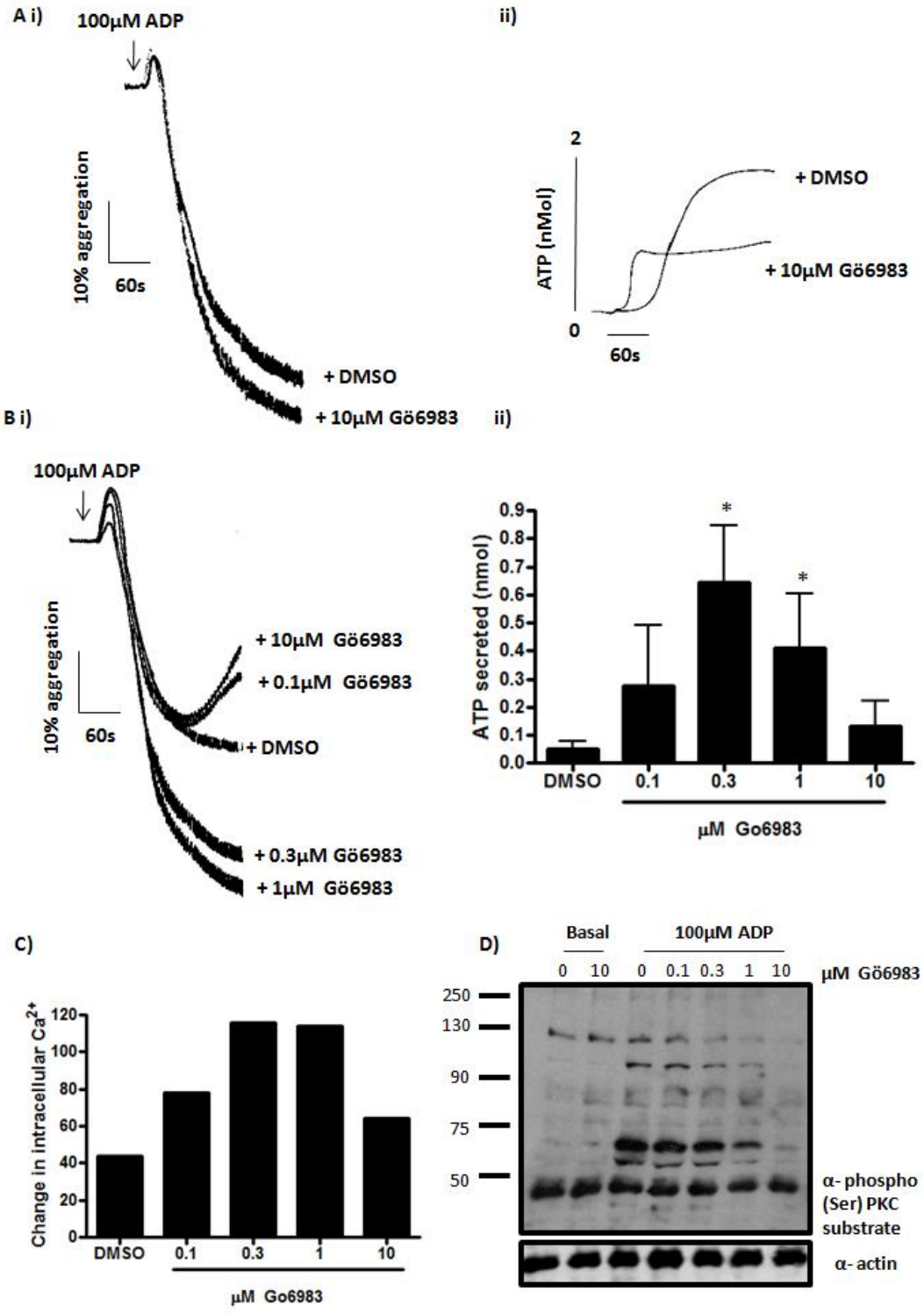


Figure 5.12. Effect of Gö6983 on platelet activation in human PRP and washed platelets in response to stimulation by ADP. (A) Human PRP and (B) human ADP-sensitive washed platelets were stimulated with 100µM ADP in the presence and absence of varying concentrations of Gö6983 (0.1-10µM) and (i) aggregation and (ii) dense granule secretion were monitored as previously described. (C) Effect of Gö6983 on intracellular calcium levels following ADP stimulation. Human washed platelets were loaded with FURA-2-AM, fluorescence measured and calcium levels calculated as

previously described. (D) Effect of the Gö6983 on PKC substrate phosphorylation in ADP stimulated human washed platelets. Whole cell lysates were resolved by SDS-PAGE and subject to western blot analysis. Phosphorylation of a PKC consensus sequence was detected using α-phospho(ser)-PKC substrate antibody, following activation by ADP (100µM) in the presence of Gö6983 (0.1 -10µM). Actin was used as a loading control. Platelets were incubated for 3 minutes with inhibitor(s) or DMSO prior to ADP stimulation. Representative traces shown, data are presented as mean ± SEM, n=3. *indicates $p \leq 0.05$ in comparison to controls.

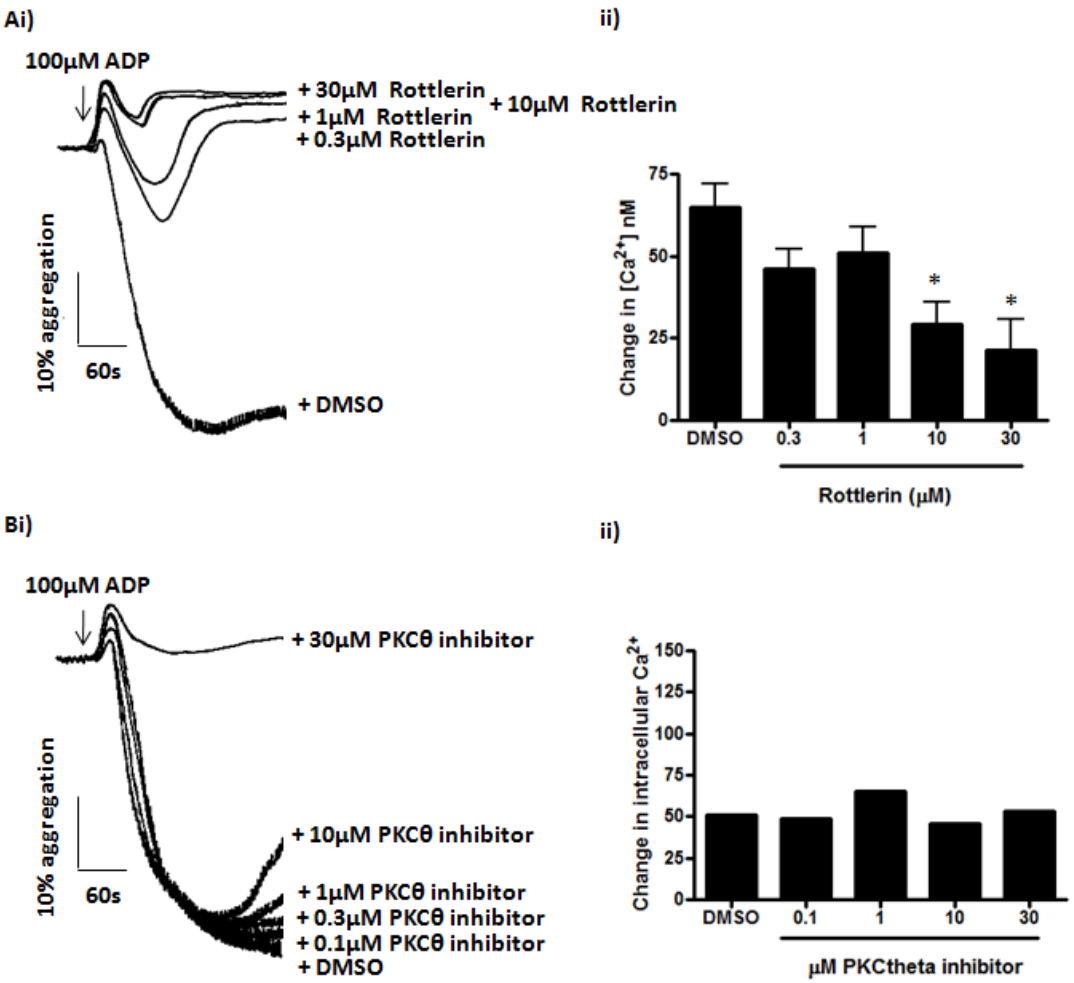


Figure 5.13. Effect of the PKCδ inhibitor, Rottlerin, and PKCθ inhibitor on aggregation and levels of intracellular Calcium of human washed platelets in response to stimulation by ADP. (A) The effect of Rottlerin and (B) PKCθ inhibitor on (i) aggregation in response to stimulation by ADP. Human ADP-sensitive washed platelets were stimulated with 100µM ADP in the presence and absence of varying concentrations of Rottlerin (0.3µM, 1µM, 10µM and 30µM) or PKCθ inhibitor (0.1µM, 0.3µM, 1µM, 10µM and 30µM). Aggregation was monitored as previously described. (ii) Effect of Rottlerin or PKCθ inhibitor on intracellular calcium levels following ADP stimulation. Human washed platelets were loaded with FURA-2-AM, fluorescence measured and calcium levels calculated as previously described. Platelets were incubated for 3 minutes with inhibitor(s) or DMSO prior to ADP stimulation. Representative traces shown, n=3.

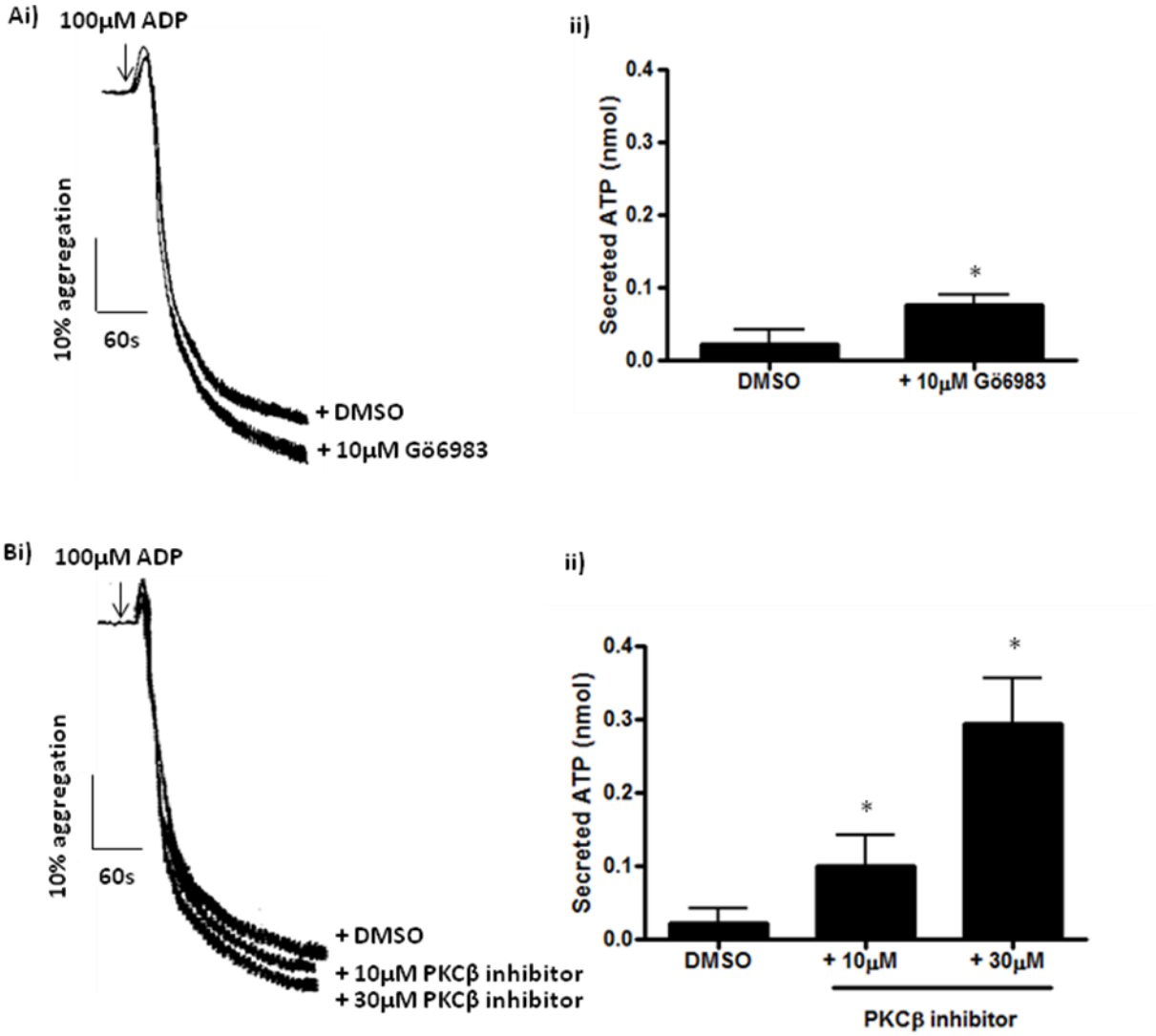


Figure 5.14. Effect of PKC inhibition on aggregation, and dense granule secretion in mouse PRP response to stimulation by ADP. WT mouse PRP was stimulated with 100µM ADP in the presence and absence of (A) Gö6983 (10µM) and (B) PKCβ inhibitor (10-30µM) and (i) aggregation and (ii) dense granule secretion were monitored as previously described. Platelets were incubated for 3 minutes with inhibitor(s) or DMSO prior to ADP stimulation. Representative traces shown. n=3

5.3 DISCUSSION

The PKC superfamily is well documented as a key regulator of the process of platelet activation, but the general view of an overall positive role for PKC is now considered to be too simplistic. Studies looking at the roles of the individual isoforms of PKC have highlighted both positive and negative regulatory roles for the kinase in several processes, required for platelet activation and thrombus formation, downstream of several platelet agonists (Shattil and Brass 1987; King and Rittenhouse 1989; Ryu, Kim et al. 1990; Toullec, Pianetti et al. 1991; Walker and Watson 1993; Wilkinson, Parker et al. 1993; Yoshioka, Shirakawa et al. 2001; Quinton, Kim et al. 2002; Tabuchi, Yoshioka et al. 2003; Murugappan, Tuluc et al. 2004; Harper and Poole 2007; Strehl, Munnix et al. 2007; Hall, Harper et al. 2008; Pears, Thornber et al. 2008; Harper and Poole 2009; Konopatskaya, Gilio et al. 2009; Nagy, Bhavaraju et al. 2009; Gilio, Harper et al. 2010; Harper, Molkentin et al. 2010; Harper and Poole 2010; Harper and Poole 2010). However, the role for the PKC superfamily in the regulation of ADP-induced platelet activation has been relatively uncharacterised and very little is known about the function of the individual isoforms of PKC in ADP dependent platelet signalling. Although ADP, compared to collagen and thrombin, is considered to be a relatively weak platelet agonist, it is essential in the positive feedback mechanism that helps maintain the process of platelet activation. In an attempt to differentiate the net effect of the PKC superfamily on ADP activation from any stimulatory and inhibitory actions for PKC, the effect of a range of concentrations of the pan-PKC inhibitors Ro31-8220 and Ro31-8425 on platelet activation by ADP was investigated. Both Ro31-8220 and Ro31-8245 are believed to be broad spectrum PKC inhibitors with similar relative affinities for the

individual PKC isoforms (Wilkinson, Parker et al. 1993; Liu and Heckman 1998), both inhibitors show the same effect and extent of overall PKC inhibition supporting that the observations made are a PKC specific effect. Interestingly however, the potentiation of ADP-induced dense granule secretion and aggregation that was observed in the presence of Ro31-8220 and Ro31-8425 was only seen in the presence of submaximal concentrations of the inhibitor, where inhibition of PKC appears to be incomplete. It is possible that at these lower, submaximal concentrations of broad spectrum PKC inhibitors, Ro31-8220 and Ro31-8425, some isoforms of PKC are less effected by the inhibitor in comparison to others, enabling the different roles of the isoforms of the PKC superfamily to be dissected or that phosphorylation of certain substrates is more sensitive to PKC inhibition.

Some pharmacological reagents have been shown to have a reduced bioavailability in plasma (Strehl, Munnix et al. 2007), where it is thought that, as a result of plasma protein binding, the acting concentration of the compound is effectively lowered in the blood. As shown in figure 5.1, comparison of the PAR-1-dependent responses, a process that is known to require PKC activity, in PRP (plasma containing) and washed platelets (plasma removed) confirmed that the broad spectrum PKC inhibitor, Ro31-8220, had reduced bioavailability in PRP in comparison to washed platelets. A 100-fold higher concentration (100µM) of the inhibitor than that used in washed platelets (1µM) was required for complete inhibition of dense granule secretion in PRP (figure 5.1), and even then this high concentration was not sufficient to achieve the same extent of inhibition of platelet aggregation observed in washed platelets. Potentiation of ADP-induced dense granule secretion and aggregation is observed following treatment with 10µM Ro31-8220 in PRP. Interestingly, although 10µM Ro31-8220 is a maximally effective concentration in

washed platelets, the free concentration is reduced by over 100 fold in PRP due to protein binding.

The data presented in this chapter highlights a novel observation that submaximal PKC inhibition causes a marked potentiation in the processes of dense granule secretion and platelet aggregation. Following activation by low concentrations of ADP (3μM) in PRP and high concentrations of ADP in washed platelets, the extent of dense granule secretion is increased and reversible aggregation is converted to sustained aggregation in the presence of the submaximal PKC inhibition, highlighting a negative regulatory role for PKC in ADP-induced platelet activation. Interestingly, in PRP following platelet activation by high concentrations of ADP (100μM), partial blockade of PKC increases the initial rate of onset of dense granule secretion although this is followed by a diminished overall secretory response, which is consistent with the existence of both inhibitory and stimulatory actions of the PKC superfamily in ADP-induced platelet signalling. Interestingly this potentiation effect is not seen downstream of PAR-1 receptor stimulation possibly because the PAR receptor initiates activation through just a Gq-dependent pathway. Consistent with this, potentiation of platelet activation is also absent in platelets solely activated through the $P2Y_1$ ADP receptor, which also signals through a Gq-dependent pathway (figure 5.4).

These results suggest the PKC superfamily has two opposing regulatory roles in platelet activation downstream of ADP, an inhibitory role that is sensitive to partial inhibition of PKC, and an activatory role which is sensitive to maximal PKC inhibition. These results also provide the first indication that broad spectrum PKC inhibitors can be used to selectively modulate one of these opposing roles.

These results raise the question of why these broad spectrum PKC inhibitors have differential and opposing effects at different concentrations. It is possible that this is due to the different PKC isoforms having different sensitivities to the broad spectrum inhibitors Ro31-8220 and Ro31-8425 and/or differential dose response relationships for PKC substrate phosphorylation. As a result, these opposing roles for PKC could be due to the action of two (or more) of the different isoforms of PKC. It is widely published in the literature that the individual isoforms of PKC can have different roles in the several processes that underlie platelet activation, in particular several negative roles in the regulation of platelet activation have been identified for some of the novel isoforms of PKC, whilst to date only positive roles have been identified for the classical isoforms.

Using PRP from mice deficient in the novel isoforms that were available to us, potentiation of ADP-induced platelet activation was observed in mice deficient PKCε in comparison to WT controls (figure 5.10), highlighting a negative role for PKCε, in the regulation of ADP-induced platelet activation in mouse platelets. Treatment of PKCε-deficient mouse platelets with a submaximal concentration of Ro31-8220 partially reduced this potentiated dense granule secretion, demonstrating a positive role for one or more of the PKC isoforms in ADP-induced secretion in mouse platelets, but also demonstrating a possible role for another of the PKC isoforms in the negative regulation of ADP-induced dense granule secretion in mouse platelets as secretion is not completely reduced to the levels seen in inhibitor Ro31-8220 treated WT platelets.

Taking these observations into account and in view of the fact that PKCε is not thought to expressed in human platelets, these results reveal species-specific functions of the individual PKC isoforms which suggests the observed potentiation must also be mediated by another PKC isoform, particularly in human platelets. Consequently, the study was

extended to the use of various PKC isoform selective inhibitors. In an attempt to ensure inhibitor isoform selectivity the range of concentrations of the inhibitors used included concentrations that were lower than previously described in human washed platelets (Harper and Poole 2010). Interestingly however, no potentiation of aggregation, dense granule secretion or changes in intracellular calcium levels in human washed platelets was identified in the presence of the PKCδ inhibitor, rottlerin, although concerns over the effectiveness of this inhibitor exist, or in the presence of the PKCθ inhibitor, suggesting that these novel isoforms do not play a role in the negative regulation of ADP-induced platelet activation in human platelets.

Potentiation, however was observed following treatment of both human PRP and washed platelets (figure 5.11 and 5.12) and WT mouse PRP (Figure 5.14) with the PKCβ inhibitor and also with Gö6983, which primarily inhibits the classical isoforms of PKC, implicating PKCβ in the observed potentiation. These results highlight a novel role for the classical isoform PKCβ in the negative regulation of ADP-induced platelet activation in human platelets and a negative role in combination with the novel isoform PKCε in mouse platelets.

Having identified PKCβ and PKCε to be involved in the negative regulation of ADP-induced platelet activation, the mechanism behind the regulation is still unknown. Pre-treatment of platelets with the cyclooxygenase inhibitor indomethacin and the αIIbβ3 inhibitor, integrilin, confirmed a critical role for both TxA_2 formation and outside-in signalling in the ability of ADP to stimulate sustained aggregation and marked dense granule secretion in human citrated plasma. Interestingly additional treatment of the platelets with submaximal concentrations of Ro31-8220 partially rescued the indomethacin induced inhibition, indicating that, although it may contribute, enhanced

TxA_2 formation unlike outside-in signalling is not essential for potentiation, and therefore PKC works to negatively regulate ADP-induced platelet activation independently of TxA_2 formation.

It is possible that the potentiation is due to the requirement of and synergy between the $P2Y_1$ and $P2Y_{12}$ signalling pathways, particularly as platelet activation downstream of PAR-1 receptor stimulation, a solely Gq-dependent pathway is not potentiated in the presence of partial PKC inhibition. Use of inhibitors of the two ADP G-protein coupled receptors, found that signalling through $P2Y_{12}$ (G_i) but not $P2Y_1$ (G_q) is essential for the potentiation, although this may be mediated through the synergy of the two receptors as the synergy is associated with an increase in intracellular Ca^{2+}. At first, these results seem to be at odds with previous reports that $P2Y_{12}$ signalling potentiates thrombin induced calcium mobilisation and that inhibition of the $P2Y_{12}$ receptor inhibits ADP-induced calcium mobilisation (Hardy, Jones et al. 2004; van der Meijden, Schoenwaelder et al. 2008). However, these observations refer to two different mechanisms of calcium regulation. It is known that there is a synergy between $P2Y_{12}$ and Gq-coupled receptors upstream of phospholipase C activation and therefore calcium responses are increased as a result of increased phospholipase C activity and hence IP_3 production, it is likely that this regulation of Ca^{2+} is essential for ADP-induced platelet activation. Interestingly, PKC has been shown to have both inhibitory and stimulatory effects on platelet Ca^{2+} mobilisation and the levels of intracellular Ca^{2+}. For example PKC and individual PKC isoforms have been shown to negatively regulate intracellular Ca^{2+} following GPVI and PAR signalling (Harper and Poole 2010; Harper and Poole 2011). Here, an elevation in intracellular Ca^{2+} following both submaximal and maximal PKC inhibition (figure 5.7, 5.9) and classical isoform and PKCβ inhibition was observed (figure 5.11, 5.12) supporting

published data that PKC suppresses agonist-induced Ca^{2+} signalling (Strehl, Munnix et al. 2007). This increase in intracellular Ca^{2+} levels has been attributed to inhibition of the plasma membrane Ca^{2+} ATPase (PMCA) pump (Enyedi, Verma et al. 1996; Wan, Zabe et al. 2003), which is involved in the extrusion of intracellular calcium and has been shown to be positively regulated by PKC. Inhibition of PMCA activation prevents Ca^{2+} extrusion and provides a mechanism to explain the PKC attributed increase in intracellular Ca^{2+} which potentially underlies the increase in the extent of platelet activation observed here following partial PKC inhibition downstream of ADP. Unfortunately attempts to investigate PKC induced PMCA activation were unsuccessful (data not shown).

In addition to a negative role in the regulation of ADP-induced platelet activation, the data presented here also highlights a positive role for PKC in the regulation of platelet activation, as inhibition of both aggregation and dense granule secretion occurs following maximal PKC inhibition. Inhibition of dense granule secretion by high concentrations of Ro31-8220 has been reported downstream of multiple platelet agonists suggesting a common target or group of targets for the different isoforms of PKC (Walker and Watson 1993; Atkinson, Stafford et al. 2001). Multiple proteins, including components of the SNARE complex and the vesicular trafficking machinery, which are essential for the processes of dense granule secretion, have been shown to be phosphorylated and activated in a PKC-dependent manner, including SNAP-23, syntaxin 4 and Munc18c (Reed, Houng et al. 1999; Chung, Polgar et al. 2000; Dent, Kato et al. 2002; Barclay, Craig et al. 2003; Polgar, Lane et al. 2003). It is possible that it is the inhibition of one or more of these events that results in the inhibition of dense granule secretion and hence aggregation downstream of ADP in the presence of maximal PKC inhibition.

To enable the maintenance of haemostasis it is essential that powerful inhibitory pathways exist to prevent unwanted and excessive platelet activation within the intact circulation. ADP, although a relatively weak agonist in comparison to major agonists such as collagen and thrombin, is a key feedback mediator which is essential for sustaining the processes of platelet activation and thrombus formation. The release of low levels of ADP from damaged cells is therefore potentially very dangerous as this could give rise to unwanted thrombus formation. The results presented here, highlight a novel, PKC regulated negative regulatory pathway that could exist to prevent unwanted thrombus formation.

The data presented in this chapter identifies both positive and negative regulatory roles for members of the PKC superfamily and demonstrates a previously unrecognised role for members of the PKC superfamily, specifically PKCβ and PKCε in the negative regulation of ADP-induced platelet activation in human and mouse platelets. This data provides further evidence for differential regulatory roles for the individual PKC isoforms, demonstrating that members of the PKC superfamily act to negatively regulate several processes involved in platelet activation following stimulation by ADP. This is the first report of an inhibitory role for a classical PKC isoform in the process of platelet activation..

This observed potentiation and negative role for PKC in the regulation of platelet activation has further importance and ramifications outside of the understanding of platelet regulatory pathways. As members of the PKC superfamily are found to be key regulators in a variety of cell types and associated with several diseases (Gustafson

2003), PKC is an attractive target for a wide-range of therapies, including as a possible antithrombotic therapy. The potentiation of dense granule secretion and hence platelet aggregation downstream of low concentrations of ADP in the presence of PKC inhibition, therefore has implications for the safe use of PKC-targeted therapies.

CHAPTER 6

THE ROLE OF PKCθ IN PLATELET ACTIVATION AND THROMBUS FORMATION

6.1 INTRODUCTION

The novel isoforms of PKC are thought to play relatively minor stimulatory and inhibitory roles in the regulation of platelet activation. This includes an inhibitory role for PKCε in the regulation of ADP-induced activation of mouse platelets as shown in the previous Chapter (chapter 5). Quantification of the PKC isoforms in Chapter 3 identified PKCθ as the most highly expressed isoform in both human and mouse platelets, with copy numbers significantly higher than those of the other isoforms that are expressed in platelets, PKCα, β, δ and ε.

Understanding the contribution of PKCθ to the regulation of platelet activation is complicated as certain aspects such as its role in GPVI signalling are controversial as both positive and negative roles have been described when studying platelets from PKCθ-null mice (Soriani, Moran et al. 2006; Hall, Harper et al. 2008; Cohen, Braiman et al. 2009; Harper and Poole 2009; Nagy, Bhavaraju et al. 2009; Gilio, Harper et al. 2010; Cohen, Braiman et al. 2011). In the investigation of GPVI signalling, Hall et al (2008) reported that aggregation and secretion to CRP (1 and 5μg/ml) were not altered in PKCθ-null mice, whereas Nagy et al (2009) observed inhibition of aggregation to higher concentrations of CRP (10 and 20μg/ml) using mice from the same source and background. Unexpectedly, however, Harper and Poole (2009) later described potentiation of dense granule secretion in PKCθ-null mice using the same concentrations of CRP (1 and 5μg/ml) as in

the Hall et al (2008) study. Furthermore, Kunapuli and Jin then described potentiation of secretion to a low concentration of CRP (2µg/ml) in PKCθ-null platelets with inhibition at a higher concentration of CRP (10µg/ml). The use of different experimental conditions and different batches of CRP (each batch has a different activity) means that these studies are not directly comparable and it is possible that these varying factors account for the apparent differences in GPVI responses especially with the concentration-dependent response as reported by Kunapuli and Jin. As no obvious robust role for PKC has been identified in GPVI signalling, these studies might suggest that PKCθ has a relatively minor role in supporting platelet activation by CRP with the net effect being governed by the experimental conditions and CRP concentration.

Interestingly a positive role for PKCθ in aggregation and secretion in response to stimulation by thrombin and the PAR-4 peptide has been described (Cohen, Braiman et al. 2009; Nagy, Bhavaraju et al. 2009) whereas Soriani et al do not describe any role for PKCθ (Soriani, Moran et al. 2006). In contrast, there is general agreement that PKCθ is required for outside-in signalling through integrin αIIbβ3 as adhesion and filopodial extension on fibrinogen are reduced in PKCθ-deficient mice as described by two publications from different laboratories (Soriani, Moran et al. 2006; Hall, Harper et al. 2008).

The overall role of PKCθ in platelet aggregation at arterial shear is also controversial. Hall et al (2008) and Gilio et al (2010) have reported larger aggregates on collagen in PKCθ deficient mice under shear in contrast to WT controls although both publications come from the same laboratory. In contrast Nagy et al (2009), using a $FeCl_3$ injury model, reported reduced thrombus formation and increased time to occlusion in $PKC\theta^{-/-}$ mice in comparison to WT mice. This positive role for PKCθ is consistent with the observation of

Cohen et al (2009) who reported an increased bleeding time using a tail bleeding assay in PKCθ$^{-/-}$ mice relative to controls (Cohen, Braiman et al. 2009). On the other hand, Soriani et al (2006) did not observe an increase in the tail bleeding time in the PKCθ$^{-/-}$ mice. The various studies investigating PKCθ are summarised in Table 6.1.

In addition to studies using mouse platelets, isoform- specific inhibitors of PKCθ have been used in the study of human platelet activation by Gilio et al (2010) and in Chapter 4. As with the studies detailed using knock-out mouse models, unlike Gilio et al who identified a negative role for PKCθ in human platelet GPVI signalling, this was not identified in the data described in Chapter 4, where a PKCθ inhibitor identified relatively minor inhibitory effects on GPVI and PAR agonist induced platelet aggregation and dense granule secretion but did identify a significant role for PKCθ in the positive regulation of CLEC-2 signalling.

6.1.1 AIMS

In this chapter, the role of PKCθ in platelet activation to several platelet agonists and in haemostasis was investigated using PKCθ$^{-/-}$ mice (Sun, Arendt et al. 2000) using our standard experimental laboratory conditions. This is important for comparison with other experiments from our laboratory in light of the differences reported in the literature. In addition, the role of PKCθ$^{-/-}$ mice in platelet activation by CLEC-2 was examined given the inhibitory effect of the PKCθ inhibitor reported in Chapter 4.

PKCθ								
Publication:	**(Soriani, Moran et al. 2006)**	**(Hall, Harper et al. 2008)**	**(Nagy, Bhavaraju et al. 2009)**		**(Harper and Poole 2009**	**(Kunapuli and Jin) Response to (Harper and Poole 2009)**	**(Cohen, Braiman et al. 2009)**	**(Gilio, Harper et al. 2010)**
Mouse Background	C57BL/6J	C57BL/6J	C57BL/6J (from Jackson Labs)		C57BL/6J	C57BL/6J (from Jackson Labs)	C57BL/6 J and BALB/C	C57BL/6J
Platelet Prep:	Washed Platelets.	Washed platelets and *Whole Blood	Washed platelets		Washed platelets	Washed platelets	Whole blood	Whole blood
Anticoagulant	ACD	Heparin *Heparin/PPACK	Sodium citrate		Sodium citrate	Sodium citrate	Lactated ringers solution	PPACK/Heparin and fragmin mix.
Spins (if required)	90g for 10 mins then 16000g for 7 sec.	200g for 8 mins then, 1000g for 5 mins	100g for 10 mins then 400g for 10 mins		100g for 10 mins then 400g for 10 mins	100g for 10 mins then 400g for 10 mins then		
Treatment with:	No treatment	+ 10μM indomethacin	+0.1μM apyrase (aggregation and secretion also +/- 10μM indomethacin)		+ 10μM indomethacin, +0.1μM apyrase	+ 10μM indomethacin		
Agonists		**GPVI**	**GPVI**	**PAR**	**GPVI**	**GPVI**	**GPVI**	**GPVI**
Aggregation	-	No role (at 0.5, 1 and 5μg/ml CRP or 5 and 30μg/ml collagen)	Positive role (10μg/ml CRP and 10μg/ml collagen) No role (20μg/ml CRP or collagen)	Positive role(to 100μM AYPGKF and 0.1U/ml Thrombin, No role to 200μM AYPGKF and 0.2U/ml thrombin)	Negative	-	Positive role (0.1U/ml Thrombin)	-
Dense granule secretion.	-	No role (1 and 5μg/ml CRP, 5 and 30μg/ml Collagen)	Positive role (10μg/ml CRP and 10μg/ml collagen) No role (20μg/ml CRP or collagen)	Positive role (to 100μM AYPGKF and 0.1U/ml Thrombin, No role to 200μM AYPGKF or 0.2U/ml thrombin)	Negative role (at low concentrations of CRP, 1μg/ml and 5μg/ml) No role at high (10μg/ml)	Negative role at 2μg/ml No role at 5μg/ml CRP	-	-
Alpha granule secretion	-	Negative (1μg/ml and 5μg/ml CRP)	Positive role (to 10μg/ml CRP)	Positive role (to 100μM AYPGKF)	-	-	Positive (0.1U/ml Thrombin)	Negative under flow (100μg/ml collagen)
Inside-out signalling (αIIbβ3 activation)	No role	Negative role to 1μg/ml CRP and no role at 5ug/ml CRP	Positive role (to 10μg/ml CRP)	Positive role (to 100μM AYPGKF)	-	-	-	-

Outside-in signalling (Spreading on fibrinogen)	Positive role on (100ug/ml fib)	Small positive role, adhesion and degree of filopodia generation	-		-	-	-	-
Spreading on collagen	No role	No role in spreading on CRP or collagen	-		-	-	-	-
Filopodia development	Positive (on fibrinogen)	Positive (fibrinogen)	-		-	-	-	-
Thromboxane A2 formation	-	-	Positive (10µg/ml CRP and collagen)	Positive role (100µM AYPGFK and 0.05U/ml Thr)	-	-	-	
Intracellular Ca^{2+}	-	-	-	-	-	-	-	Negative under flow (100µg/ml collagen)
Coagulation (PS exposure on collagen under flow)	-	-	-		-	-	-	Negative under flow (100µg/ml collagen)
Thrombus formation on collagen under flow *in vitro*	-	Negative as measured using surface area coverage.	-		-	-	-	Negative
Thrombus formation in vivo (foecal occlusion)	-	-	Positive role (only just), KO showed prolonger occlusion time and failure to form stable thrombi.		-	-	-	-
Thrombus formation in vivo (Tail Bleeding)	No bleeding phenotype (data not shown and method unknown)	-	-		-	-	Positive role, prolonged bleeding observed following removal of 5mm tip then immersed in saline.	-

Table 6.1 The role of PKCθ in platelet activation. Comparison of the published data for PKCθ in platelet activation and haemostasis using PKCθ-/- mice.

6.2 RESULTS

6.2.1 Expression of the PKC isoforms in PKCθ$^{-/-}$ mice

Mice deficient in PKCθ (Sun, Arendt et al. 2000) were indistinguishable from littermate WT controls for up to 30 weeks and had similar platelet count and size (data not shown). The expression of the major PKC isoforms, α, β, δ and ε was similar in the PKCθ$^{-/-}$ platelets to controls and no PKCθ was detected in PKCθ$^{-/-}$ platelets (Figure 6.1).

6.2.2 Functional analysis of PKCθ$^{-/-}$ mouse platelets.

6.2.2.1 The role of PKCθ in GPVI induced platelet activation.

Aggregation, dense and α-granule secretion were monitored in platelets resuspended in a modified Tyrodes-HEPES buffer in response to concentrations of CRP that gave submaximal (0.3μg/ml) and maximal (1μg/ml) aggregation. There was no significant difference in aggregation or secretion to CRP between the PKCθ$^{-/-}$ mice and WT littermate controls CRP (Figure 6.2). There was also no significant difference in aggregation or secretion in response to low and intermediate concentrations of collagen (3 and 10μg/ml) as shown in Figure 6.3. These results therefore suggest that PKCθ plays a relatively minor role in GPVI signalling.

6.2.2.2 The role of PKCθ in thrombin induced platelet activation.

The role of PKCθ in the response to thrombin was also investigated. As with GPVI signalling, no significant difference was observed in the rate and extent of platelet aggregation and granule secretion in PKCθ$^{-/-}$ mouse washed platelets in comparison to WT controls following stimulation by thrombin (Figure 6.4).

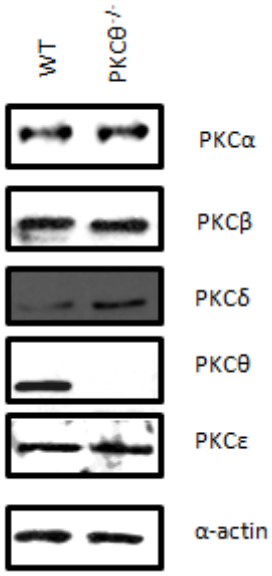


Figure 6.1. PKC isoform expression in PKCθ$^{-/-}$ mouse platelets. Equal amounts of washed platelets from WT and PKCθ$^{-/-}$ were separated by SDS-PAGE and the expression levels of PKCα, β, δ, θ and ε determined by western blot using anti-sera specific to the individual isoforms of PKC. Actin was used as a loading control.

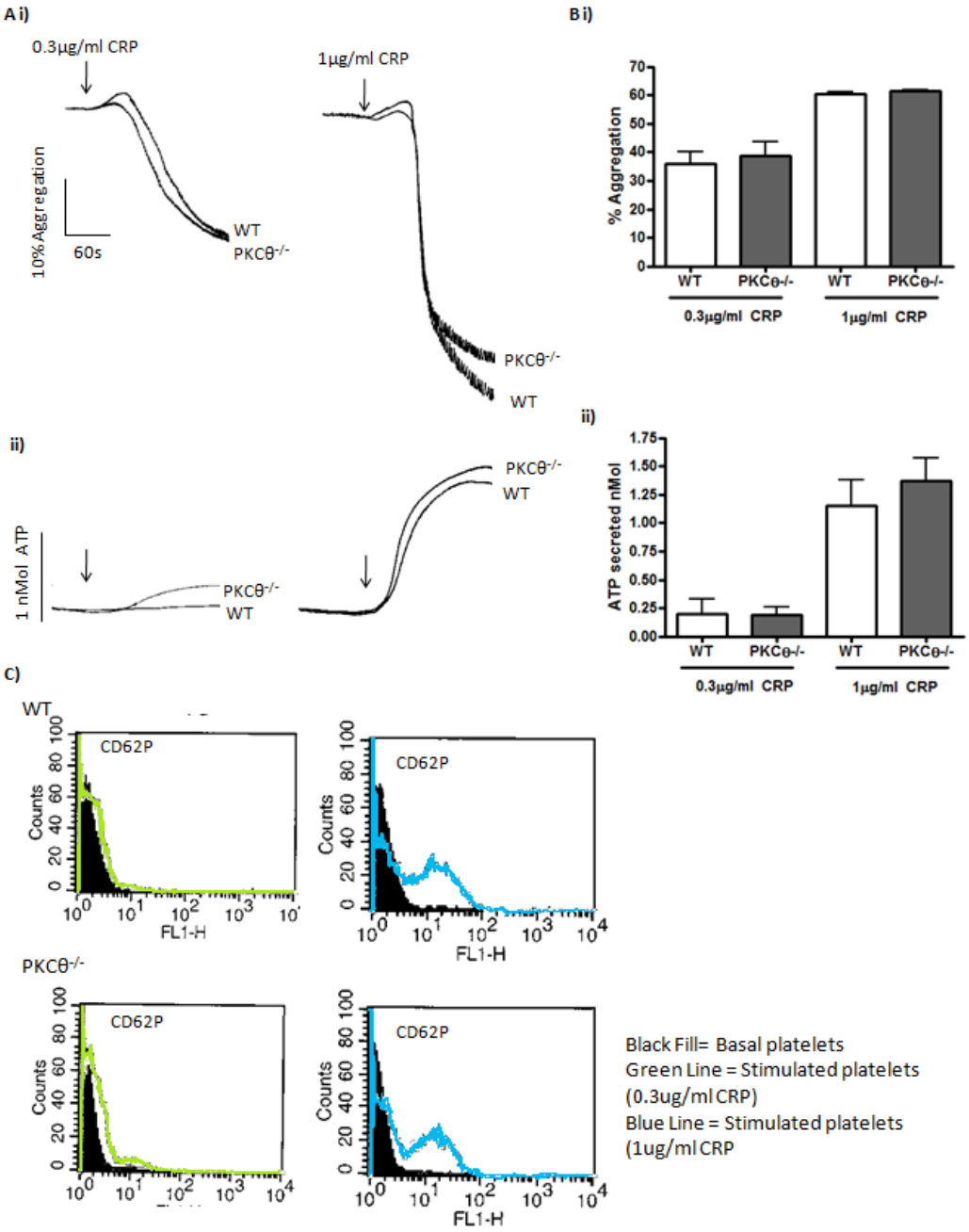


Figure 6.2. The role of PKCθ in GPVI induced platelet activation. Mouse washed platelets from PKCθ$^{-/-}$ mice or wild-type littermate controls were stimulated with 0.3 or 1µg/ml CRP and aggregation and alpha and dense granule secretion were monitored. (i) Aggregation was measured by optical aggregometry. (ii) Dense granule secretion was measured by monitoring ATP secretion using luminometry. (A) Traces representative of n=3 are shown. (B) Results are average + S.E.M. for n=3. * indicates $p<0.05$ in comparison to controls. (C) Representative traces of flow cytometric analysis of P-selectin surface exposure, n=3. Black fill = basal platelets, green line = platelets stimulated with 0.3µg/ml, blue line = platelets stimulated with 1µg/ml.

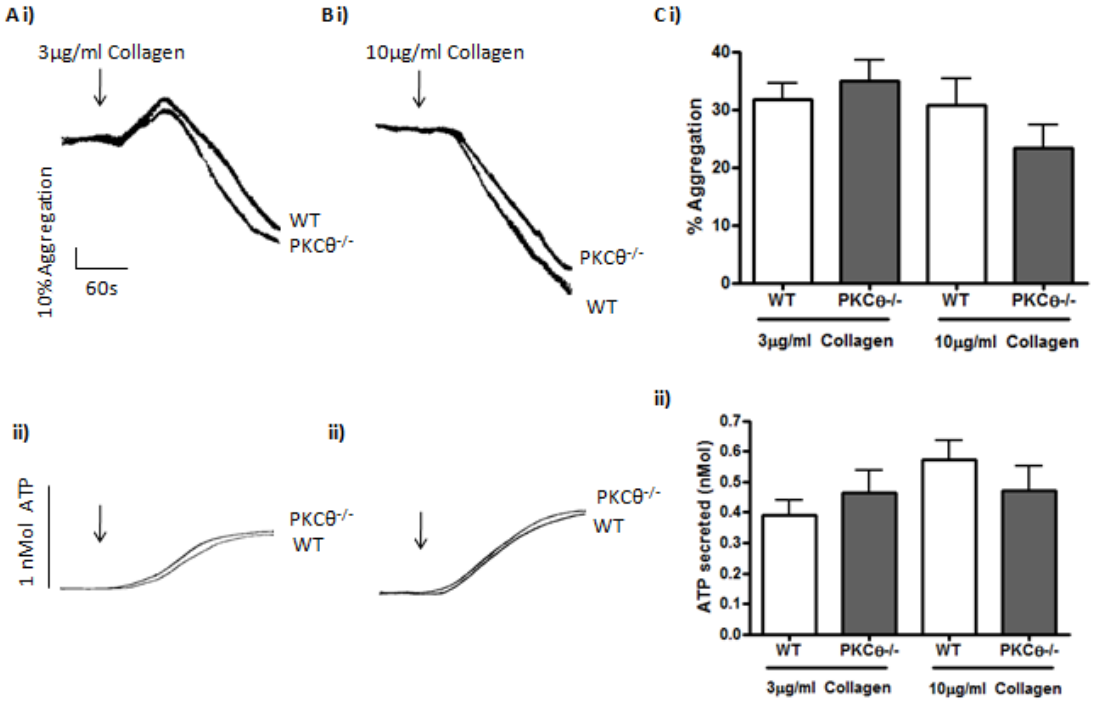


Figure 6.3. The role of PKCθ in collagen induced platelet activation. Mouse washed platelets from PKCθ$^{-/-}$ mice or wild-type littermate controls were stimulated with A) 3 or B) 10µg/ml Collagen and aggregation and dense granule secretion were monitored. (i) Aggregation was measured by optical aggregometry. (ii) Dense granule secretion was measured by monitoring ATP secretion using luminometry. (A and B) Traces representative of n=3 are shown. (C) Results are average + S.E.M. for n=3. No data was significantly different from control $p<0.05$.

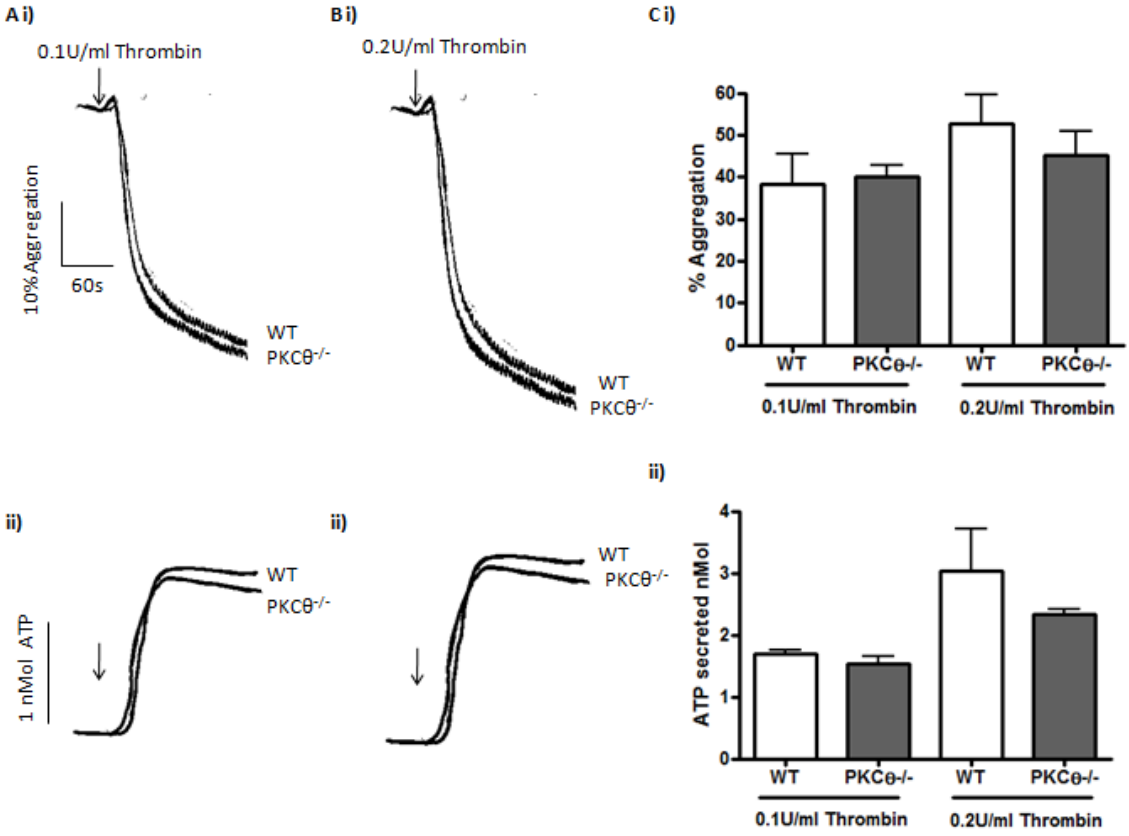


Figure 6.4. The role of PKCθ in PAR induced platelet activation. Mouse washed platelets from PKCθ$^{-/-}$ mice or wild-type littermate controls were stimulated with (A) 0.1 or (B) 0.2U/ml Thrombin and aggregation and dense granule secretion were monitored. (i) Aggregation was measured by optical aggregometry. (ii) Dense granule secretion was measured by monitoring ATP secretion using luminometry. (A and B) Traces representative of n=3 are shown. (C) Results are average + S.E.M. for n=3. No data was significantly different from control $p<0.05$.

6.2.2.3 The role of PKCθ in CLEC-2 induced platelet activation.

A role for PKCθ in the positive regulation of platelet activation by CLEC-2 was reported in Chapter 4 using a PKCθ-specific inhibitor in human platelets. In the present Chapter, the role of the novel PKC isoform was further investigated using PKCθ-deficient mouse platelets following stimulation using a CLEC-2 specific antibody and the exogenous CLEC-2 receptor agonist, the snake venom toxin rhodocytin. Unexpectedly, given the results with the PKCθ inhibitor in human platelets, there was no difference in aggregation or dense granule secretion between the PKCθ$^{-/-}$ and WT littermate controls following stimulation with either the CLEC-2 antibody (3 and 10μg/ml) or rhodocytin (30 nM) as shown in Figure 6.5.

In view of this apparent discrepancy, the effect of the PKCθ inhibitor was investigated in both WT control and PKCθ$^{-/-}$ mouse platelets, as the PKCθ$^{-/-}$-deficient platelets provide a system to investigate any actions of the inhibitor not due to inhibition of PKCθ. In WT mouse platelets the PKCθ inhibitor caused a reduction in both aggregation and secretion to rhodocytin although the extent of inhibition was notably smaller than that observed in human platelets (Figure 6.6A) which indicates a species difference between mouse and human platelet responses to rhodocytin. Use of PKCθ$^{-/-}$ platelets also enables the specificity of the PKCθ inhibitor to be determined. As in human and WT mouse platelets, the PKCθ inhibitor induced a dose-dependent partial reduction in dense granule secretion and small inhibition of aggregation to rhodocytin in PKCθ$^{-/-}$-deficient platelets with increasing concentrations of the inhibitor (Figure 6.6B). However no difference in either process was observed at low concentrations (0.1μM) of the inhibitor. These results suggest that at higher concentrations the PKCθ inhibitor is acting non-specifically, as no difference is observed in rhodocytin induced platelet activation between WT and

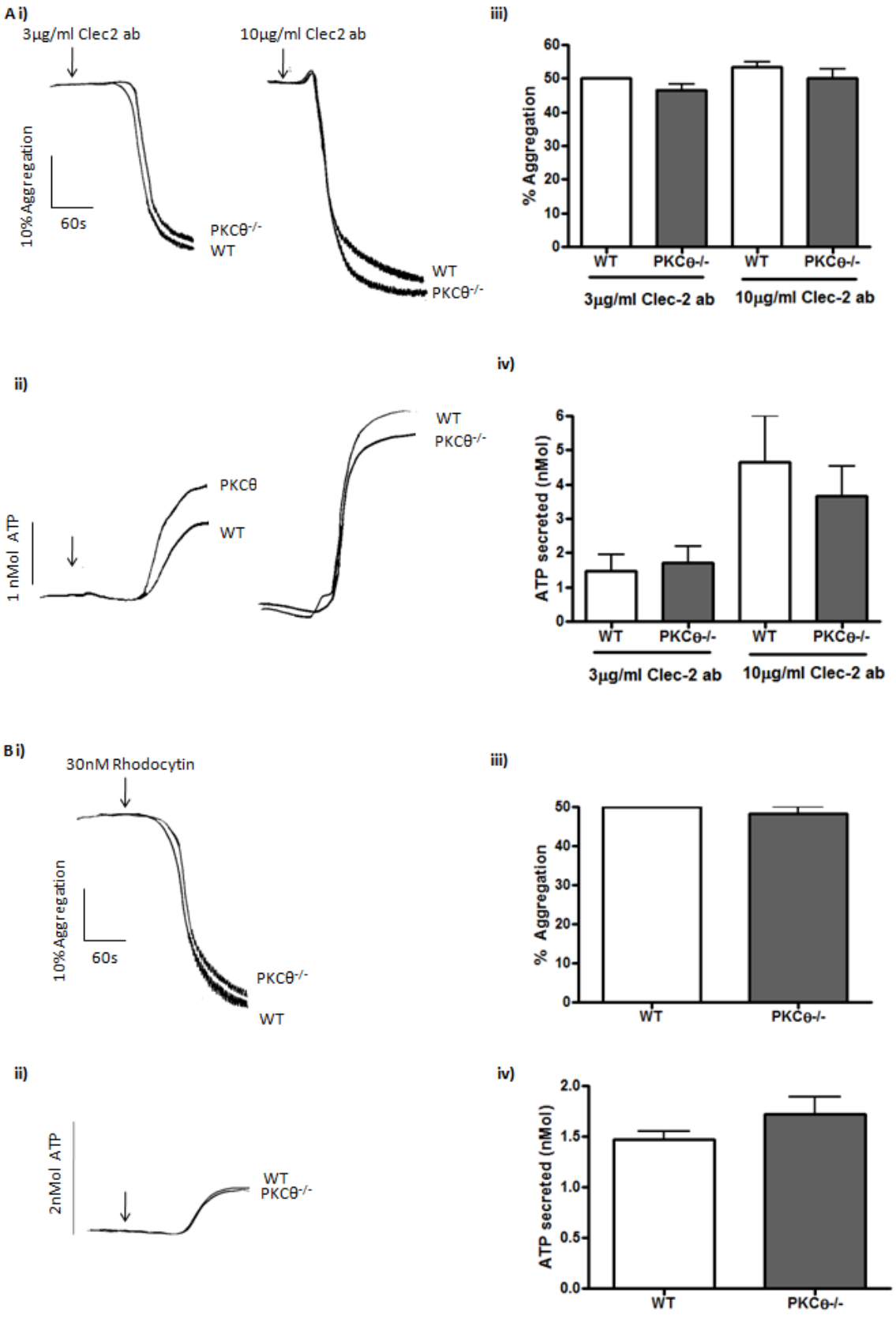


Figure 6.5. The role of PKCθ in CLEC-2 induced platelet activation. Mouse washed platelets from PKCθ$^{-/-}$ mice or wild-type littermate controls were stimulated with (A) 3 or 10µg/ml CLEC-2 antibody or (B) 30nM Rhodocytin and aggregation and dense granule

secretion were monitored. (i) Aggregation was measured by optical aggregometry. (ii) Dense granule secretion was measured by monitoring ATP secretion using luminometry. traces representative of n=3 are shown. (iii and iv) Results are average + S.E.M. for n=3. No data was significantly different from control $p<0.05$..

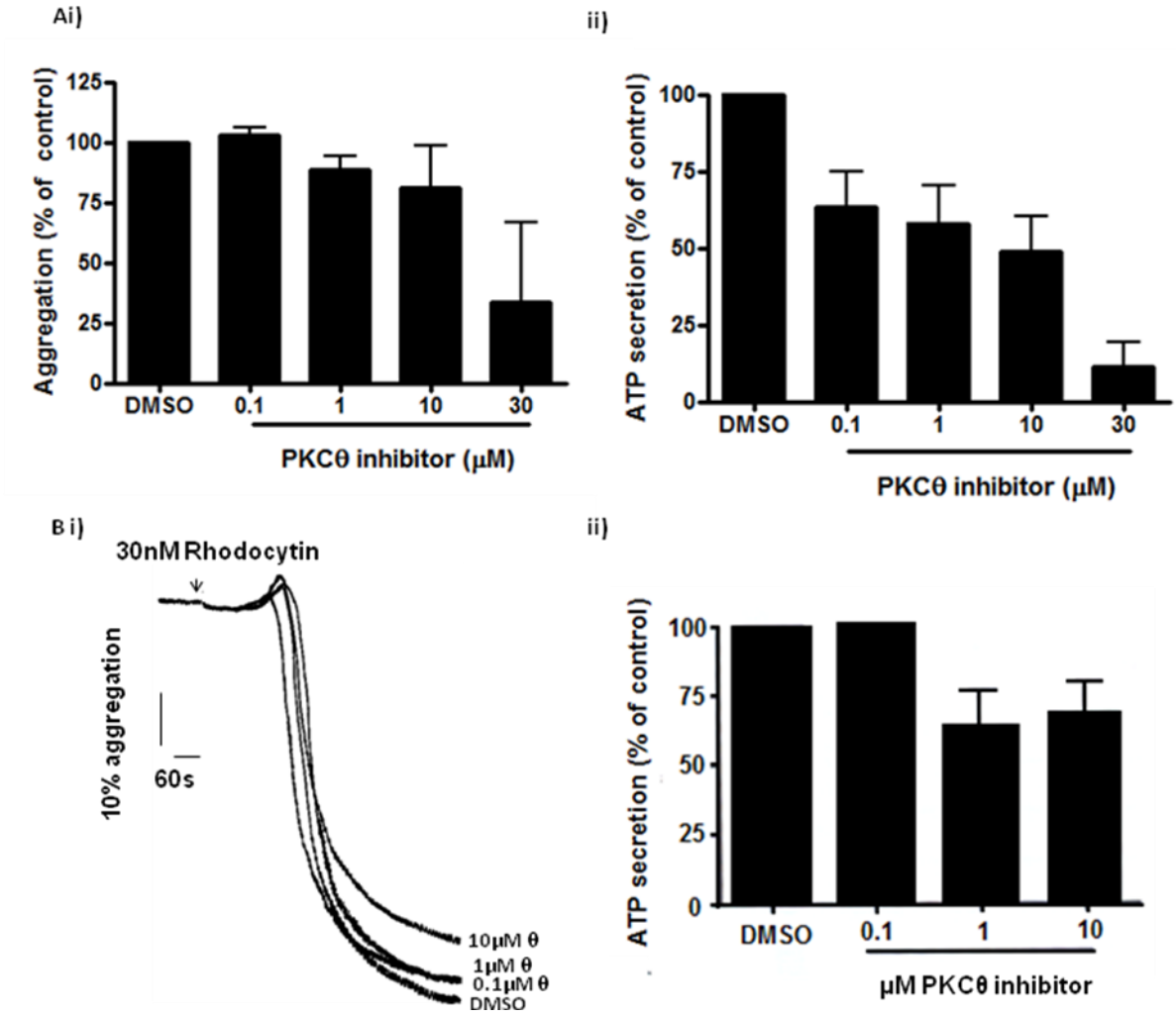


Figure 6.6. Effect of the PKCθ inhibitor on PKCθ$^{-/-}$ mouse platelet activation. Washed mouse platelets from WT and PKCθ$^{-/-}$ mice were pretreated with varying concentrations of the PKCθ inhibitor and stimulated with A) and B) 30nM rhodocytin and aggregation and dense granule secretion were monitored. A) Dose response curve for WT mouse platelets showing i) aggregation and ii) dense granule secretion expressed as percentages of the untreated control condition following treatment with increasing concentrations of PKCθ inhibitor (0.1, 1, 10, 30μM). B) PKCθ$^{-/-}$ mouse platelets i) representative traces of the effect of inhibitors on aggregation (optical light transmission) and ii) dense granule secretion expressed as percentages of the untreated control condition, mean ± SEM. ($n \geq 2$ due to limited mouse availability).

PKCθ$^{-/-}$deficient platelets. However, as 0.1μM of the PKCθ inhibitor appears to have no effect on rhodocytin-induced platelet activation in PKCθ$^{-/-}$ platelets, it would appear that at this concentration the inhibitor may be acting specifically.

However, due to the inter-species differences observed downstream of rhodocytin induced signalling, rhodocytin may not be the best agonist in which to determine whether or not the inhibitor is working specifically. Preliminary studies (n≥2) using the inhibitor looking at the effect on collagen (5μg/ml) induced mouse platelet responses identified a very similar dose response curve for the inhibitor when comparing responses from WT mouse platelets to human platelets (Figure 6.7A). Analysis of the inhibitor dose response curve in PKCθ$^{-/-}$ deficient mouse platelets downstream of collagen (figure 6.7B) also identified that at low inhibitor concentrations (0.1μM), the PKCθ inhibitor may be acting specifically as there is a greater effect on WT compared to PKCθ$^{-/-}$ null platelets following stimulation by both collagen and rhodocytin.

6.2.2.4 The role of PKCθ in spreading on fibrinogen.

The ability of PKCθ$^{-/-}$ mouse washed platelets to perform outside-in signalling via αIIbβ3 was determined. Washed platelets from either PKCθ$^{-/-}$ or WT mice were exposed to fibrinogen coated coverslips (100μg/ml) and left to adhere, unattached platelets were then washed away and the coverslips fixed for analysis (Figure 6.8). In support of previously published data, platelets from PKCθ deficient mice platelets showed a significant reduction in adhesion to and therefore surface area coverage of the fibrinogen coated coverslips in comparison to WT control platelets. Additionally filopodia generation (rather than lamellipodia) also appears reduced in PKCθ$^{-/-}$ platelets in support of Hall et al (2008). These results support a positive regulatory role for PKCθ

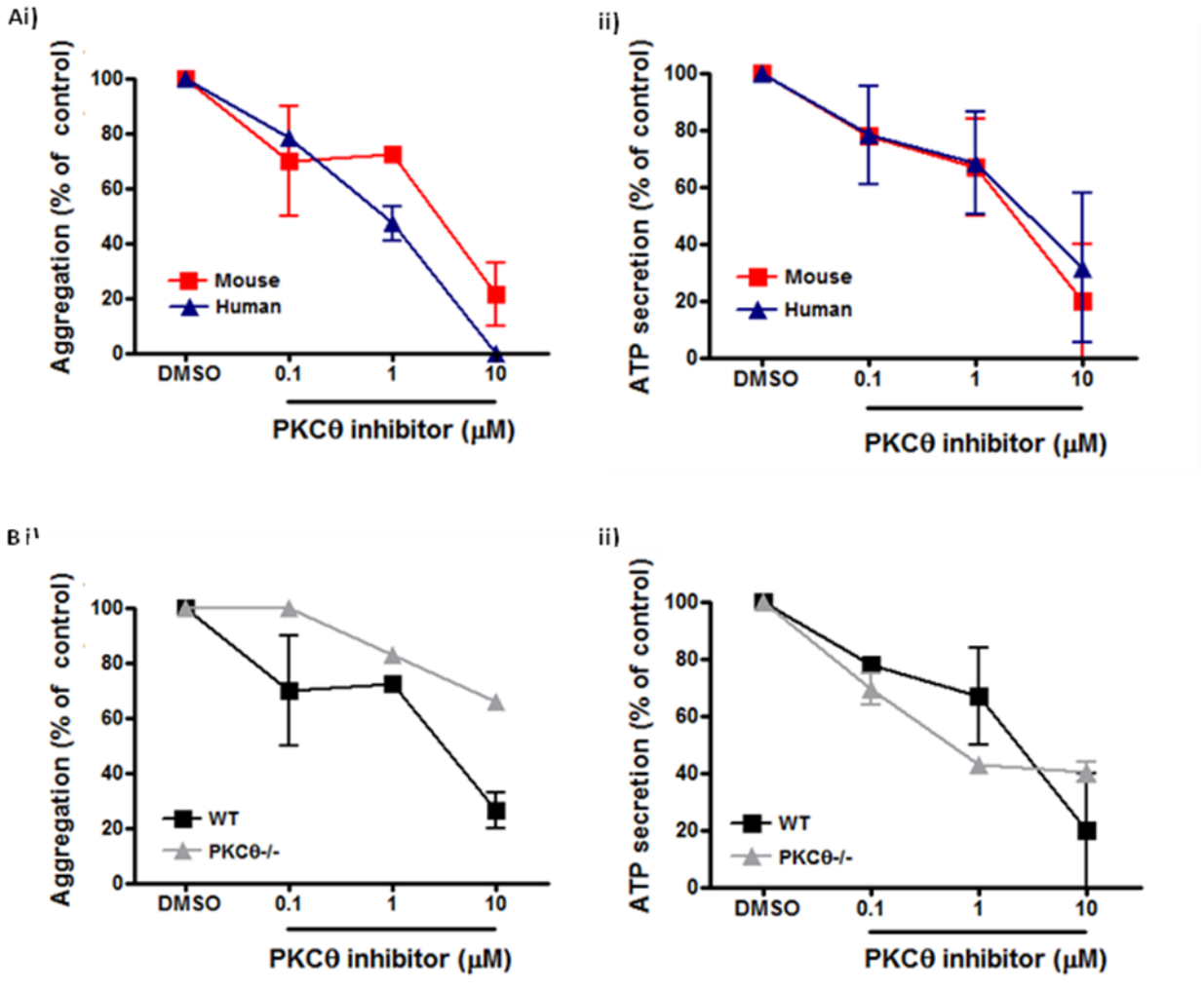


Figure 6.7. Effect of the PKCθ inhibitor on human and WT and PKCθ$^{-/-}$ mouse collagen induced platelet activation. Washed human and mouse WT and PKCθ$^{-/-}$ platelets were pretreated with varying concentrations of the PKCθ inhibitor and stimulated with 5μg/ml collagen and (i) aggregation and (ii) dense granule secretion were monitored. A) Dose response curve for WT mouse platelets (red) compared to human platelets (blue). B) Dose response curve for PKCθ$^{-/-}$ (grey) compared to WT mouse platelets (black). Results expressed as percentages of the untreated control condition following treatment with increasing concentrations of PKCθ inhibitor (0.1, 1, 10, 30μM). mean ± SEM. ($n \leq 2$ due to limited mouse availability).

in outside-in signalling, filopodial generation and adhesion to fibrinogen.

6.2.3 PKCθ in thrombus formation *in vitro* and *in vivo.*

It has been previously published by Hall et al (2008) and Gilio et al (2010) that lack of PKCθ in mouse platelets causes an increase in the formation of aggregates and number of larger stable thrombi formed on collagen under high arteriolar shear rates *in vitro*. In contrast, here, analysis of aggregate formation on a collagen coated surface under flow in both WT and PKCθ$^{-/-}$ mouse whole blood, showed similar formation of stable aggregates on collagen with a surface area coverage of approximately 27% in the presence and absence of PKCθ (Figure 6.9A). This *in vitro* phenotype, further correlates to a normal bleeding phenotype in support of that observed by Soriani et al, as no difference in the amount and rate of blood loss is observed during a tail bleeding assay when comparing PKCθ$^{-/-}$ to WT mice (Figure 6.9B) .

6.3 DISCUSSION

The study presented in this chapter, investigating the role for PKCθ in platelet activation in mice, was only able to identify a robust phenotype for the isoform in the positive regulation of outside-in signalling and filopodial generation downstream of αIIbβ3 in support of observations made by Soriani et al (2006). Interestingly, under the experimental conditions used (as detailed in the methods section, chapter 2), no differences in the rate or extent of aggregation and dense granule secretion downstream of several platelet agonists, including GPVI, CLEC-2 and PAR receptor agonists was observed when comparing platelets from PKCθ$^{-/-}$ mice with WT controls

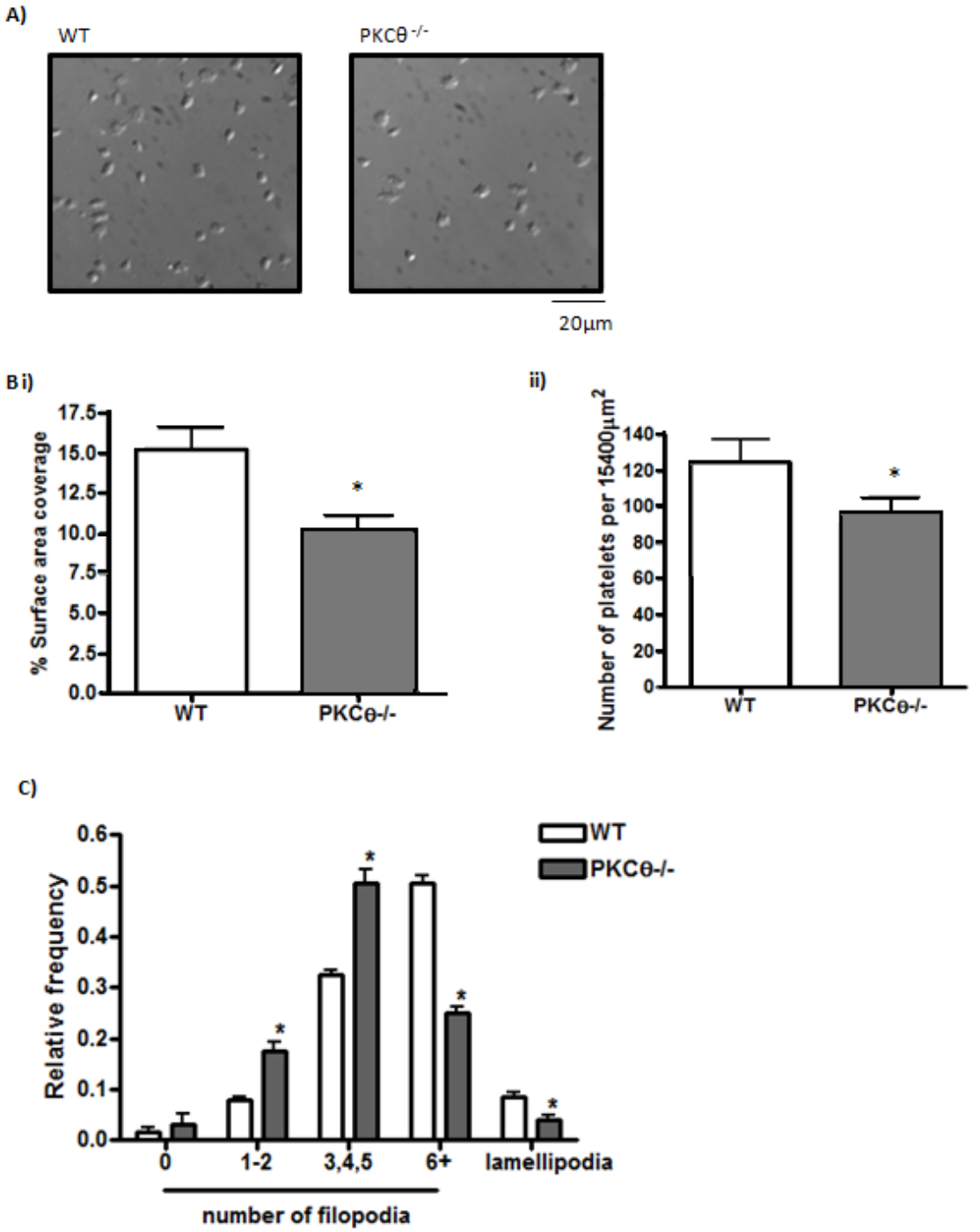


Figure 6.8. Defect in fibrinogen spreading in PKCθ$^{-/-}$ mouse platelets. Washed platelets from WT and PKCθ$^{-/-}$ mice were exposed to fibrinogen coated coverslips. A) Representative phase-contrast images after 4 min. Images were taken under oil immersion. Original magnification, ×63. B) Image J analysis on 3 separate images per mouse i) Surface area coverage, presented as a percentage. ii) Number of platelets adhered per mm^2 C) Filopodia number was counted for each visible platelet and the number of platelets with none, few (1–2), some (3–5) or many (6+) filopodia were expressed as relative frequency (proportion of the total number of platelets in view). Results are mean + S.E.M. for n=3, * indicates $p<0.05$ in comparison to controls.

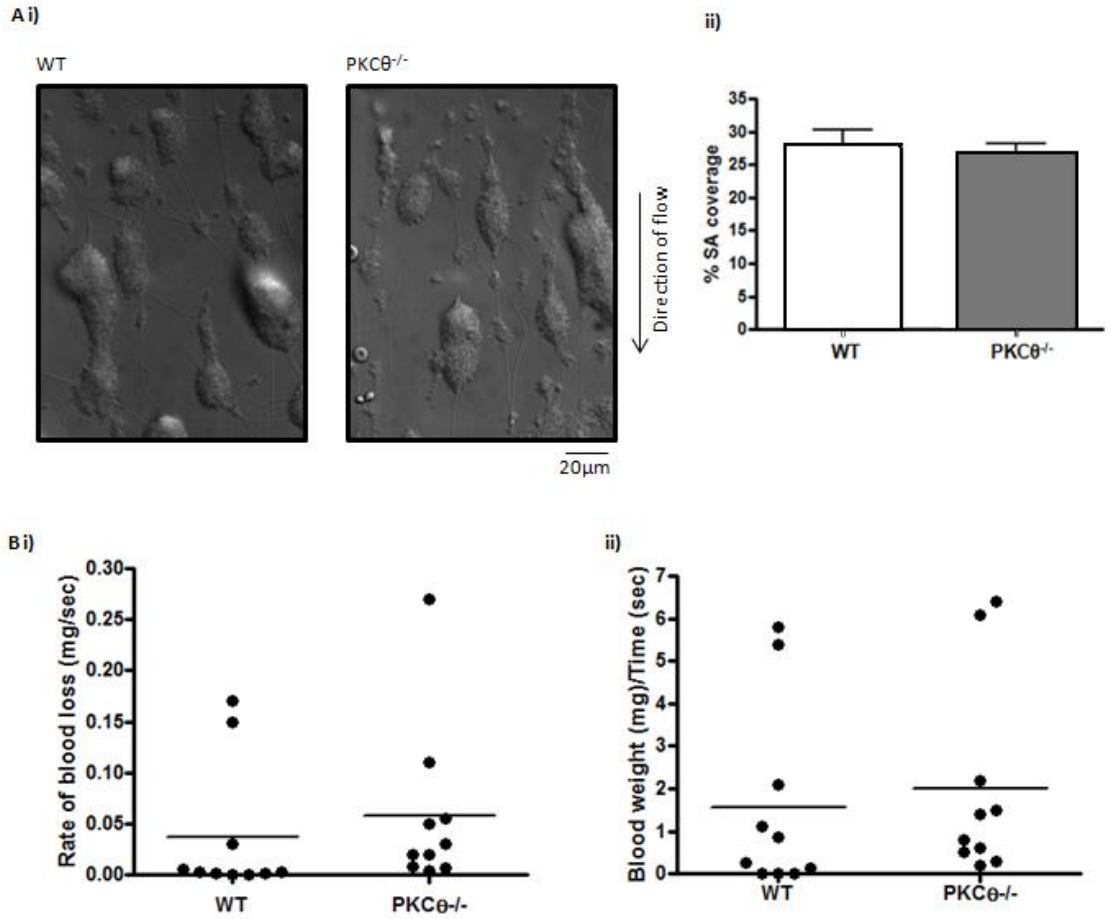


Figure 6.9. Thrombus formation in PKCθ$^{-/-}$ mice. (A) Heparin/PPACK-anticoagulated blood from WT or PKCθ$^{-/-}$ mice was passed over collagen (shear rate 1000 s^{-1}). (i) Representative phase-contrast images after 4 min. Images were taken under oil immersion. Original magnification, ×63. (ii) Surface area coverage with thrombi, calculated using 3 separate images per mouse, mean ± SEM. (B) Thrombus formation and haemostasis monitored *in vivo* using a tail bleeding assay. Tail bleeding was monitored following removal of the terminal 3mm of the mouse tail. Dot plots show (i) tail bleeding expressed as weight of blood lost in 20 min as a ratio of total mouse weight and (ii) tail bleeding expressed as rate of blood lost, (n = 10). Means are represented by the horizontal lines. This work was carried out with (A) S. Severin and (B) B. Finney.

which further extrapolates to a lack of a role for the isoform in aggregate formation on collagen under arterial flow rates and lack of a bleeding phenotype.

The apparent lack of robust phenotype in PKCθ$^{-/-}$ platelets downstream of GPVI and PAR receptor signalling is in contrast to other observations in the literature. As previously described there is current debate over the role for PKCθ in GPVI induced platelet activation, although as a middle ground there is a general consensus that PKCθ plays a negative role at lower concentrations of CRP (1-2µg/ml) and a positive role at higher concentrations (10µg/ml) (Harper and Poole 2009; Kunapuli and Jin 2009; Harper and Poole 2010). However, the data presented in this chapter, in addition to other publications (Soriani, Moran et al. 2006; Hall, Harper et al. 2008), challenges this idea. It is possible that these differences may be due to the range of different experimental conditions that are used hinting towards a non-robust and relatively minor role for PKCθ. This is further supported by the observation of no difference in the formation of aggregates on collagen phenotype under *in vitro* flow conditions in PKCθ deficient mouse platelets compared to WT. Previously published work from Nagy et al and Cohen et al, have highlighted a positive role for PKCθ in the regulation of platelet aggregation and dense granule secretion, although Soriani et al did observe no role for PKCθ in PAR receptor induced inside-out signalling. Similar to the observations observed downstream of GPVI signalling, no role was identified for PKCθ in the regulation of thrombin induced platelet aggregation and dense granule secretion. These differences could indicate a relatively minor role for PKCθ in PAR receptor signalling that is not robust.

Despite only identifying a positive role for PKCθ in the regulation of outside-in signalling, minor roles for the isoform in other platelet processes, could not be ruled out and so the effects of loss of PKCθ on thrombus formation *in vivo*, was determined by monitoring tail

bleeding times. Previous studies, using several different methods, have failed to reach a general conclusion as to the role for PKCθ in thrombus formation as both positive and no regulatory roles have been identified (Soriani, Moran et al. 2006; Cohen, Braiman et al. 2009; Nagy, Bhavaraju et al. 2009). In support of Soriani et al, the studies presented in this chapter observed no bleeding phenotype in PKCθ deficient mice in comparison to WT controls. This is further supported by the lack of observation for a major role for PKCθ in several platelet receptor signalling pathways, with the exception of αIIbβ3 signalling (also observed by Soriani et al, 2006) and the lack of effect on the formation of aggregates under flow *in vitro*.

Normal bleeding and aggregate formation in PKCθ$^{-/-}$ platelets occurs despite the reduction in outside-in signalling which is likely as PKCθ$^{-/-}$ platelets appear to retain some αIIbβ3 outside-in signalling which is likely to be enhanced by other mechanisms, compensating for the reduction in platelet-platelet contacts and enabling normal thrombus formation and haemostasis. As PKCθ$^{-/-}$ platelets do not appear to have altered GPVI signalling they are likely to be capable to integrin activation via inside-out signalling leading to stable adhesion of platelets through binding of α2β1 and vWF ensuring thrombus formation is maintained despite the lack of outside-in signalling (Watson, Auger et al. 2005).

Despite their advantages, the use of mouse models in the study and understanding of human proteins and cell function can have limitations, especially when taking into consideration the fundamental biological differences between the two species. In the study presented here, the results produced following analysis of platelet aggregation and dense granule secretion in platelets from PKCθ$^{-/-}$ mice and WT mice provided similar results as those achieved using a PKCθ inhibitor in human platelets (see Chapter 4), in

that no major roles were identified for the isoform in the processes of platelet aggregation and dense granule secretion downstream of GPVI or PAR receptor stimulation. Interestingly a positive role for PKCθ was identified using the PKCθ inhibitor in rhodocytin-induced platelet signalling in human platelets. Unexpectedly however, no difference in aggregation or dense granule secretion was observed in PKCθ$^{-/-}$ mouse platelets following stimulation by either 30nM rhodocytin or a specific CLEC-2 antibody in comparison to WT mouse platelets. Interestingly, increasing concentrations of the PKCθ inhibitor caused a dose dependent reduction in both aggregation and secretion in WT mouse platelets downstream of rhodocytin (30nM) although not to the extent seen in human platelets (Chapter 4 Figures 4.11 and 4.12). Taken together, these findings highlight inter-species differences in the regulation of rhodocytin-induced platelet activation between human and mouse platelets. The difference between human and mouse platelets has several possible explanations. The first is that the observation made in human platelets is due to a non-specific effect of the PKCθ inhibitor and is therefore not paralleled in PKCθ deficient mouse platelets. Alternatively, there is also the possibility that in mouse platelets the role normally taken by PKCθ in human platelets is taken over by another PKC isoform such as PKCε which is expressed in mouse but not detectable in human platelets. Studies using PKCε$^{-/-}$ mice have identified a positive role for the isoform in GPVI and a negative role in ADP signalling, functions that must be performed by another isoform in human platelets (Pears, Thornber et al. 2008; Unsworth, Smith et al. 2011). A caveat to all of this, however, is the assumption that the PKCθ inhibitor will interact with the human and mouse PKCθ forms with similar affinity. Given the similarity between the two proteins (≥95% homology) it is unlikely that the two proteins would interact differently with the inhibitor but it cannot be ruled out.

Study of the PKCθ inhibitor in PKCθ null mice enables the specificity of the PKCθ inhibitor to be determined. These studies revealed that both aggregation and dense granule secretion were inhibited with increasing concentrations of the inhibitor downstream of rhodocytin and collagen, suggesting non-specific, PKCθ independent effects at higher inhibitor concentrations. However, studies using PKCθ$^{-/-}$ platelets identified no effect on rhodocytin or collagen-induced platelet responses in the presence of 0.1μM of the PKCθ inhibitor, suggesting at this concentration the inhibitor is acting specifically.

Studies using mouse models and isoform-specific inhibitors in human platelets have identified robust and dominant positive roles for the classical isoforms of PKC in several processes involved in platelet activation and thrombus formation. In comparison the data presented here identifies that it is likely that the novel isoform PKCθ plays a relatively minor role in the regulation of several processes involved in platelet activation and thrombus formation in mouse platelets, a trait which is somewhat unexpected given its high expression levels. The results presented here suggests that with the exception of a positive regulatory role in αIIbβ3 outside-in signalling, and adhesion and filopodial generation and extension on fibrinogen, there is either no other major role for PKCθ in the regulation of platelet activation and thrombus formation and/or redundancy exists between PKCθ and another of the PKC isoforms expressed in mouse platelets.

CHAPTER 7

THE INTERACTION BETWEEN PKCθ AND PKCε IN MOUSE PLATELETS.

7.1 INTRODUCTION

As shown in the previous Chapter, PKCθ plays a relatively minor role in supporting platelet activation notably downstream of integrin αIIbβ3. In comparison, the novel isoform PKCε has also been shown to have small roles in supporting activation of mouse platelets by collagen through phosphorylation of the FcR γ-chain and increased binding and activation of the tyrosine kinase Syk (Pears, Thornber et al. 2008) and also in the negative regulation of platelet activation by ADP (as detailed in Chapter 5) (Unsworth, Smith et al. 2011).

One explanation for these relatively minor roles for the two novel isoforms in regulating platelet activation is that functional redundancy exists between the two in mouse platelets which not only accounts for their relatively minor roles but also the lack of requirement for PKCε in human platelets. To address this, as both PKCε deficient and PKCθ deficient mice were available, mice that were double-deficient in PKCε and PKCθ were generated and characterised. Mice deficient in multiple classical isoforms of PKC have been previously reported (Liu, Chen et al. 2009) although platelet function was not investigated. This is however, first report of mice lacking two or more of the novel isoforms of PKC.

7.1.1 AIMS

These studies were performed in order to determine whether any redundant functions existed between the two novel isoforms PKCθ and PKCε in mouse platelet activation and haemostasis and whether loss of both isoforms resulted in a more major phenotype than those observed in mice deficient in just PKCθ or PKCε. The processes involved in platelet activation, platelet aggregation, dense granule secretion, and spreading on fibrinogen were characterised, in addition to haemostasis and thrombosis in the $PKC\theta^{-/-}/\varepsilon^{-/-}$ mice in comparison to WT and $PKC\theta^{-/-}$ control mouse platelets.

7.2 RESULTS

$PKC\varepsilon^{-/-}$ male mice are sterile whereas $PKC\theta^{-/-}$ mice can be bred as homozygotes. Further, the viability of $PKC\varepsilon^{-/-}$ is far below that expected by Mendelian genetics (K. Thornber personal communication). In consideration of this, $PKC\theta^{-/-}/\varepsilon^{-/-}$ mice were therefore generated by first generating $PKC\theta^{-/-}/\varepsilon^{+/-}$ mice from available $PKC\theta^{-/-}$ (purchased from Jackson Labs) and $PKC\varepsilon^{+/-}$ mice (Castrillo, Pennington et al. 2001) and breeding male and females with this genotype (Figure 7.1). As with $PKC\varepsilon^{-/-}$ mice, the number of $PKC\theta^{-/-}/\varepsilon^{-/-}$ mice born was significantly lower than the expected Mendelian genetics (approximately 1/20). This breeding plan does not lead to ($PKC\theta^{+/+}/\varepsilon^{+/+}$) littermate controls, but $PKC\theta^{-/-}/\varepsilon^{+/+}$ ($PKC\theta^{-/-}$) littermate mice and C57/BL6 age-matched WT mice were used as controls when investigating platelet responses, in some experiments $PKC\varepsilon^{-/-}$ mice are included for comparison, but these mice were unfortunately not litter or age-matched, although they were from the same colony that the double-deficient mice were bred from.

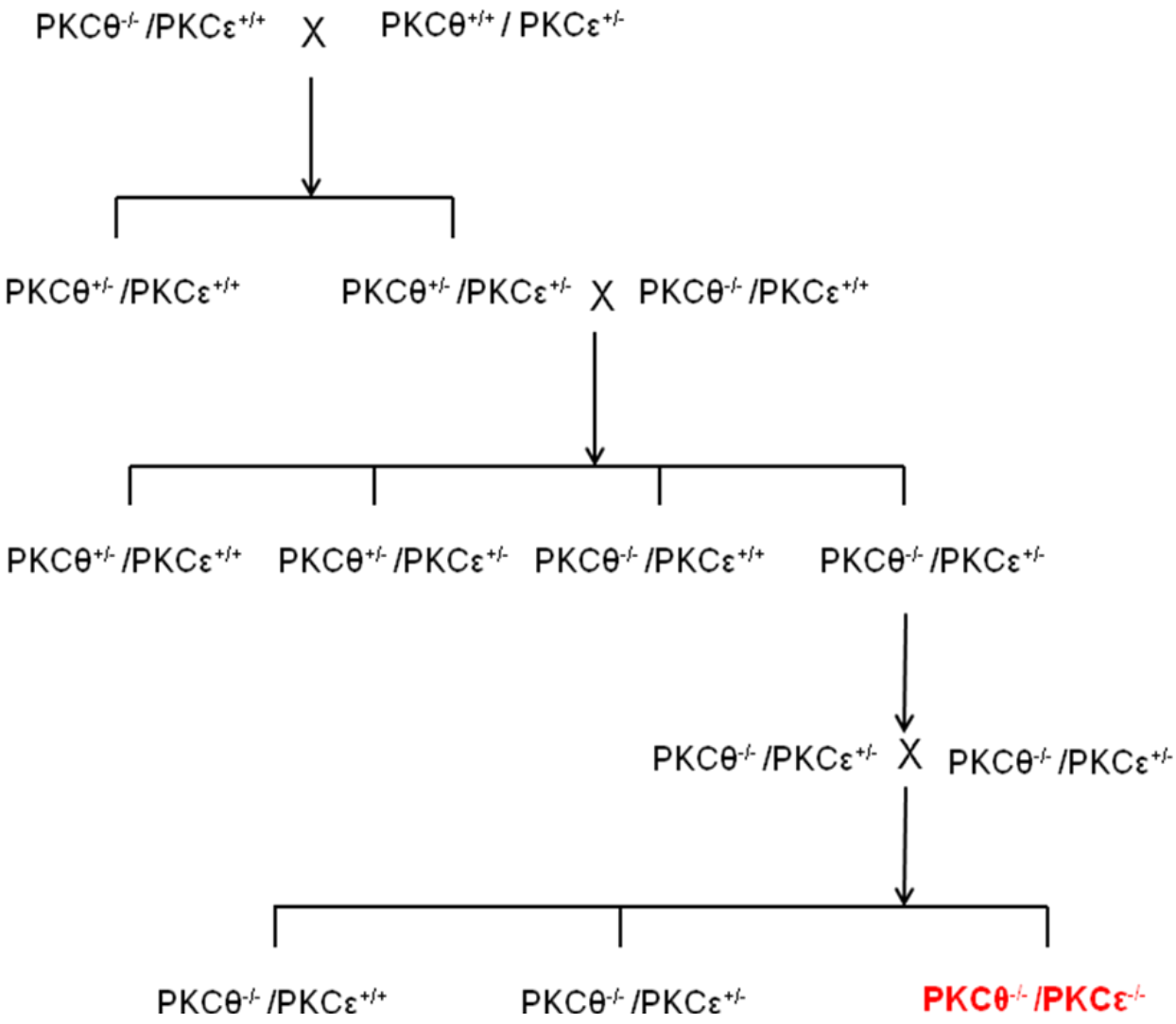


Figure 7.1. Breeding of PKCθ$^{-/-}$/ε$^{-/-}$ mice

7.2.1 Expression of the PKC isoforms in the PKCθ$^{-/-}$/ε$^{-/-}$ mice

Mice deficient in both PKCθ and ε were indistinguishable from littermate controls and survived for up to 30 weeks which was the longest time point studied. The platelet count and size were similar to those in C57/BL6 wild type mice and in mice deficient in either isoform of PKC (Figure 7.2; and data not shown).

Further, the expression of the major PKC isoforms, α, β and δ were not altered in the PKCθ$^{-/-}$/ε$^{-/-}$ platelets in comparison to controls as determined by western blotting (Figure 7.3). As expected, no expression of PKCθ, and PKCθ and ε was observed in platelets from the PKCθ$^{-/-}$ and PKCθ$^{-/-}$ ε$^{-/-}$ mice, respectively (Figure 7.3).

7.2.2 PKCθ$^{-/-}$/ε$^{-/-}$ platelets express normal levels of GPVI, αIIbβ3, and GPIb.

To ensure that any observations made in the PKCθ$^{-/-}$/ε$^{-/-}$ mouse platelets is not due to a change in the level of expression of GPVI, GPIb or αIIbβ3, the level of expression of these cell surface proteins, was analysed using flow cytometry (Figure 7.4) and was found to be similar in the PKCθ$^{-/-}$/ε$^{-/-}$, PKCθ$^{-/-}$ and WT platelets.

7.2.3 Functional studies on PKCθ$^{-/-}$ ε$^{-/-}$ mouse platelets.

7.2.3.1 PKCθ$^{-/-}$/ε$^{-/-}$ platelets show normal signalling downstream of thrombin.

Similar to the observations made by Pears et al (2008) and in the previous chapter for the single PKCθ-null mice, aggregation and dense granule secretion induced by a concentration of thrombin (0.1U/ml) that induces near maximal aggregation, were similar in platelets from the double PKCθ$^{-/-}$/ε$^{-/-}$ mice, PKCθ$^{-/-}$ littermate controls and age-matched background WT controls (Figure 7.5). This demonstrates no redundancy

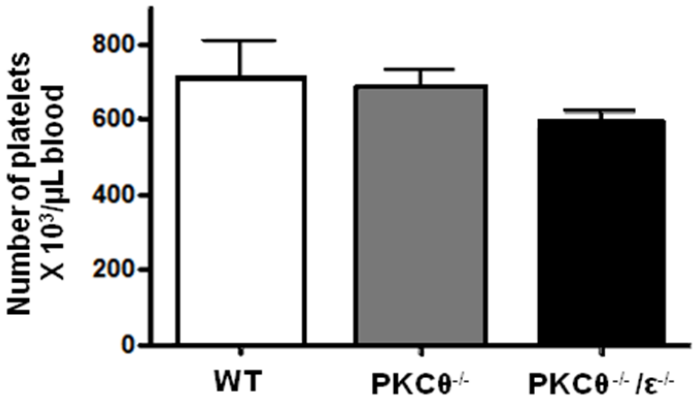


Figure 7.2. PKCθ$^{-/-}$/ε$^{-/-}$ platelet number is indistinguishable from controls. Platelet count was determined from anticoagulated whole blood samples from WT, PKCθ$^{-/-}$ and PKCθ$^{-/-}$/ε$^{-/-}$ mice. Results are average + S.E.M. for n=3.

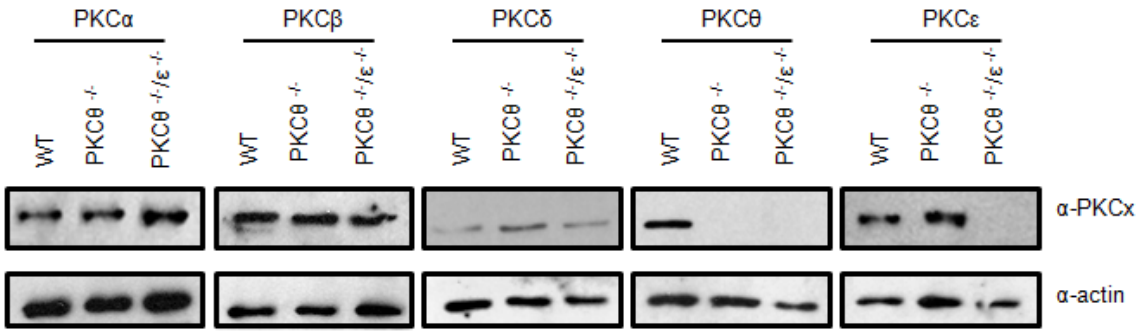


Figure 7.3. Other PKC isoform expression is normal in PKCθ$^{-/-}$/ε$^{-/-}$ mouse platelets. Equal amounts of washed platelets from WT, PKCθ$^{-/-}$ or PKCθ$^{-/-}$/ε$^{-/-}$ mice were subjected to SDS-PAGE and the expression levels of PKCα, β, δ, θ and ε determined by western blot using anti-sera specific to the individual isoforms of PKC. Actin was used as a loading control. Representative images shown, n=3.

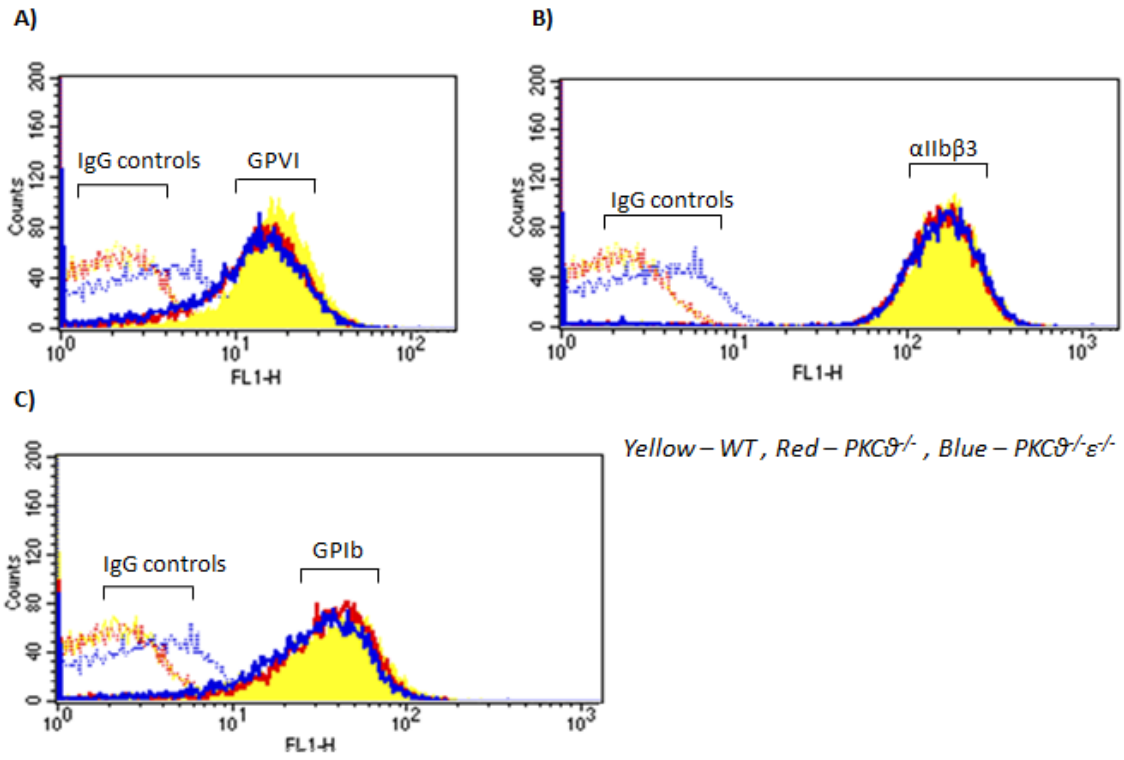


Figure 7.4. PKCθ$^{-/-}$/ε$^{-/-}$ platelets express similar levels of membrane surface receptors to controls. Expression levels of surface glycoproteins. PKCθ$^{-/-}$, PKCθ$^{-/-}$/ε$^{-/-}$ and WT washed platelets were incubated with FITC-labelled antisera against (A) GPVI, (B) integrin $\alpha_{IIb}\beta_3$ and (C) GPIb and expression levels analysed by flow cytometry. A non-specific IgG control was included. WT: yellow, PKCθ$^{-/-}$: red, PKCθ$^{-/-}$/ε$^{-/-}$: blue. n=3.

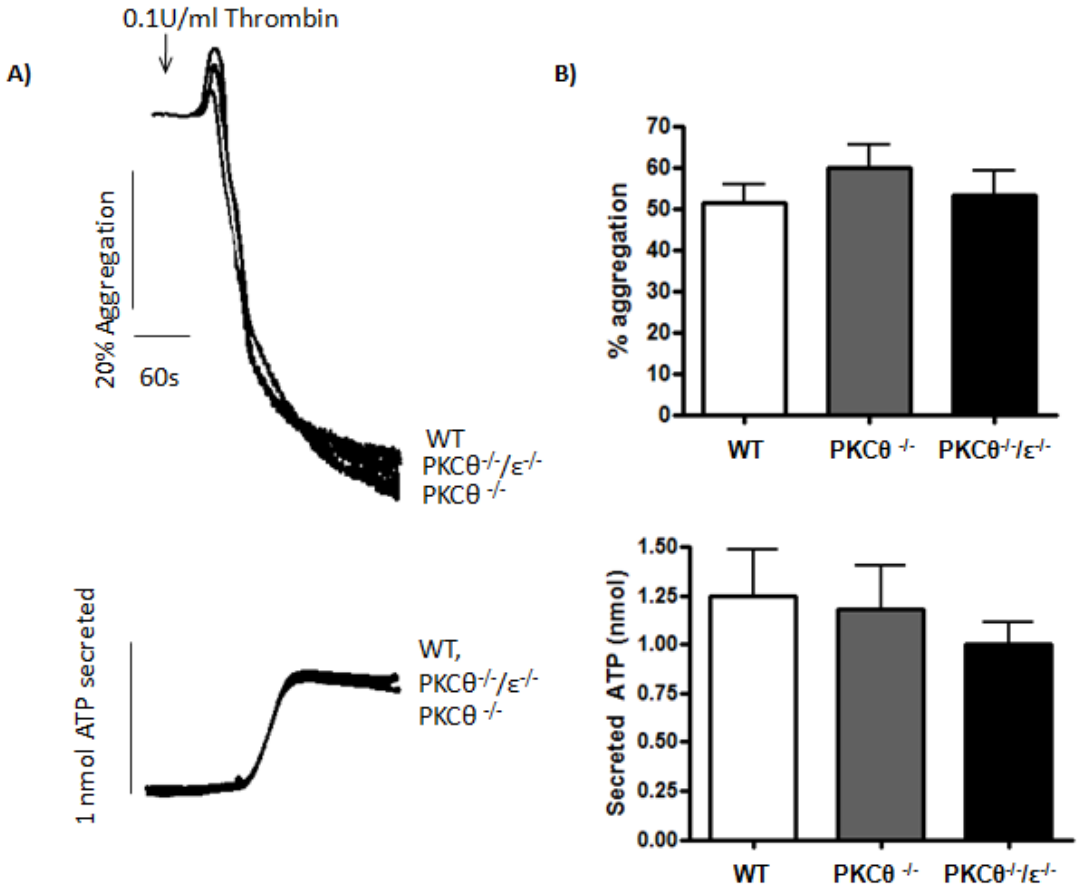


Figure 7.5. The role of PKCθ and PKCε in thrombin induced platelet activation *in vitro*. Washed platelets from WT, PKCθ$^{-/-}$ or PKCθ$^{-/-}$/ε$^{-/-}$ mice were stimulated with 0.1U/ml thrombin. Aggregation was measured by optical aggregometry. Dense granule secretion was measured by monitoring ATP secretion using luminometry. (A) Traces typical of n=3 are shown. (B) Results are average ± S.E.M. for n=3. Data was not significantly different following analysis by t-test.

between the two isoforms, with neither isoform functionally compensating for the absence of the other isoform.

7.2.3.2 PKCθ$^{-/-}$/ε$^{-/-}$ platelets show reduced GPVI signalling, similar to PKCε$^{-/-}$ null platelets

In contrast to thrombin induced responses, PKCθ$^{-/-}$/ε$^{-/-}$ mouse platelets show a delay in the onset and reduction in the extent of platelet aggregation and dense granule secretion to the GPVI agonist CRP (0.3µg/ml) compared to PKCθ$^{-/-}$ littermate and WT background controls (Figure 7.6). This minor defect in GPVI induced platelet activation is similar to that observed in the single PKCε-null mice as previously reported (Pears, Thornber et al. 2008). Direct comparison of low and intermediate CRP responses (1µg/ml and 3µg/ml, different CRP batch and so different potency and concentrations) of PKCθ$^{-/-}$ /ε$^{-/-}$ mouse platelets to PKCε$^{-/-}$ mouse platelets highlighted similar levels of inhibition in both of the null platelets (Figure 7.7 preliminary data, n=2). This therefore demonstrates that there is no redundancy between the two novel isoforms in supporting platelet activation to GPVI.

7.2.3.3 PKCθ$^{-/-}$/ε$^{-/-}$ platelets show reduced TxA_2 signalling, similar to both PKCε$^{-/-}$ and PKCθ$^{-/-}$ single deficient platelets

Preliminary studies looking at the role for the two novel isoforms in secondary mediator signalling, downstream of ADP and the TxA_2 analog U46619 (Figure 7.8, n≤2) identified no difference in ADP-induced (100µM) platelet activation in PKCθ$^{-/-}$/ε$^{-/-}$ mouse platelets compared to WT, similar to that observed in PKCθ$^{-/-}$ mouse platelets. No dense granule secretion was observed in WT platelets or those lacking PKCθ. Interestingly, the potentiation of dense granule secretion observed in the PKCε$^{-/-}$ platelets is not seen in PKCθ$^{-/-}$/ε$^{-/-}$ platelets. This highlights a positive role for PKCθ in the regulation of ADP

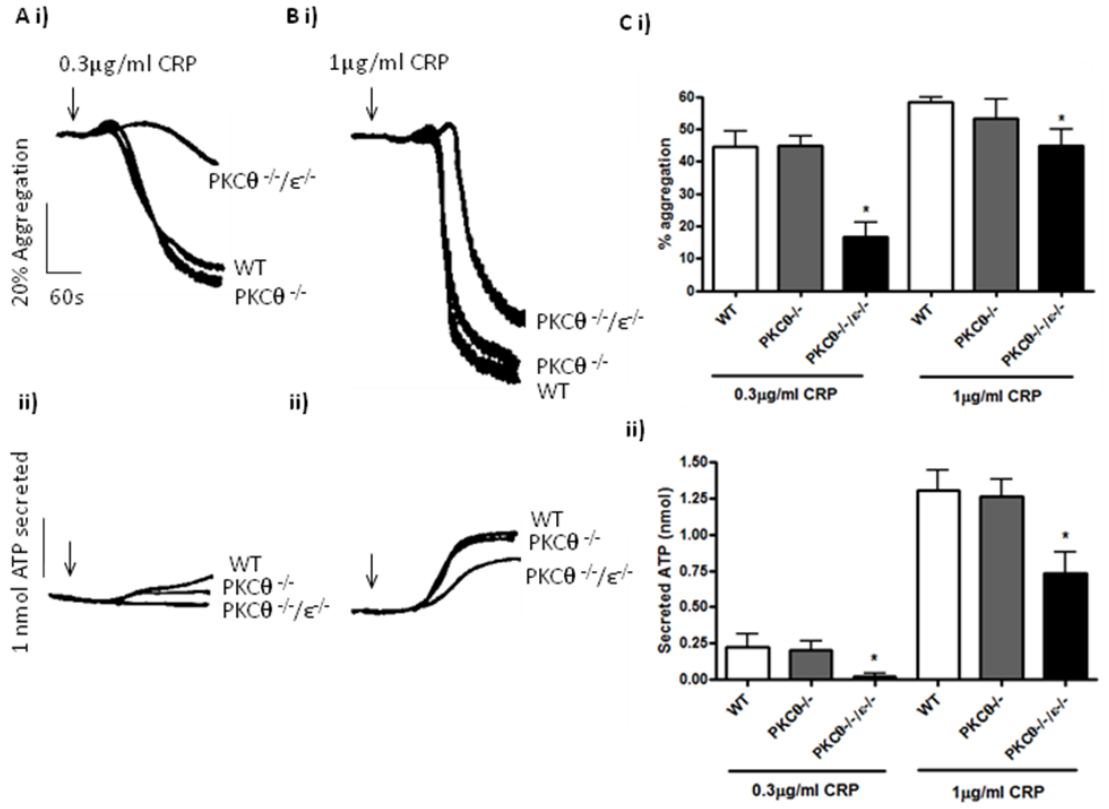


Figure 7.6 The role of PKCθ and PKCε in GPVI receptor induced platelet activation. Mouse washed platelets from PKCθ$^{-/-}$/ε$^{-/-}$, PKCθ$^{-/-}$ or wild-type (WT) mice were stimulated with (A) 0.3 or (B) 1µg/ml CRP. (i) Aggregation was measured by optical aggregometry (ii) Dense granule secretion was measured by monitoring ATP secretion using luminometry. (A and B) Traces representative of n=3 are shown. (C) Results are average + S.E.M. for n=3. * indicates $p<0.05$ in comparison to controls.

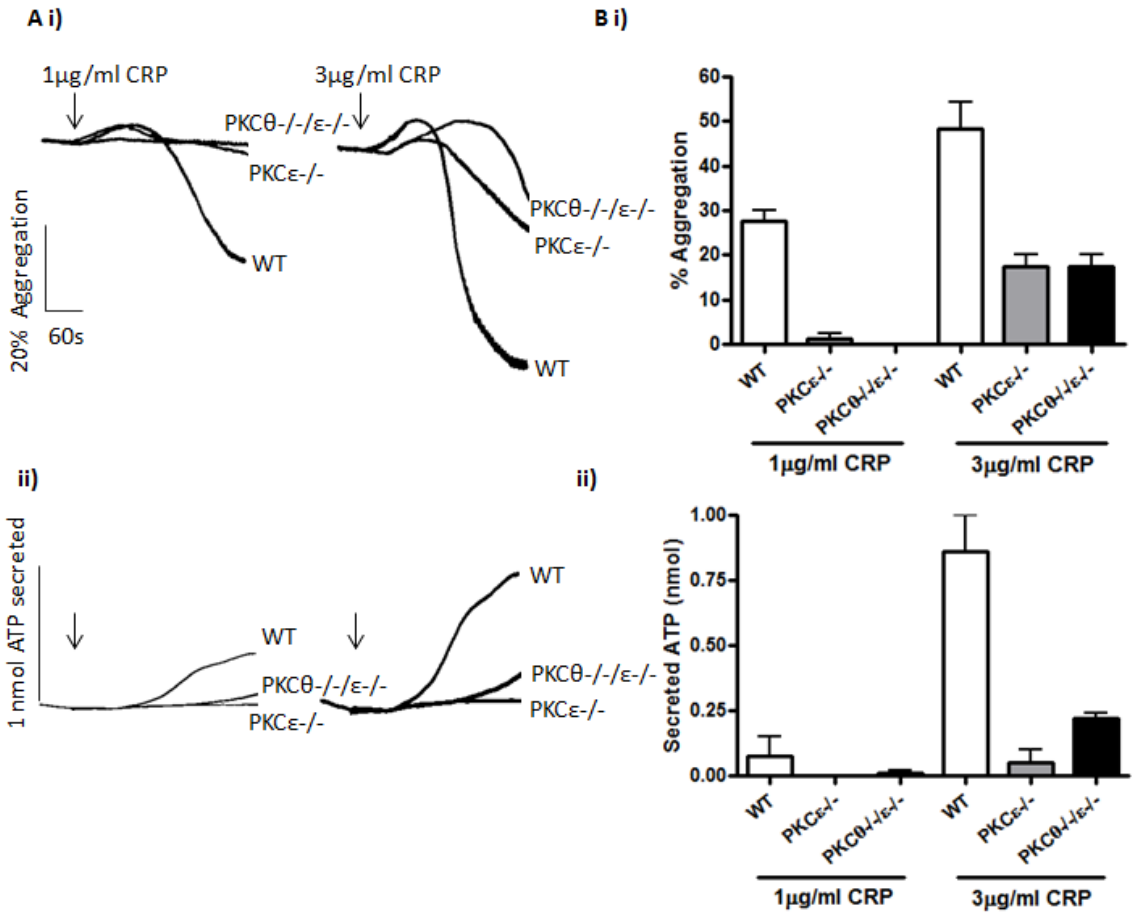


Figure 7.7. PKCθ$^{-/-}$/ε$^{-/-}$ mice like PKCε$^{-/-}$ mice show impaired GPVI signalling. Mouse washed platelets from PKCθ$^{-/-}$/ε$^{-/-}$, PKCε$^{-/-}$ or wild-type (WT) mice were stimulated with 1 or 3µg/ml CRP. (i) Aggregation was measured by optical aggregometry (ii) Dense granule secretion was measured by monitoring ATP secretion using luminometry. (A) Traces representative of n≤2 are shown. (B) Results are average + S.E.M. . (*n* ≤ 2 due to limited mouse availability).

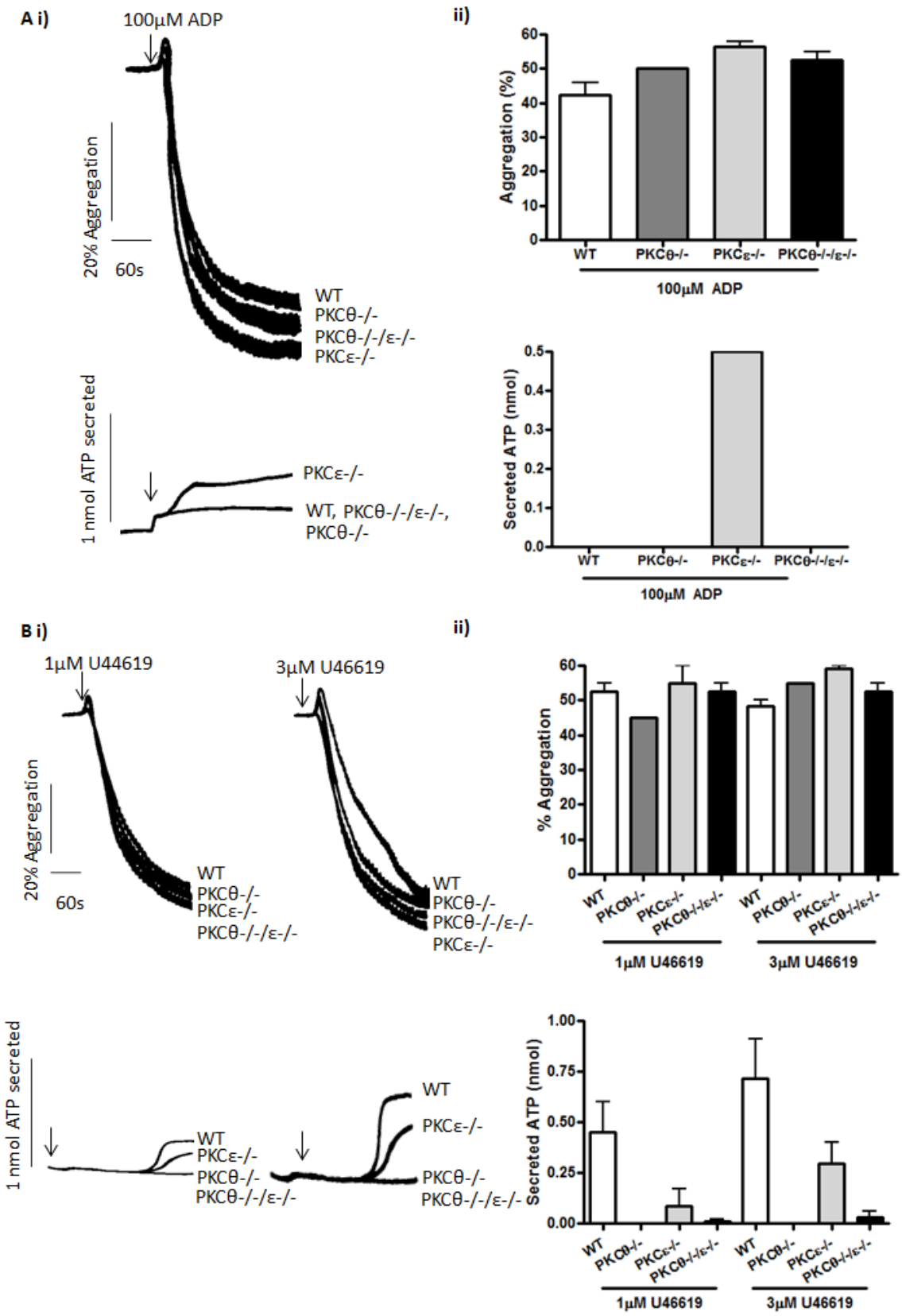


Figure 7.8. **PKC novel isoforms play positive roles in thromboxane A2 induced platelet activation.** Mouse PRP (A) and washed platelets (B) from PKCθ$^{-/-}$/ε$^{-/-}$, PKCε$^{-/-}$, PKCθ$^{-/-}$ or wild-type (WT) mice were stimulated with (A) 100µM ADP and (B) 1 or 3µM U46619.

Aggregation was measured by optical aggregometry and dense granule secretion was measured by monitoring ATP secretion using luminometry. (i) Traces representative of n≤2 are shown. (ii) Results are average + S.E.M. for n≤2.

-induced dense granule secretion which opposes the PKCε phenotype. In addition significant inhibition of platelet secretion in response to U46619 (1μM and 3μM) stimulation was observed in the absence of either of the single novel isoforms and in PKCθ$^{-/-}$/ε$^{-/-}$ mouse platelets. Unfortunately low mouse numbers prevented further studies.

7.2.3.3 PKCθ$^{-/-}$/ε$^{-/-}$ platelets show reduced adhesion to fibrinogen, similar to PKCθ$^{-/-}$ platelets.

A positive role for PKCθ in adhesion and spreading on fibrinogen has been reported by several groups (Soriani, Moran et al. 2006; Hall, Harper et al. 2008) and was also confirmed in the previous Chapter. In contrast, there was no significant effect on spreading on fibrinogen in PKCε null mouse platelets (Pears, Thornber et al. 2008). PKCθ$^{-/-}$/ε$^{-/-}$ platelets showed a reduction in adhesion and filopodia generation on fibrinogen relative to WT control which was not significantly different to that observed in PKCθ$^{-/-}$ platelets (Figure 7.9). These results therefore suggest that PKCε does not play a role in the outside-in signalling and adhesion to fibrinogen that is redundant with PKCθ.

7.2.3.4 Absence of both PKCθ and PKCε markedly attenuates thrombus formation on collagen under arteriolar shear.

The results presented in this chapter so far, suggest different isoform-specific rather than redundant roles for the two novel PKC isoforms, PKCθ and PKCε, in supporting platelet secretion, aggregation and spreading. To determine whether the loss of both isoforms had a cumulative effect on platelet function, platelet aggregation under arteriolar shear was investigated by flowing whole blood over immobilised collagen. Despite the defect in GPVI receptor signalling in PKCε-null mouse platelets, there is no

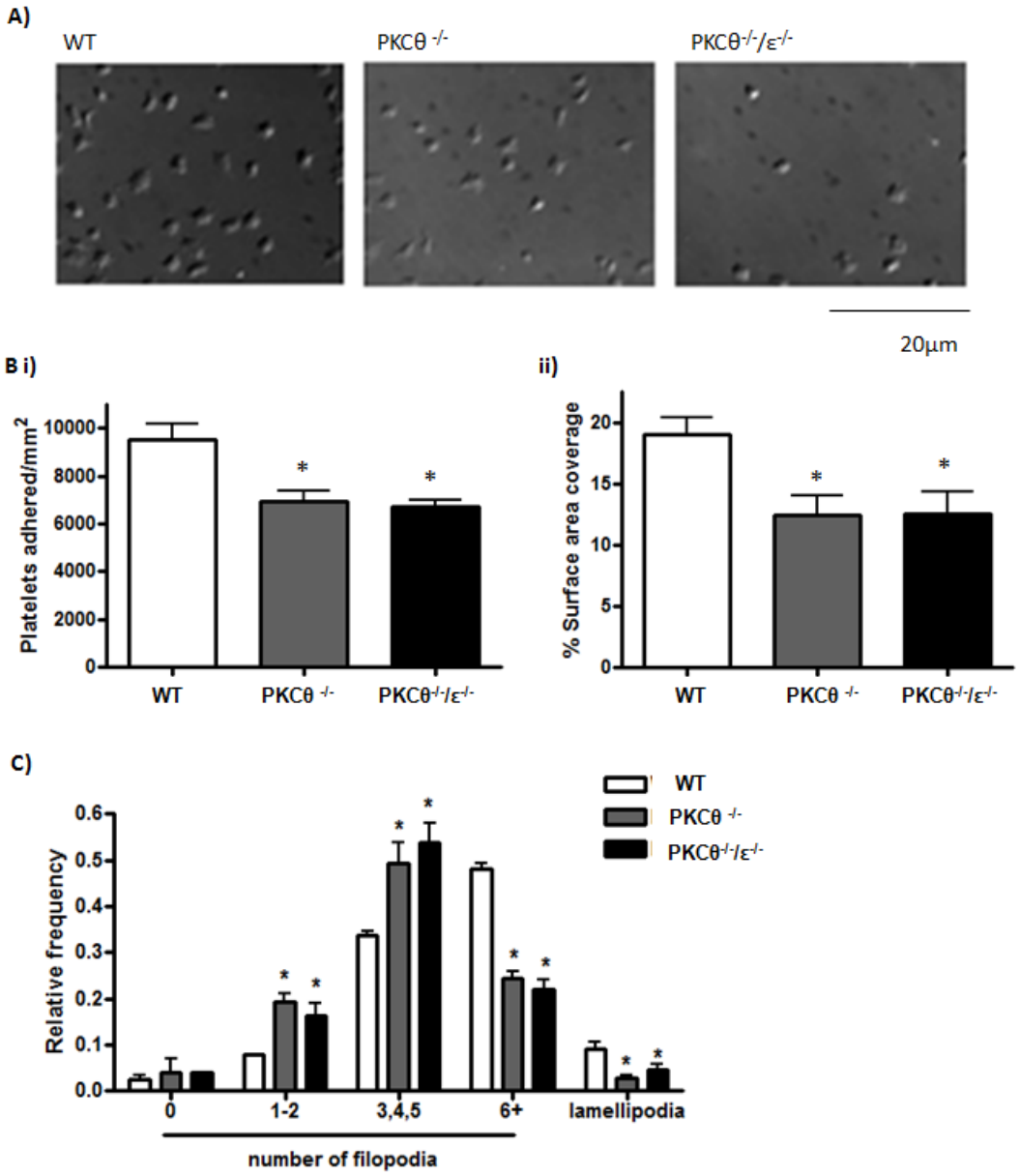


Figure 7.9. PKCθ$^{-/-}$/ε$^{-/-}$ mice platelets adhesion and spreading on fibrinogen. Washed platelets from WT, PKCθ$^{-/-}$ or PKCθ$^{-/-}$/ε$^{-/-}$ mice were exposed to fibrinogen coated coverslips. A) Representative phase-contrast images after 4 min. Images were taken under oil immersion. Original magnification, ×63 Bi) number of platelets adhered per mm^2, calculated by counting the number of cells adhered in 3 separate images per mouse ii) Surface area coverage, presented as a percentage, calculated using Image J analysis on 3 separate images per mouse. C) Filopodia number was counted for each visible platelet and the number of platelets with none, few (1–2), some (3–5) or many (6+) filopodia were expressed as relative frequency (proportion of the total number of platelets in view). At least 100 platelets of each type were scored. Results are mean + S.E.M. for n=3, * indicates $p<0.05$ in comparison to WT controls. P values are given in comparison to WT controls.

defect in aggregate formation on collagen under arteriolar shear (Pears, Thornber et al. 2008). Similarly despite a deficiency in spreading on fibrinogen and hence reduced outside-in signalling no significant difference in thrombus formation on collagen under high arteriolar shear is observed in the single PKCθ$^{-/-}$ mouse platelets (Figure 7.10). In contrast, there was a significant reduction in adhesion and platelet aggregation to a collagen-coated surface in PKCθ$^{-/-}$/ε$^{-/-}$ platelets relative to WT and PKCθ$^{-/-}$ platelets (Figure 7.8). Interestingly, the ability to spread on collagen under static conditions remains unaffected in PKCθ$^{-/-}$/ε$^{-/-}$ compared to WT platelets (preliminary data). These results indicate that the process of platelet aggregation under flow conditions markedly relies upon the combined effect of the two novel isoforms, PKCθ and PKCε.

7.2.4 Increased bleeding in PKCθ$^{-/-}$/ε$^{-/-}$ mice.

To see whether the defect in platelet aggregation under shear *in vitro* PKCθ$^{-/-}$/ε$^{-/-}$ deficient mice extrapolates to a loss of function *in vivo*, the tail bleeding times of the PKCθ$^{-/-}$/ε$^{-/-}$ deficient mice in comparison to controls was monitored (Figure 7.11) as was tail bleeding in the PKCε$^{-/-}$ single mice, as this has not been previously reported. As expected, given the lack of phenotype of thrombus formation *in vitro*, no significant difference was observed between WT and either the PKCε$^{-/-}$ or PKCθ$^{-/-}$ single isoform null mice. In contrast tail bleeding was significantly increased in the double deficient PKCθ$^{-/-}$/ε$^{-/-}$ mice, with both the amount of blood lost and the rate of blood loss significantly increased (Figure 7.10). This highlights a cumulative requirement for PKCθ and PKCε in supporting platelet activation in mice.

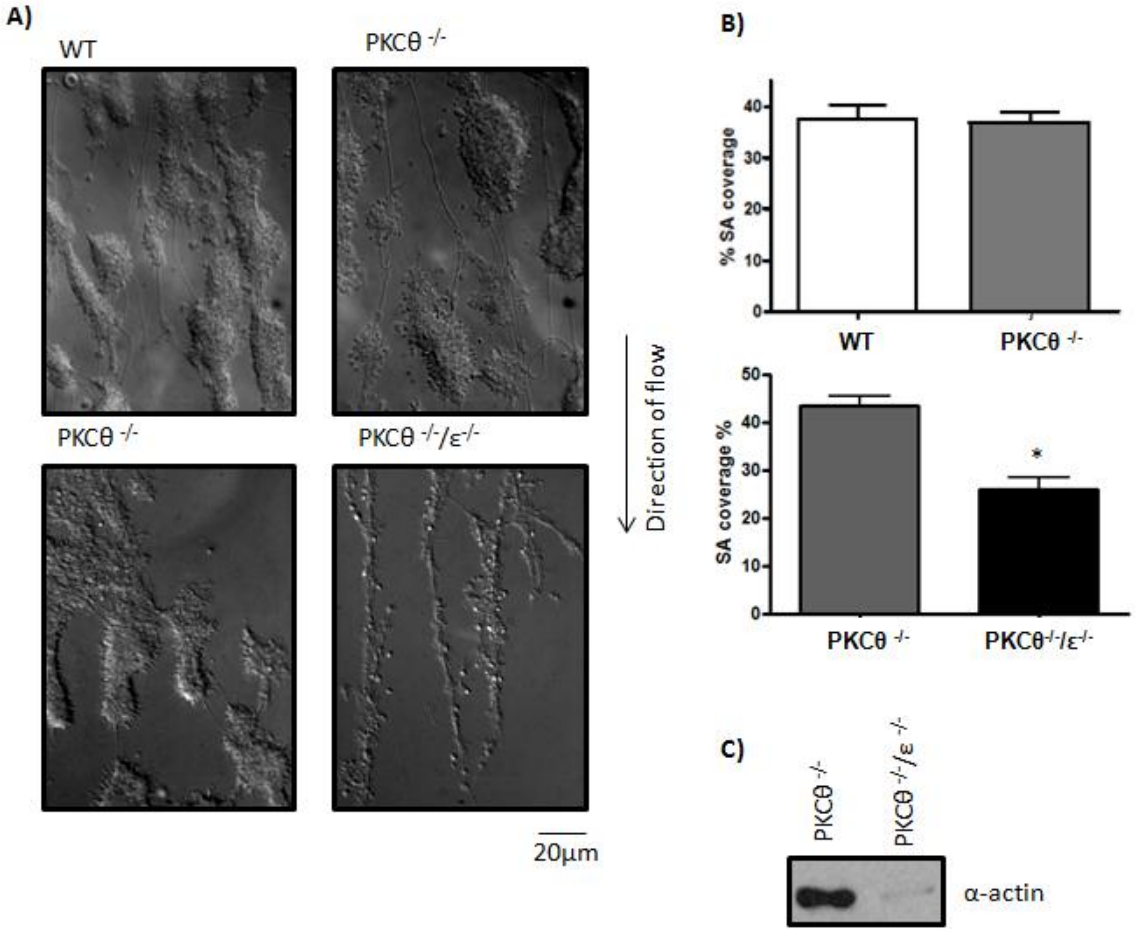
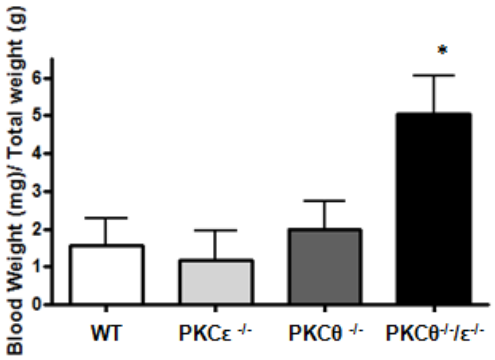
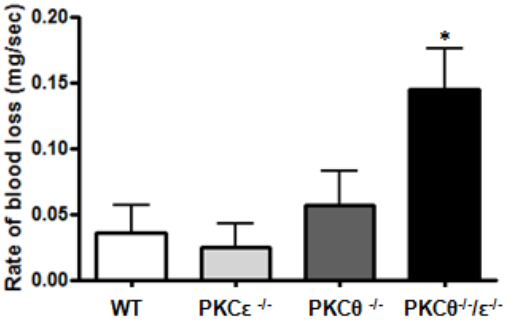


Figure 7.10. Aggregation of PKCθ$^{-/-}$/ε$^{-/-}$ platelets on collagen under arteriolar shear. Heparin/PPACK-anticoagulated blood from WT, PKCθ$^{-/-}$ or PKCθ$^{-/-}$/ε$^{-/-}$ mice was passed over collagen (shear rate 1000 s^{-1}). (A) Representative phase-contrast images after 4 min. Images were taken under oil immersion. Original magnification, ×63. (B) Surface area coverage with thrombi, calculated using 3 separate images per mouse, mean ± SEM. (C) Contents of the capillaries were lysed, subjected to SDS PAGE and western blot for actin used to determine relative protein levels. This work was carried out with L.Navarro-Nunez.

A i)



ii)



B

Genotype	Number of mice which bled to 15% blood vol within 20mins
WT	1/10
PKCε-/-	1/6
PKCθ-/-	1/10
PKCθ-/-ε-/-	5/6

Figure 7.11. Thrombus formation in PKCθ$^{-/-}$/ε$^{-/-}$ mice *in vivo*. Tail bleeding monitored following removal of the terminal 3mm of the mouse tail. (Ai) Tail bleeding expressed as weight of blood lost in 20 min as a ratio of total mouse weight. (Aii) Tail bleeding expressed as rate of blood lost. (B) Table summarising the number of mice which bled to 15% blood volume within 20mins at which point the experiment had to be stopped. (n = 10 for WT, 6 for PKCε$^{-/-}$, 10 for PKCθ$^{-/-}$, and 6 for PKCθ$^{-/-}$/ε$^{-/-}$). * indicates $p<0.05$ in comparison to WT controls. P values are given in comparison to WT controls. This work was carried out with B.Finney.

7.3 DISCUSSION

Genetic studies using mice deficient in the classical isoforms of PKC, PKCα and PKCβ, have highlighted very clear and dominant positive roles for these isoforms, in particular PKCα, in several of processes involved in platelet activation and thrombus formation (Konopatskaya, Gilio et al. 2009; Gilio, Harper et al. 2010). In comparison studies using mice deficient in the novel isoforms of PKC expressed in mouse platelets, PKCθ, PKCδ and PKCε, have highlighted relatively minor or in some cases negative roles for these isoforms in the regulation of platelet activation and haemostasis (Hall, Harper et al. 2008; Pears, Thornber et al. 2008; Chari, Getz et al. 2009; Cohen, Braiman et al. 2009; Harper and Poole 2009; Kunapuli and Jin 2009; Nagy, Bhavaraju et al. 2009; Gilio, Harper et al. 2010; Harper and Poole 2010). For example, mice deficient in either of the classical isoforms, PKCα and PKCβ, show a significant reduction in aggregate and thrombus formation on collagen under shear in comparison to WT controls (Konopatskaya, Gilio et al. 2009; Gilio, Harper et al. 2010). In contrast platelets deficient in PKCε (Pears, Thornber et al. 2008) show no difference in thrombus formation on collagen whilst PKCδ$^{-/-}$ platelets show increased aggregate formation in comparison to WT controls (Gilio, Harper et al. 2010). PKCθ has also been shown to play a negative role in thrombus formation on collagen (Hall, Harper et al. 2008; Gilio, Harper et al. 2010), although no difference was observed here and in Chapter 6 using PKCθ$^{-/-}$ mouse platelets. However, despite this PKCθ has also been reported to play a positive role in thrombus formation (Cohen, Braiman et al. 2009; Nagy, Bhavaraju et al. 2009) with Cohen et al (2009) reporting increase tail bleeding times in mice deficient in PKCθ although this is at odds with data presented here in this thesis and with the observations of Soriani et al (2006). Interestingly in the present study, a strong defect in thrombus formation is observed in

mouse platelets deficient in both of the novel isoforms, PKCθ and PKCε suggesting that although individually the novel isoforms PKCε and PKCθ play relatively minor roles in regulating platelet activation they can act in combination to support thrombus formation on collagen and haemostasis presumably because of synergy in their individual roles in regulating GPVI responses and outside–in signalling through αIIβ3, respectively.

Lack of an effect on aggregate and thrombus formation under shear in the absence of just PKCε (Pears, Thornber et al. 2008), is consistent with reports that show unaltered thrombus formation on collagen in mice that have reduced levels of the GPVI-FcRγ complex, (Best, Senis et al. 2003). This is most likely due to only partial inhibition of collagen induced signalling, which may be compensated for under arteriolar shear, as aggregate formation can be driven by the release of secondary mediators and the interaction and activation of other platelet signalling pathways.

Data presented here showing normal aggregate formation under shear in the PKCθ deficient mice, despite the reduction in αIIbβ3 outside-in signalling, is most likely due to the platelets partly retaining some outside-in signalling through αIIbβ3 which is further enhanced by other compensating mechanisms. Studies in chapter 6 show that PKCθ$^{-/-}$ unlike PKCε$^{-/-}$ mouse platelets have unaffected GPVI-induced aggregation and dense granule secretion indicating that PKCθ$^{-/-}$ platelets are capable of integrin activation. Intracellular signalling by GPVI stimulates αIIbβ3 integrin activation leading to stable adhesion of platelets through binding of collagen through α2β1 and the binding of vWF. Additionally Hall et al have shown increased inside-outside signalling in PKCθ$^{-/-}$ mouse platelets following GPVI stimulation (Hall, Harper et al. 2008). Either of these mechanisms could help maintain the GPVI induced aggregatory response, despite the reduction in αIIbβ3 outside-in signalling (Watson, Auger et al. 2005). Both PKCε$^{-/-}$ and

PKCθ$^{-/-}$ single isoform deficient mice also undergoes normal haemostasis, as demonstrated by no alteration in tail bleeding times, further indicating that compensatory mechanisms that allow normal aggregate formation under flow are sufficient to allow normal haemostasis.

In contrast, despite functional deficits in platelet aggregation to GPVI and αIIbβ3 outside-in signalling, that can be overcome and compensated for in the single deficient mice, the double deficient PKCθ$^{-/-}$/ε$^{-/-}$ mice show a significant reduction in aggregate formation under flow that further extrapolates to a significant increase in the rate and extent of tail bleeding times and abnormal haemostasis. It is likely that this phenotype is a result of the combined effects of the loss of the individual isoforms. It is possible that reduced GPVI signalling is unable to stimulate the required levels of inside-out signalling required for stable adhesion and aggregate formation, rendering the platelets incapable of compensating for the reduction in αIIbβ3 outside-in signalling. Additionally, it has also been shown that αIIbβ3 outside-in signalling is required for adhesion and spreading on GPVI agonists (Watson, Auger et al. 2005), and it is possible that in combination these functional losses prevent stable thrombus formation in PKCθ$^{-/-}$/ε$^{-/-}$ mouse platelets which is unable to support normal haemostasis resulting in increased tail bleeding.

The marked reduction in the ability of PKCθ$^{-/-}$/ε$^{-/-}$ mouse platelets to form normal aggregates on collagen and undergo normal haemostasis could also be due to possible defects in secondary mediator and positive feedback signalling, preventing the enhancement of platelet activation. Preliminary studies looking at ADP and TxA_2 signalling identified a significant inhibition of platelet secretion following stimulation by U46619 in the absence of either of the single novel isoforms and in the double deficient mouse platelets and a possible positive role for PKCθ in the regulation of ADP-induced

dense granule secretion. As the U46619-induced signalling is similarly reduced in the single deficient platelets, this is unlikely to underlie the defect in thrombus formation seen in the double deficient mice, although it may have a slight additive effect in combination with the other observed defects. The lack of ADP-induced secretion in combination with the reduced GPVI responses could also underlie the reduction in thrombus formation and haemostasis observed in the PKCθ$^{-/-}$/ε$^{-/-}$ double deficient mouse platelets. Unfortunately low mouse numbers prevented any further study of this.

The severe reduction in thrombus formation and haemostasis observed in the PKCθ$^{-/-}$/ε$^{-/-}$ mice could also be due to alteration to the procoagulant properties of the platelets. The platelet procoagulant response is a dependent and a direct response of GPVI induced activation of collagen adhered platelets (Heemskerk, Vuist et al. 1997; Nieswandt, Brakebusch et al. 2001). GPVI signalling (which is enhanced by αIIbβ3 outside-in signalling) enhances the process of coagulation (Heemskerk, Kuijpers et al. 2005) which involves the exposure of phosphatidylserine (PS) at the platelet surface membrane which acts as a platform for the recruitment of other procoagulant factors to enable thrombin formation (Heemskerk, Bevers et al. 2002; Munnix, Strehl et al. 2005). Reduced GPVI signalling as observed in the PKCθ$^{-/-}$/ε$^{-/-}$ mice could underlie this observation. Paradoxically, Harper et al have observed increased GPVI-induced increases in PS exposure, a marker of procoagulant activity in PKCθ$^{-/-}$ mice (Gilio, Harper et al. 2010), implicating PKCθ in the negative regulation of coagulation. However the same study also saw a significant increase in thrombus formation on collagen in comparison to WT controls which is at odds with the data presented here where no difference is observed. The role for PKCε in the regulation of platelet procoagulation however, is unknown. Interestingly PKCθ$^{-/-}$/ε$^{-/-}$ mouse platelets like mouse platelets deficient in either of the

classical isoforms, PKCα and PKCβ, show a decrease in GPVI signalling, and PKCα$^{-/-}$ and PKCβ$^{-/-}$ platelets also show decreased procoagulant activity that is associated with the significant reduction in GPVI induced platelet activation and thrombus formation. Although PKCε mice show normal aggregate formation under flow on collagen, a slight reduction in GPVI dependent procoagulant activity as a result of loss of PKCε in combination with the loss of PKCθ functions provides a possible mechanism that explains the inability of PKCθ$^{-/-}$/ε$^{-/-}$ mouse platelets to perform normal haemostasis and thrombosis.

Current opinion favours that the major positive regulatory role in platelet activation belongs to the classical isoform PKCα, supported by PKCβ, whilst the novel isoforms PKCθ and PKCε are considered to play relatively minor or negative regulatory roles. It is therefore interesting to note, that whilst PKCα$^{-/-}$ mouse platelets, like PKCθ$^{-/-}$/ε$^{-/-}$ platelets, show defects in GPVI induced platelet activation and aggregation under shear conditions, PKCα$^{-/-}$ platelets are able to undergo normal haemostasis as no difference in tail bleeding is observed (Konopatskaya, Gilio et al. 2009). PKCθ$^{-/-}$/ε$^{-/-}$ mice in comparison show a severe defect in their ability to undergo normal haemostasis, suggesting important roles for the two isoforms in the compensatory mechanisms that rescued haemostasis in PKCα$^{-/-}$ mice to normal levels.

The data presented here demonstrates a robust functional synergy between the novel isoforms PKCε and PKCθ in platelet aggregation under flow conditions and thrombus formation *in vivo*, suggesting that the differential roles of the single isoforms combine to have a marked positive effect on the processes of platelet activation and thrombus

formation. The work presented in this chapter demonstrates a major positive role for the two novel isoforms in the regulation of platelet activation and haemostasis that has so far been underestimated. Together this data therefore suggests that the two novel isoforms work in combination with the classical isoforms PKCα and PKCβ to positively regulate platelet activation and thrombus formation.

CHAPTER 8.

DISCUSSION

A central aim of platelet research is to identify the pathways that are required for the processes of thrombosis and haemostasis. Understanding the roles of cellular receptors, adhesive proteins and regulatory proteins involved in platelet-vessel interaction, platelet activation and thrombus formation is crucial for the development of new anti-platelet drugs. Protein kinase C (PKC) activity is a key regulator of many signal transduction pathways in platelets and is therefore a putative target for antithrombotic therapies.

PKC is thought to be a key mediator in several platelet agonist induced signalling pathways that regulate platelet activation. Pharmacological inhibitor studies and transgenic mutant mouse models presented here have identified that both positive and negative roles for PKC in the regulation of platelet activation exist but also that the role for PKC is isotype-dependent, with the isoforms of PKC displaying different expression patterns and regulatory roles in both human and mouse platelets.

An essential overall positive role for the PKC superfamily in the regulation of GPVI, CLEC-2, and PAR receptor induced platelet activation was identified as both aggregation and dense granule secretion were shown to be inhibited following activation in the presence of maximally active concentrations of broad spectrum PKC inhibitors. Pharmacological studies in human platelets identified major positive roles for the classical isoforms of PKC in the regulation of GPVI, CLEC-2 and PAR receptor induced platelet activation, most likely with PKCα playing the dominant positive role that is supported by PKCβ. In contrast the same studies identified relatively minor positive roles for PKCδ and PKCθ in response

to most of these agonists, thereby suggesting the classical isoforms act as the key players, supported by the novel isoforms, in several agonist specific PKC regulated processes. Interestingly however, use of a putative PKCθ-specific inhibitor identified a significant positive role for PKCθ in the regulation of rhodocytin-induced platelet activation in human platelets although this was not observed in mouse platelets deficient in PKCθ or in WT mouse platelets treated with low concentrations of the inhibitor. Interestingly rhodocytin-induced responses were also inhibited in WT mouse platelets at higher concentrations of the inhibitor, although not to the extent seen in human platelets. This provides evidence for inter-species differences in rhodocytin induced responses. PKCθ inhibitor dependent inhibition of rhodocytin and collagen –induced platelet aggregation and secretion seen in the PKCθ -deficient platelets to an extent greater than that observed in WT platelets identifies non-specific effects of the inhibitor at higher concentrations. However, analysis of the inhibitor dose response curve also revealed that at low inhibitor concentrations (0.1μM), the PKCθ inhibitor may be acting specifically. In human platelets this inhibitor concentration (0.1μM) caused slight inhibition of collagen-induced responses at lower collagen concentrations but not responses induced by thrombin or CRP (suggesting a minor role for PKCθ downstream of α2β1 but not GPVI). In contrast at 0.1μM of the PKCθ inhibitor, significant inhibition of rhodocytin-induced responses in human platelets occurs, suggesting that CLEC-2 induced responses, especially secretion, do appear to be dependent on PKCθ in human platelets, but not in mouse platelets. Interestingly this also highlights possible further differences between CLEC-2 and GPVI or collagen-induced platelet signalling pathways and their modes of regulation.

Studies using broad spectrum inhibitors of PKC identified both positive and negative regulatory roles for the PKC superfamily in the regulation of ADP-induced platelet activation, following maximal and submaximal inhibition of PKC respectfully. If submaximal doses of PKC inhibitors have very different effects from maximal concentrations this has implications for the use of these broad spectrum inhibitors in a clinical context as the bioavailability and effective concentration of drugs within the bloodstream may differ from the prescribed starting concentration. As this was observed in PRP, PRP containing extracellular calcium and washed platelets, this also highlights the level of PKC activity as a key regulator of platelet activation by ADP in both low and normal Ca^{2+} containing plasma. Unlike in citrated plasma, in plasma containing millimolar concentrations of extracellular Ca^{2+}, ADP is only able to induce transient aggregation and is unable to stimulate dense granule secretion. It is therefore possible that this lack of response to ADP is due to low levels of endogenous PKC activation in Ca^{2+} containing plasma. The novel negative role for PKC, specifically PKCβ, and PKCβ and PKCε in human and mouse platelets respectively, in ADP-induced platelet dense granule secretion and aggregation was also shown to be related to an increase in levels of intracellular calcium highlighting a potential mechanism that underlies this negative regulation. This is also the first description of a negative regulatory role for a classical PKC isoform, which so far have only been identified to have positive regulatory roles in the several major platelet agonist signalling pathways.

Transgenic 'knock-out' mice deficient in PKCθ confirmed a positive regulatory role for PKCθ in αIIbβ3 outside-in signalling. However, in contrast to previous publications no other platelet activation phenotype for $PKC\theta^{-/-}$ mouse platelets was identified suggesting that PKCθ plays relatively minor roles in other processes involved in platelet activation

and thrombus formation. There are several conflicting reports as to the role for PKCθ in platelet activation following stimulation by several agonists and in the regulation of thrombus formation. It is possible that these differences may be due to the different strains of mice, experimental conditions and protocols used by different laboratories, suggesting non-robust and relatively minor roles for PKCθ in several platelet processes. In contrast the defect in αIIbβ3 outside-in signalling observed in PKCθ deficient mouse platelets has been observed by three independent laboratories under different sets of conditions indicating a robust phenotype. However, despite this lack of a major phenotype for PKCθ, a functional redundancy was identified between the novel isoform and PKCε in platelet aggregation on collagen under flow conditions which further extrapolates to a bleeding defect *in vivo* using mouse platelets deficient in both PKCθ and PKCε. These studies identified a major positive role for the two novel isoforms together that works in combination with the classical isoforms PKCα and PKCβ, to positively regulate platelet activation, thrombus formation and haemostasis in mouse platelets.

Quantification of the five major PKC isoforms expressed in human and mouse platelets identified varying levels of expression for the different isoforms that are significantly different between species. The varying expression levels of the different isoforms in the same species does not appear to correlate with functional significance, suggesting that other factors must be involved in the regulation and functionality of the different PKC isoforms. Of concern is the difference in isoform expression levels and patterns between species as mice are frequently used as models for the understanding of human platelet function and these differences may have implications for any conclusions drawn using mouse models. For example although the novel isoform PKCθ appears to be the most

highly expressed in both human and mouse platelets it is expressed at much higher levels in mouse compared to human platelets. With these significant differences, can we be sure that the isoforms function in the same way between species?

Several of the studies described in this thesis involve the use of pharmacological inhibitors, caution should be taken when using these, as many inhibitors will target other proteins other than their presumed target. For example the broad spectrum inhibitor Ro31-8220 has been shown to act on other targets (Beltman, McCormick et al. 1996) and the previously considered classical isoform inhibitor Gö6976 has been shown to also inhibit the tyrosine kinase Syk (Getz, Mayanglambam et al. 2011). As syk is a key mediator in tyrosine kinase linked signalling pathways, such as GPVI and CLEC-2 signalling, Gö6976 was not included in the studies here and has significant implications for previously drawn conclusions in other studies. Preliminary experiments (Appendix-1) identified that in support of Gilio et al (2010), GPVI-induced aggregation and dense granule secretion was significantly ablated following treatment with low concentrations of Gö6976 in comparison to low concentrations of the broad spectrum inhibitor Ro31-8425 (Appendix-1A). Gilio et al (2010), concluded that this is due to the classical isoforms of PKC playing major positive roles in GPVI signalling. However, more recently it has been demonstrated that Gö6976 inhibits syk activity in human platelets (Getz, Mayanglambam et al. 2011) A detailed analysis of PKC substrate phosphorylation following GPVI stimulation showed an abolishment of total PKC activity even at low concentrations of Gö6976, inhibition that is significantly greater than that achieved using low concentrations of Ro31-8425, consistent with inhibition of an upstream regulator (syk) of GPVI signalling (Appendix 1B). In further support of this Gö6976, does not cause the same extent of inhibition following G protein-coupled receptor induced responses,

signalling that is not Syk kinase dependent (Appendix 1C). If inhibitor cross-reactivity exists between different kinases, there is the possibility that the PKC isoform-specific inhibitors may show cross-reactivity with the different PKC isoforms (especially as they share similar catalytic domains), thereby reducing the likelihood that the isoform-specific inhibitors are acting specifically. For these reasons, the data obtained from all the inhibitors used should focus on the results achieved at the low inhibitor concentrations as this minimises the possibility of non-specific target binding and hopefully finds a good balance between specificity and efficacy. There are however, many benefits as to the use of pharmacological inhibitors, as consistency in the concentrations used can be guaranteed, leading to reproducible results. The data presented in Chapters 4 and 5, show that such inhibitors have been useful in highlighting both essential and non-essential roles for the PKC superfamily and the individual PKC isoforms in specific agonist signalling pathways.

In addition to pharmacological methods, the use of transgenic null mice models has become a powerful approach in the understanding of the events that underlie platelet activation. Mouse models however, also have limitations as disruption of a particular gene and genetic modification can lead to secondary consequences, including developmental and compensatory changes in expression of related genes that could affect the results obtained, for example it has been shown that PKC$\alpha^{-/-}$ mice appear to show a marked reduction in their dense granule biogenesis in platelets. Functional differences between human and mouse platelets also exists, including differences in cell surface receptor expression, for example mouse platelets do not express functional PAR1 receptor like human platelets, mouse platelets also show a difference in several functional responses, including spreading on fibrinogen or collagen and as shown in

Chapter 1, the expression patterns and levels of the PKC isoforms also appear considerably different in mouse compared to human platelets. These physiological differences between the two species have implications for the use of murine platelets in the study and understanding of PKC induced platelet activation and in the development of anti-platelet drugs. Nevertheless, the use of mutant murine platelets has been invaluable in the understanding of the different roles for the individual isoforms of PKC as equivalent studies are not possible in human platelets due them not being genetically tractable and due to the lack of suitable specific inhibitors.

The data presented in this thesis identifies key roles for the PKC superfamily and several of its isoforms in the regulation of platelet activation, thrombus formation and haemostasis (summary diagram is provided in Figure 8.1). Further establishing the kinase family as a key regulator and central mediator or many platelet signalling pathways. Despite this, there are several implications for the use of PKC and its individual isoforms as a drug target for the development of anti-platelet therapies as both positive and negative roles for the superfamily have been identified, as inhibition of the negative roles of PKC and subsequent potentiation of activity could have undesirable consequences restricting the safe use of PKC-targeted antithrombotic therapies. Further understanding is needed in regards to PKC isoform-specific activation and regulation in addition to identification of isoform-specific substrates and interacting partners. As neither the pharmacological or genetic studies currently available can provide complete understanding, a combination of the two will most likely provide the most effective method for understanding PKC and its role in the regulation of platelet activation and haemostasis.

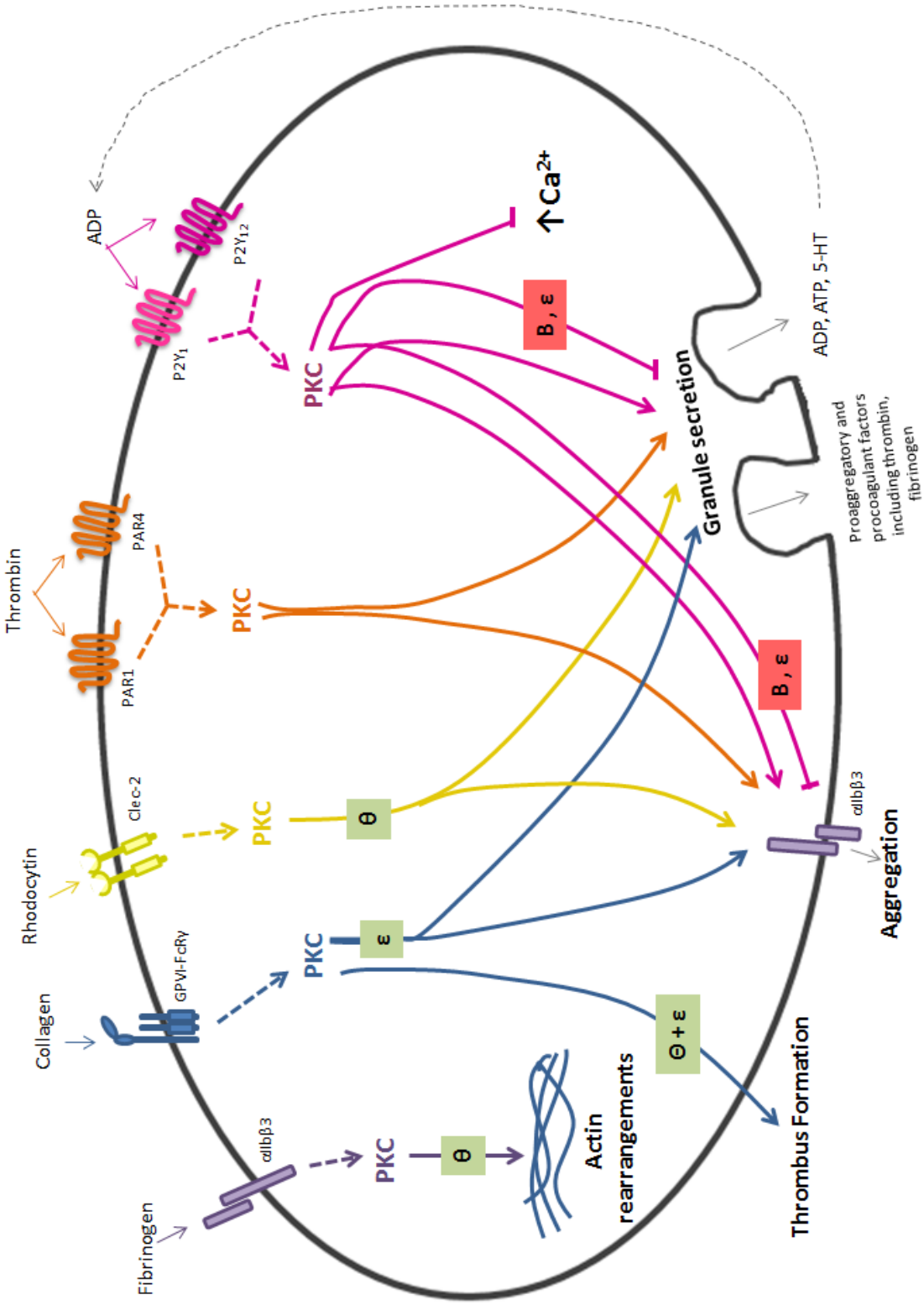


Figure 8.1. Proposed roles for the individual roles of PKC in agonist induced platelet activation as described in this thesis. Platelets are activated by several platelet agonists including; collagen, thrombin, clec-2, ADP and fibrinogen which stimulate a variety of G protein-coupled and tyrosine kinase linked receptor pathways, leading to the activation of PKC. PKC and its isoforms are implicated in many platelet responses including

aggregation, granule secretion, changes in intracellular calcium levels, actin rearrangements and thrombus formation. ‘个’ indicates general positive roles for the PKC superfamily, ‘T’ indicates general negative roles for the PKC superfamily. Green boxes indicate positive roles for a particular PKC isoform, red boxes indicate negative roles for a particular PKC isoform.

REFERENCES

Arias-Salgado, E. G., S. Lizano, et al. (2003). "Src kinase activation by direct interaction with the integrin beta cytoplasmic domain." Proc Natl Acad Sci U S A **100**(23): 13298-13302.

Asselin, J., J. M. Gibbins, et al. (1997). "A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C gamma2 in platelets independent of the integrin alpha2beta1." Blood **89**(4): 1235-1242.

Asselin, J., C. G. Knight, et al. (1999). "Monomeric (glycine-proline-hydroxyproline)10 repeat sequence is a partial agonist of the platelet collagen receptor glycoprotein VI." Biochem J **339 (Pt 2)**: 413-418.

Atkinson, B. T., M. J. Stafford, et al. (2001). "Signalling events underlying platelet aggregation induced by the glycoprotein VI agonist convulxin." Eur J Biochem **268**(20): 5242-5248.

Bain, J., H. McLauchlan, et al. (2003). "The specificities of protein kinase inhibitors: an update." Biochem J **371**(Pt 1): 199-204.

Bain, J., L. Plater, et al. (2007). "The selectivity of protein kinase inhibitors: a further update." Biochem J **408**(3): 297-315.

Barclay, J. W., T. J. Craig, et al. (2003). "Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis." J Biol Chem **278**(12): 10538-10545.

Behnke, O. (1968). "An electron microscope study of the megacaryocyte of the rat bone marrow. I. The development of the demarcation membrane system and the platelet surface coat." J Ultrastruct Res **24**(5): 412-433.

Beltman, J., F. McCormick, et al. (1996). "The selective protein kinase C inhibitor, Ro-31-8220, inhibits mitogen-activated protein kinase phosphatase-1 (MKP-1) expression, induces c-Jun expression, and activates Jun N-terminal kinase." J Biol Chem **271**(43): 27018-27024.

Bergmeier, W., M. Oh-Hora, et al. (2009). "R93W mutation in Orai1 causes impaired calcium influx in platelets." Blood **113**(3): 675-678.

Best, D., Y. A. Senis, et al. (2003). "GPVI levels in platelets: relationship to platelet function at high shear." Blood **102**(8): 2811-2818.

Bird, G. S., O. Aziz, et al. (2004). "Mechanisms of phospholipase C-regulated calcium entry." Curr Mol Med **4**(3): 291-301.

Blair, P. and R. Flaumenhaft (2009). "Platelet alpha-granules: basic biology and clinical correlates." Blood Rev **23**(4): 177-189.

Blake, R. A., G. L. Schieven, et al. (1994). "Collagen stimulates tyrosine phosphorylation of phospholipase C-gamma 2 but not phospholipase C-gamma 1 in human platelets." FEBS Lett **353**(2): 212-216.

Born, G. V. (1972). "Current ideas on the mechanism of platelet aggregation." Ann N Y Acad Sci **201**: 4-12.

Born, G. V. (1972). "The functional physiology of blood platelets." Adv Exp Med Biol **34**: 3-21.

Boyles, J., J. E. Fox, et al. (1985). "Organization of the cytoskeleton in resting, discoid platelets: preservation of actin filaments by a modified fixation that prevents osmium damage." J Cell Biol **101**(4): 1463-1472.

Brass, L. F. (2003). "Thrombin and platelet activation." Chest **124**(3 Suppl): 18S-25S.

Braun, A., J. E. Gessner, et al. (2009). "STIM1 is essential for Fcgamma receptor activation and autoimmune inflammation." Blood **113**(5): 1097-1104.

Buensuceso, C. S., A. Obergfell, et al. (2005). "Regulation of outside-in signaling in platelets by integrin-associated protein kinase C beta." J Biol Chem **280**(1): 644-653.

Bynagari, Y. S., B. Nagy, Jr., et al. (2009). "Mechanism of activation and functional role of protein kinase Ceta in human platelets." J Biol Chem **284**(20): 13413-13421.

Castrillo, A., D. J. Pennington, et al. (2001). "Protein kinase Cepsilon is required for macrophage activation and defense against bacterial infection." J Exp Med **194**(9): 1231-1242.

Cattaneo, M., C. Gachet, et al. (2002). "Adenosine diphosphate(ADP) does not induce thromboxane A2 generation in human platelets." Blood **99**(10): 3868-3869; author reply 3869-3870.

Cavallini, L. and A. Alexandre (1994). "Ca2+ efflux from platelets. Control by protein kinase C and the filling state of the intracellular Ca2+ stores." Eur J Biochem **222**(2): 693-702.

Chari, R., T. Getz, et al. (2009). "Protein kinase C[delta] differentially regulates platelet functional responses." Arterioscler Thromb Vasc Biol **29**(5): 699-705.

Chung, S. H., J. Polgar, et al. (2000). "Protein kinase C phosphorylation of syntaxin 4 in thrombin-activated human platelets." J Biol Chem **275**(33): 25286-25291.

Clemetson, J. M., J. Polgar, 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." J Biol Chem **274**(41): 29019-29024.

Clemetson, K. J., J. L. McGregor, et al. (1982). "Characterization of the platelet membrane glycoprotein abnormalities in Bernard-Soulier syndrome and comparison with normal by surface-labeling techniques and high-resolution two-dimensional gel electrophoresis." J Clin Invest **70**(2): 304-311.

Cohen, P. (1999). "The development and therapeutic potential of protein kinase inhibitors." Curr Opin Chem Biol **3**(4): 459-465.

Cohen, S., A. Braiman, et al. (2011). "Protein kinase C-theta in platelet activation." FEBS Lett **585**(20): 3208-3215.

Cohen, S., A. Braiman, et al. (2009). "PKCtheta is required for hemostasis and positive regulation of thrombin-induced platelet aggregation and alpha-granule secretion." Biochem Biophys Res Commun **385**(1): 22-27.

Cosemans, J. M., B. F. Iserbyt, et al. (2008). "Multiple ways to switch platelet integrins on and off." J Thromb Haemost **6**(8): 1253-1261.

Crosby, D. and A. W. Poole (2002). "Interaction of Bruton's tyrosine kinase and protein kinase Ctheta in platelets. Cross-talk between tyrosine and serine/threonine kinases." J Biol Chem **277**(12): 9958-9965.

Crosby, D. and A. W. Poole (2003). "Physical and functional interaction between protein kinase C delta and Fyn tyrosine kinase in human platelets." J Biol Chem **278**(27): 24533-24541.

Cywin, C. L., G. Dahmann, et al. (2007). "Discovery of potent and selective PKC-theta inhibitors." Bioorg Med Chem Lett **17**(1): 225-230.

Daniel, J. L., C. Dangelmaier, et al. (1998). "Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets." J Biol Chem **273**(4): 2024-2029.

Davies, S. P., H. Reddy, et al. (2000). "Specificity and mechanism of action of some commonly used protein kinase inhibitors." Biochem J **351**(Pt 1): 95-105.

Davis, P. D., C. H. Hill, et al. (1989). "Potent selective inhibitors of protein kinase C." FEBS Lett **259**(1): 61-63.

Dean, W. L., D. Chen, et al. (1997). "Regulation of platelet plasma membrane Ca2+-ATPase by cAMP-dependent and tyrosine phosphorylation." J Biol Chem **272**(24): 15113-15119.

Dent, J., K. Kato, et al. (2002). "A prototypic platelet septin and its participation in secretion." Proc Natl Acad Sci U S A **99**(5): 3064-3069.

Dorn, G. W., 2nd (1989). "Distinct platelet thromboxane A2/prostaglandin H2 receptor subtypes. A radioligand binding study of human platelets." J Clin Invest **84**(6): 1883-1891.

Dowling, M. R., E. C. Josefsson, et al. (2010). "Platelet senescence is regulated by an internal timer, not damage inflicted by hits." Blood **116**(10): 1776-1778.

Dupont, A., P. Fontana, et al. (2003). "An intronic polymorphism in the PAR-1 gene is associated with platelet receptor density and the response to SFLLRN." Blood **101**(5): 1833-1840.

Ebbe, S. (1976). "Biology of megakaryocytes." Prog Hemost Thromb **3**: 211-229.

Enyedi, A., B. Sarkadi, et al. (1986). "Demonstration of two distinct calcium pumps in human platelet membrane vesicles." J Biol Chem **261**(20): 9558-9563.
Enyedi, A., A. K. Verma, et al. (1996). "Protein kinase C activates the plasma membrane Ca2+ pump isoform 4b by phosphorylation of an inhibitory region downstream of the calmodulin-binding domain." J Biol Chem **271**(50): 32461-32467.
Ezumi, Y., K. Shindoh, et al. (1998). "Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets." J Exp Med **188**(2): 267-276.
Falati, S., C. E. Edmead, et al. (1999). "Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor gamma-chain, Fyn, and Lyn to activate human platelets." Blood **94**(5): 1648-1656.
FitzGerald, G. A. (1991). "Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists." Am J Cardiol **68**(7): 11B-15B.
Flaumenhaft, R. (2003). "Molecular basis of platelet granule secretion." Arterioscler Thromb Vasc Biol **23**(7): 1152-1160.
Flaumenhaft, R., K. Croce, et al. (1999). "Proteins of the exocytotic core complex mediate platelet alpha-granule secretion. Roles of vesicle-associated membrane protein, SNAP-23, and syntaxin 4." J Biol Chem **274**(4): 2492-2501.
Frojmovic, M., K. Longmire, et al. (1990). "Long-range interactions in mammalian platelet aggregation. II. The role of platelet pseudopod number and length." Biophys J **58**(2): 309-318.
Gachet, C., B. Hechler, et al. (1997). "Activation of ADP receptors and platelet function." Thromb Haemost **78**(1): 271-275.
Getz, T. M., A. Mayanglambam, et al. (2011). "Go6976 abrogates GPVI-mediated platelet functional responses in human platelets through inhibition of Syk." J Thromb Haemost.
Gibbins, J., J. Asselin, et al. (1996). "Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets." J Biol Chem **271**(30): 18095-18099.
Gibbins, J. M. (2004). "Platelet adhesion signalling and the regulation of thrombus formation." J Cell Sci **117**(Pt 16): 3415-3425.
Gilio, K., M. T. Harper, et al. (2010). "Functional divergence of platelet protein kinase C (PKC) isoforms in thrombus formation on collagen." J Biol Chem **285**(30): 23410-23419.
Ginsberg, M. H., A. Partridge, et al. (2005). "Integrin regulation." Curr Opin Cell Biol **17**(5): 509-516.
Giuliano, S., W. S. Nesbitt, et al. (2003). "Bidirectional integrin alphaIIbbeta3 signalling regulating platelet adhesion under flow: contribution of protein kinase C." Biochem J **372**(Pt 1): 163-172.
Gruner, S., M. Prostredna, et al. (2003). "Multiple integrin-ligand interactions synergize in shear-resistant platelet adhesion at sites of arterial injury in vivo." Blood **102**(12): 4021-4027.
Grynkiewicz, G., M. Poenie, et al. (1985). "A new generation of Ca2+ indicators with greatly improved fluorescence properties." J Biol Chem **260**(6): 3440-3450.
Gschwendt, M., S. Dieterich, et al. (1996). "Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes." FEBS Lett **392**(2): 77-80.
Gschwendt, M., H. J. Muller, et al. (1994). "Rottlerin, a novel protein kinase inhibitor." Biochem Biophys Res Commun **199**(1): 93-98.
Gustafson, A. P. F. a. W. C. (2003). Protein Kinase C in Disease Cancer, Methods in Molecular Biology.
Hall, K. J., M. T. Harper, et al. (2008). "Genetic analysis of the role of protein kinase Ctheta in platelet function and thrombus formation." PLoS One **3**(9): e3277.
Hall, K. J., M. L. Jones, et al. (2007). "Coincident regulation of PKCdelta in human platelets by phosphorylation of Tyr311 and Tyr565 and phospholipase C signalling." Biochem J **406**(3): 501-509.

Han, J., C. J. Lim, et al. (2006). "Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3." Curr Biol **16**(18): 1796-1806.
Handagama, P. J., J. N. George, et al. (1987). "Incorporation of a circulating protein into megakaryocyte and platelet granules." Proc Natl Acad Sci U S A **84**(3): 861-865.
Hardy, A. R., P. B. Conley, et al. (2005). "P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms." Blood **105**(9): 3552-3560.
Hardy, A. R., M. L. Jones, et al. (2004). "Reciprocal cross-talk between P2Y1 and P2Y12 receptors at the level of calcium signaling in human platelets." Blood **104**(6): 1745-1752.
Harper, M. T., J. D. Molkentin, et al. (2010). "Protein kinase C alpha enhances sodium-calcium exchange during store-operated calcium entry in mouse platelets." Cell Calcium **48**(6): 333-340.
Harper, M. T. and A. W. Poole (2007). "Isoform-specific functions of protein kinase C: the platelet paradigm." Biochem Soc Trans **35**(Pt 5): 1005-1008.
Harper, M. T. and A. W. Poole (2009). "PKCtheta in platelet activation." Blood **114**(2): 489-491; author reply 491-482.
Harper, M. T. and A. W. Poole (2010). "Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation." J Thromb Haemost **8**(3): 454-462.
Harper, M. T. and A. W. Poole (2010). "Protein kinase Ctheta negatively regulates store-independent Ca2+ entry and phosphatidylserine exposure downstream of glycoprotein VI in platelets." J Biol Chem **285**(26): 19865-19873.
Harper, M. T. and A. W. Poole (2011). "PKC inhibition markedly enhances Ca2+ signaling and phosphatidylserine exposure downstream of protease-activated receptor-1 but not protease-activated receptor-4 in human platelets." J Thromb Haemost **9**(8): 1599-1607.
Harper, M. T. and S. O. Sage (2006). "PAR-1-dependent pp60src activation is dependent on protein kinase C and increased [Ca2+]: evidence that pp60src does not regulate PAR-1-dependent Ca2+ entry in human platelets." J Thromb Haemost **4**(12): 2695-2703.
Harrison, P. and E. M. Cramer (1993). "Platelet alpha-granules." Blood Rev **7**(1): 52-62.
Hartley, P. S. (2007). "Platelet senescence and death." Clin Lab **53**(3-4): 157-166.
Hartwig, J. and J. Italiano, Jr. (2003). "The birth of the platelet." J Thromb Haemost **1**(7): 1580-1586.
Hartwig, J. H. (1992). "Mechanisms of actin rearrangements mediating platelet activation." J Cell Biol **118**(6): 1421-1442.
Hartwig, J. H. and M. DeSisto (1991). "The cytoskeleton of the resting human blood platelet: structure of the membrane skeleton and its attachment to actin filaments." J Cell Biol **112**(3): 407-425.
Hassock, S. R., M. X. Zhu, et al. (2002). "Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel." Blood **100**(8): 2801-2811.
Hathaway, D. R. and R. S. Adelstein (1979). "Human platelet myosin light chain kinase requires the calcium-binding protein calmodulin for activity." Proc Natl Acad Sci U S A **76**(4): 1653-1657.
Heemskerk, J. W., E. M. Bevers, et al. (2002). "Platelet activation and blood coagulation." Thromb Haemost **88**(2): 186-193.
Heemskerk, J. W., M. T. Harper, et al. (2011). "Unravelling the different functions of protein kinase C isoforms in platelets." FEBS Lett **585**(12): 1711-1716.
Heemskerk, J. W., M. J. Kuijpers, et al. (2005). "Platelet collagen receptors and coagulation. A characteristic platelet response as possible target for antithrombotic treatment." Trends Cardiovasc Med **15**(3): 86-92.
Heemskerk, J. W., W. M. Vuist, et al. (1997). "Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent

platelets: evidence for regulation by protein tyrosine kinase-dependent Ca2+ responses." Blood **90**(7): 2615-2625.
Hers, I., J. Donath, et al. (1998). "Differential involvement of tyrosine and serine/threonine kinases in platelet integrin alphaIIbbeta3 exposure." Arterioscler Thromb Vasc Biol **18**(3): 404-414.
Hidalgo, A. and P. S. Frenette (2009). "When integrins fail to integrate." Nat Med **15**(3): 249-250.
Holtkotter, O., B. Nieswandt, et al. (2002). "Integrin alpha 2-deficient mice develop normally, are fertile, but display partially defective platelet interaction with collagen." J Biol Chem **277**(13): 10789-10794.
Hourani, S. M. and N. J. Cusack (1991). "Pharmacological receptors on blood platelets." Pharmacol Rev **43**(3): 243-298.
Huang, J. S., S. K. Ramamurthy, et al. (2004). "Cell signalling through thromboxane A2 receptors." Cell Signal **16**(5): 521-533.
Huang, T. F., C. Z. Liu, et al. (1995). "Aggretin, a novel platelet-aggregation inducer from snake (Calloselasma rhodostoma) venom, activates phospholipase C by acting as a glycoprotein Ia/IIa agonist." Biochem J **309 (Pt 3)**: 1021-1027.
Hughes, C. E., A. Y. Pollitt, et al. (2010). "CLEC-2 activates Syk through dimerization." Blood **115**(14): 2947-2955.
Hynes, R. O. (2002). "Integrins: bidirectional, allosteric signaling machines." Cell **110**(6): 673-687.
Inoue, K., Y. Ozaki, et al. (1999). "Signal transduction pathways mediated by glycoprotein Ia/IIa in human platelets: comparison with those of glycoprotein VI." Biochem Biophys Res Commun **256**(1): 114-120.
Inoue, O., K. Suzuki-Inoue, 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): 769-780.
Italiano, J. E., Jr., P. Lecine, et al. (1999). "Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes." J Cell Biol **147**(6): 1299-1312.
Jackson, S. P., W. S. Nesbitt, et al. (2003). "Signaling events underlying thrombus formation." J Thromb Haemost **1**(7): 1602-1612.
Jackson, S. P., W. S. Nesbitt, et al. (2009). "Dynamics of platelet thrombus formation." J Thromb Haemost **7 Suppl 1**: 17-20.
Jaken, S. and P. J. Parker (2000). "Protein kinase C binding partners." Bioessays **22**(3): 245-254.
Jin, J. and S. P. Kunapuli (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): 8070-8074.
Jin, J., T. M. Quinton, et al. (2002). "Adenosine diphosphate (ADP)-induced thromboxane A(2) generation in human platelets requires coordinated signaling through integrin alpha(IIb)beta(3) and ADP receptors." Blood **99**(1): 193-198.
Judd, B. A., P. S. Myung, et al. (2000). "Hematopoietic reconstitution of SLP-76 corrects hemostasis and platelet signaling through alpha IIb beta 3 and collagen receptors." Proc Natl Acad Sci U S A **97**(22): 12056-12061.
Kahn, M. L., M. Nakanishi-Matsui, et al. (1999). "Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin." J Clin Invest **103**(6): 879-887.
Kahn, M. L., Y. W. Zheng, et al. (1998). "A dual thrombin receptor system for platelet activation." Nature **394**(6694): 690-694.
Kahner, B. N., H. Shankar, et al. (2006). "Nucleotide receptor signaling in platelets." J Thromb Haemost **4**(11): 2317-2326.
Karlsson, R., I. Lassing, et al. (1984). "The organization of microfilaments in spreading platelets: a comparison with fibroblasts and glial cells." J Cell Physiol **121**(1): 96-113.

Kato, K., T. Kanaji, et al. (2003). "The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion." Blood **102**(5): 1701-1707.

Kenney, D. M. and R. W. Linck (1985). "The cystoskeleton of unstimulated blood platelets: structure and composition of the isolated marginal microtubular band." J Cell Sci **78**: 1-22.

King, S. M. and G. L. Reed (2002). "Development of platelet secretory granules." Semin Cell Dev Biol **13**(4): 293-302.

King, W. G. and S. E. Rittenhouse (1989). "Inhibition of protein kinase C by staurosporine promotes elevated accumulations of inositol trisphosphates and tetrakisphosphate in human platelets exposed to thrombin." J Biol Chem **264**(11): 6070-6074.

Konopatskaya, O., K. Gilio, et al. (2009). "PKCalpha regulates platelet granule secretion and thrombus formation in mice." J Clin Invest **119**(2): 399-407.

Konopatskaya, O. and A. W. Poole (2010). "Protein kinase Calpha: disease regulator and therapeutic target." Trends Pharmacol Sci **31**(1): 8-14.

Kulkarni, S., S. M. Dopheide, et al. (2000). "A revised model of platelet aggregation." J Clin Invest **105**(6): 783-791.

Kunapuli, S. P. and J. G. Jin (2009). "Role of PKC theta in collagen-related peptide-induced platelet activation Response." Blood **114**(2): 491-492.

Lind, S. E. and T. P. Stossel (1982). "The microfilament network of the platelet." Prog Hemost Thromb **6**: 63-84.

Liu, Q., X. Chen, et al. (2009). "Protein kinase C{alpha}, but not PKC{beta} or PKC{gamma}, regulates contractility and heart failure susceptibility: implications for ruboxistaurin as a novel therapeutic approach." Circ Res **105**(2): 194-200.

Liu, W. S. and C. A. Heckman (1998). "The sevenfold way of PKC regulation." Cell Signal **10**(8): 529-542.

Liu, Y., S. Witte, et al. (2000). "Regulation of protein kinase Ctheta function during T cell activation by Lck-mediated tyrosine phosphorylation." J Biol Chem **275**(5): 3603-3609.

Mackay, J., G. Mensah, et al. (2004). The atlas of heart disease and stroke. Geneva, World Health Organization.

Martiny-Baron, G., M. G. Kazanietz, et al. (1993). "Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976." J Biol Chem **268**(13): 9194-9197.

Mellor, H. and P. J. Parker (1998). "The extended protein kinase C superfamily." Biochem J **332 (Pt 2)**: 281-292.

Meyers, K. M., H. Holmsen, et al. (1982). "Comparative study of platelet dense granule constituents." Am J Physiol **243**(3): R454-461.

Mills, D. C. (1996). "ADP receptors on platelets." Thromb Haemost **76**(6): 835-856.

Miranti, C. K., L. Leng, et al. (1998). "Identification of a novel integrin signaling pathway involving the kinase Syk and the guanine nucleotide exchange factor Vav1." Curr Biol **8**(24): 1289-1299.

Mochly-Rosen, D. and L. M. Kauvar (1998). "Modulating protein kinase C signal transduction." Adv Pharmacol **44**: 91-145.

Mochly-Rosen, D., H. Khaner, et al. (1991). "Identification of intracellular receptor proteins for activated protein kinase C." Proc Natl Acad Sci U S A **88**(9): 3997-4000.

Morton, L. F., P. G. Hargreaves, et al. (1995). "Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity." Biochem J **306 (Pt 2)**: 337-344.

Muik, M., I. Frischauf, et al. (2008). "Dynamic coupling of the putative coiled-coil domain of ORAI1 with STIM1 mediates ORAI1 channel activation." J Biol Chem **283**(12): 8014-8022.

Mundell, S. J., M. L. Jones, et al. (2006). "Distinct roles for protein kinase C isoforms in regulating platelet purinergic receptor function." Mol Pharmacol **70**(3): 1132-1142.
Munnix, I. C., A. Strehl, et al. (2005). "The glycoprotein VI-phospholipase Cgamma2 signaling pathway controls thrombus formation induced by collagen and tissue factor in vitro and in vivo." Arterioscler Thromb Vasc Biol **25**(12): 2673-2678.
Murugappa, S. and S. P. Kunapuli (2006). "The role of ADP receptors in platelet function." Front Biosci **11**: 1977-1986.
Murugappan, S., R. Chari, et al. (2009). "Differential regulation of threonine and tyrosine phosphorylations on protein kinase Cdelta by G-protein-mediated pathways in platelets." Biochem J **417**(1): 113-120.
Murugappan, S., H. Shankar, et al. (2005). "Molecular mechanism and functional implications of thrombin-mediated tyrosine phosphorylation of PKCdelta in platelets." Blood **106**(2): 550-557.
Murugappan, S., F. Tuluc, et al. (2004). "Differential role of protein kinase C delta isoform in agonist-induced dense granule secretion in human platelets." J Biol Chem **279**(4): 2360-2367.
Mustard, J. F., D. W. Perry, et al. (1975). "Factors responsible for ADP-induced release reaction of human platelets." Am J Physiol **228**(6): 1757-1765.
Nagy, B., Jr., K. Bhavaraju, et al. (2009). "Impaired activation of platelets lacking protein kinase C-theta isoform." Blood **113**(11): 2557-2567.
Nakahata, N. (2008). "Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology." Pharmacol Ther **118**(1): 18-35.
Nakanishi-Matsui, M., Y. W. Zheng, et al. (2000). "PAR3 is a cofactor for PAR4 activation by thrombin." Nature **404**(6778): 609-613.
Nakao, K. and A. A. Angrist (1968). "Membrane surface specialization of blood platelet and megakaryocyte." Nature **217**(5132): 960-961.
Newton, A. C. (1997). "Regulation of protein kinase C." Curr Opin Cell Biol **9**(2): 161-167.
Newton, A. C. (2003). "Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm." Biochem J **370**(Pt 2): 361-371.
Nieswandt, B., C. Brakebusch, et al. (2001). "Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen." EMBO J **20**(9): 2120-2130.
Nieswandt, B. and S. P. Watson (2003). "Platelet-collagen interaction: is GPVI the central receptor?" Blood **102**(2): 449-461.
Nixon, J. S., J. Bishop, et al. (1992). "The design and biological properties of potent and selective inhibitors of protein kinase C." Biochem Soc Trans **20**(2): 419-425.
Offermanns, S. (2006). "Activation of platelet function through G protein-coupled receptors." Circ Res **99**(12): 1293-1304.
Packham, M. A., N. L. Bryant, et al. (1989). "Effect of the concentration of Ca2+ in the suspending medium on the responses of human and rabbit platelets to aggregating agents." Thromb Haemost **62**(3): 968-976.
Pasquet, J. M., B. Gross, et al. (1999). "LAT is required for tyrosine phosphorylation of phospholipase cgamma2 and platelet activation by the collagen receptor GPVI." Mol Cell Biol **19**(12): 8326-8334.
Patel, S. R., J. H. Hartwig, et al. (2005). "The biogenesis of platelets from megakaryocyte proplatelets." J Clin Invest **115**(12): 3348-3354.
Paul, B. Z., J. Jin, et al. (1999). "Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors." J Biol Chem **274**(41): 29108-29114.
Pears, C. J., K. Thornber, et al. (2008). "Differential roles of the PKC novel isoforms, PKCdelta and PKCepsilon, in mouse and human platelets." PLoS One **3**(11): e3793.

Pelletier, A. J., S. C. Bodary, et al. (1992). "Signal transduction by the platelet integrin alpha IIb beta 3: induction of calcium oscillations required for protein-tyrosine phosphorylation and ligand-induced spreading of stably transfected cells." Mol Biol Cell **3**(9): 989-998.

Polanowska-Grabowska, R., C. G. Simon, Jr., et al. (1999). "Platelet adhesion to collagen type I, collagen type IV, von Willebrand factor, fibronectin, laminin and fibrinogen: rapid kinetics under shear." Thromb Haemost **81**(1): 118-123.

Polgar, J., W. S. Lane, et al. (2003). "Phosphorylation of SNAP-23 in activated human platelets." J Biol Chem **278**(45): 44369-44376.

Pollitt, A. Y., B. Grygielska, et al. (2010). "Phosphorylation of CLEC-2 is dependent on lipid rafts, actin polymerization, secondary mediators, and Rac." Blood **115**(14): 2938-2946.

Pollock, W. K., S. O. Sage, et al. (1987). "Stimulation of Ca2+ efflux from fura-2-loaded platelets activated by thrombin or phorbol myristate acetate." FEBS Lett **210**(2): 132-136.

Poole, A., J. M. Gibbins, 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): 2333-2341.

Poole, A. W., G. Pula, et al. (2004). "PKC-interacting proteins: from function to pharmacology." Trends Pharmacol Sci **25**(10): 528-535.

Poole, A. W. and S. P. Watson (1995). "Regulation of cytosolic calcium by collagen in single human platelets." Br J Pharmacol **115**(1): 101-106.

Protty, M. B., N. A. Watkins, et al. (2009). "Identification of Tspan9 as a novel platelet tetraspanin and the collagen receptor GPVI as a component of tetraspanin microdomains." Biochem J **417**(1): 391-400.

Pula, G., D. Crosby, et al. (2005). "Functional interaction of protein kinase Calpha with the tyrosine kinases Syk and Src in human platelets." J Biol Chem **280**(8): 7194-7205.

Pula, G., K. Schuh, et al. (2006). "PKCdelta regulates collagen-induced platelet aggregation through inhibition of VASP-mediated filopodia formation." Blood **108**(13): 4035-4044.

Pulcinelli, F. M., M. T. Ciampa, et al. (1999). "Concomitant activation of Gi protein-coupled receptor and protein kinase C or phospholipase C is required for platelet aggregation." FEBS Lett **460**(1): 37-40.

Quek, L. S., J. M. Pasquet, 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.

Quinton, T. M., S. Kim, et al. (2002). "Protein kinase C- and calcium-regulated pathways independently synergize with Gi pathways in agonist-induced fibrinogen receptor activation." Biochem J **368**(Pt 2): 535-543.

Ramanathan, G., S. Gupta, et al. (2011). "Defective Diacylglycerol-Induced Ca(2+) Entry but Normal Agonist-Induced Activation Responses in TRPC6-Deficient Mouse Platelets." J Thromb Haemost.

Ravid, K., J. Lu, et al. (2002). "Roads to polyploidy: the megakaryocyte example." J Cell Physiol **190**(1): 7-20.

Reed, G. L., M. L. Fitzgerald, et al. (2000). "Molecular mechanisms of platelet exocytosis: insights into the "secrete" life of thrombocytes." Blood **96**(10): 3334-3342.

Reed, G. L., A. K. Houng, et al. (1999). "Human platelets contain SNARE proteins and a Sec1p homologue that interacts with syntaxin 4 and is phosphorylated after thrombin activation: implications for platelet secretion." Blood **93**(8): 2617-2626.

Ren, Q., S. Ye, et al. (2008). "The platelet release reaction: just when you thought platelet secretion was simple." Curr Opin Hematol **15**(5): 537-541.

Rink, T. J. and S. O. Sage (1990). "Calcium signaling in human platelets." Annu Rev Physiol **52**: 431-449.

Rivera, J., M. L. Lozano, et al. (2009). "Platelet receptors and signaling in the dynamics of thrombus formation." Haematologica **94**(5): 700-711.

Rosado, J. A. and S. O. Sage (2000). "Protein kinase C activates non-capacitative calcium entry in human platelets." J Physiol **529 Pt 1**: 159-169.

Ryu, S. H., U. H. Kim, et al. (1990). "Feedback regulation of phospholipase C-beta by protein kinase C." J Biol Chem **265**(29): 17941-17945.

Sachs, U. J. and B. Nieswandt (2007). "In vivo thrombus formation in murine models." Circ Res **100**(7): 979-991.

Samuelsson, B., M. Goldyne, et al. (1978). "Prostaglandins and thromboxanes." Annu Rev Biochem **47**: 997-1029.

Sargeant, P. and S. O. Sage (1994). "Calcium signalling in platelets and other nonexcitable cells." Pharmacol Ther **64**(3): 395-443.

Sarratt, K. L., H. Chen, et al. (2005). "GPVI and alpha2beta1 play independent critical roles during platelet adhesion and aggregate formation to collagen under flow." Blood **106**(4): 1268-1277.

Savage, B., E. Saldivar, et al. (1996). "Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor." Cell **84**(2): 289-297.

Shattil, S. J. and L. F. Brass (1987). "Induction of the fibrinogen receptor on human platelets by intracellular mediators." J Biol Chem **262**(3): 992-1000.

Shin, Y. and T. Morita (1998). "Rhodocytin, a functional novel platelet agonist belonging to the heterodimeric C-type lectin family, induces platelet aggregation independently of glycoprotein Ib." Biochem Biophys Res Commun **245**(3): 741-745.

Soltoff, S. P. (2007). "Rottlerin: an inappropriate and ineffective inhibitor of PKCdelta." Trends Pharmacol Sci **28**(9): 453-458.

Soriani, A., B. Moran, et al. (2006). "A role for PKCtheta in outside-in alpha(IIb)beta3 signaling." J Thromb Haemost **4**(3): 648-655.

Stabel, S. and P. J. Parker (1991). "Protein kinase C." Pharmacol Ther **51**(1): 71-95.

Steinberg, S. F. (2008). "Structural basis of protein kinase C isoform function." Physiol Rev **88**(4): 1341-1378.

Strehl, A., I. C. Munnix, et al. (2007). "Dual role of platelet protein kinase C in thrombus formation: stimulation of pro-aggregatory and suppression of procoagulant activity in platelets." J Biol Chem **282**(10): 7046-7055.

Sugiyama, T., M. Okuma, et al. (1987). "A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia." Blood **69**(6): 1712-1720.

Sun, Z., C. W. Arendt, et al. (2000). "PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes." Nature **404**(6776): 402-407.

Suzuki-Inoue, K., G. L. Fuller, et al. (2006). "A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2." Blood **107**(2): 542-549.

Suzuki-Inoue, K., O. Inoue, et al. (2011). "Novel platelet activation receptor CLEC-2: from discovery to prospects." J Thromb Haemost **9 Suppl 1**: 44-55.

Suzuki-Inoue, K., Y. Kato, et al. (2007). "Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells." J Biol Chem **282**(36): 25993-26001.

Suzuki-Inoue, K., D. Tulasne, et al. (2002). "Association of Fyn and Lyn with the proline-rich domain of glycoprotein VI regulates intracellular signaling." J Biol Chem **277**(24): 21561-21566.

Tabuchi, A., A. Yoshioka, et al. (2003). "Direct demonstration of involvement of protein kinase Calpha in the Ca2+-induced platelet aggregation." J Biol Chem **278**(29): 26374-26379.

Takahara, K., R. Murray, et al. (1990). "The response to thromboxane A2 analogues in human platelets. Discrimination of two binding sites linked to distinct effector systems." J Biol Chem **265**(12): 6836-6844.

Tanaka, M., S. Sagawa, et al. (2004). "Synthesis of anilino-monoindolylmaleimides as potent and selective PKCbeta inhibitors." Bioorg Med Chem Lett **14**(20): 5171-5174.

Tomlinson, M. G., L. P. Kane, et al. (2004). "Expression and function of Tec, Itk, and Btk in lymphocytes: evidence for a unique role for Tec." Mol Cell Biol **24**(6): 2455-2466.

Tomlinson, M. G., T. Kurosaki, et al. (1999). "Reconstitution of Btk signaling by the atypical tec family tyrosine kinases Bmx and Txk." J Biol Chem **274**(19): 13577-13585.

Toullec, D., P. Pianetti, et al. (1991). "The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C." J Biol Chem **266**(24): 15771-15781.

Unsworth, A. J., H. Smith, et al. (2011). "Submaximal Inhibition of Protein Kinase C Restores ADP-induced Dense Granule Secretion in Platelets in the Presence of Ca2+." J Biol Chem **286**(24): 21073-21082.

van der Meijden, P. E., S. M. Schoenwaelder, et al. (2008). "Dual P2Y 12 receptor signaling in thrombin-stimulated platelets--involvement of phosphoinositide 3-kinase beta but not gamma isoform in Ca2+ mobilization and procoagulant activity." FEBS J **275**(2): 371-385.

Varga-Szabo, D., A. Braun, et al. (2008). "The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction." J Exp Med **205**(7): 1583-1591.

Varga-Szabo, D., A. Braun, et al. (2009). "Calcium signaling in platelets." J Thromb Haemost **7**(7): 1057-1066.

Vial, C., M. G. Rolf, et al. (2002). "A study of P2X1 receptor function in murine megakaryocytes and human platelets reveals synergy with P2Y receptors." Br J Pharmacol **135**(2): 363-372.

Vitrat, N., K. Cohen-Solal, et al. (1998). "Endomitosis of human megakaryocytes are due to abortive mitosis." Blood **91**(10): 3711-3723.

Wagner, C. L., M. A. Mascelli, et al. (1996). "Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets." Blood **88**(3): 907-914.

Walker, T. R. and S. P. Watson (1993). "Synergy between Ca2+ and protein kinase C is the major factor in determining the level of secretion from human platelets." Biochem J **289 (Pt 1)**: 277-282.

Wan, T. C., M. Zabe, et al. (2003). "Plasma membrane Ca2+-ATPase isoform 4b is phosphorylated on tyrosine 1176 in activated human platelets." Thromb Haemost **89**(1): 122-131.

Wang, Q. J. (2006). "PKD at the crossroads of DAG and PKC signaling." Trends Pharmacol Sci **27**(6): 317-323.

Watson, S. P. (1999). "Collagen receptor signaling in platelets and megakaryocytes." Thromb Haemost **82**(2): 365-376.

Watson, S. P., J. M. Auger, et al. (2005). "GPVI and integrin alphaIIb beta3 signaling in platelets." J Thromb Haemost **3**(8): 1752-1762.

Watson, S. P., J. M. Herbert, et al. (2010). "GPVI and CLEC-2 in hemostasis and vascular integrity." J Thromb Haemost **8**(7): 1456-1467.

Wendling, F. and Z. C. Han (1997). "Positive and negative regulation of megakaryocytopoiesis." Baillieres Clin Haematol **10**(1): 29-45.

Wilkinson, S. E., P. J. Parker, et al. (1993). "Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C." Biochem J **294 (Pt 2)**: 335-337.

Wonerow, P., A. Obergfell, et al. (2002). "Differential role of glycolipid-enriched membrane domains in glycoprotein VI- and integrin-mediated phospholipase Cgamma2 regulation in platelets." Biochem J **364**(Pt 3): 755-765.

Woodside, D. G., A. Obergfell, et al. (2001). "Activation of Syk protein tyrosine kinase through interaction with integrin beta cytoplasmic domains." Curr Biol **11**(22): 1799-1804.

Woodside, D. G., A. Obergfell, et al. (2002). "The N-terminal SH2 domains of Syk and ZAP-70 mediate phosphotyrosine-independent binding to integrin beta cytoplasmic domains." J Biol Chem **277**(42): 39401-39408.

Woulfe, D., J. Yang, et al. (2001). "ADP and platelets: the end of the beginning." J Clin Invest **107**(12): 1503-1505.

Yacoub, D., J. F. Theoret, et al. (2006). "Essential role of protein kinase C delta in platelet signaling, alpha IIb beta 3 activation, and thromboxane A2 release." J Biol Chem **281**(40): 30024-30035.

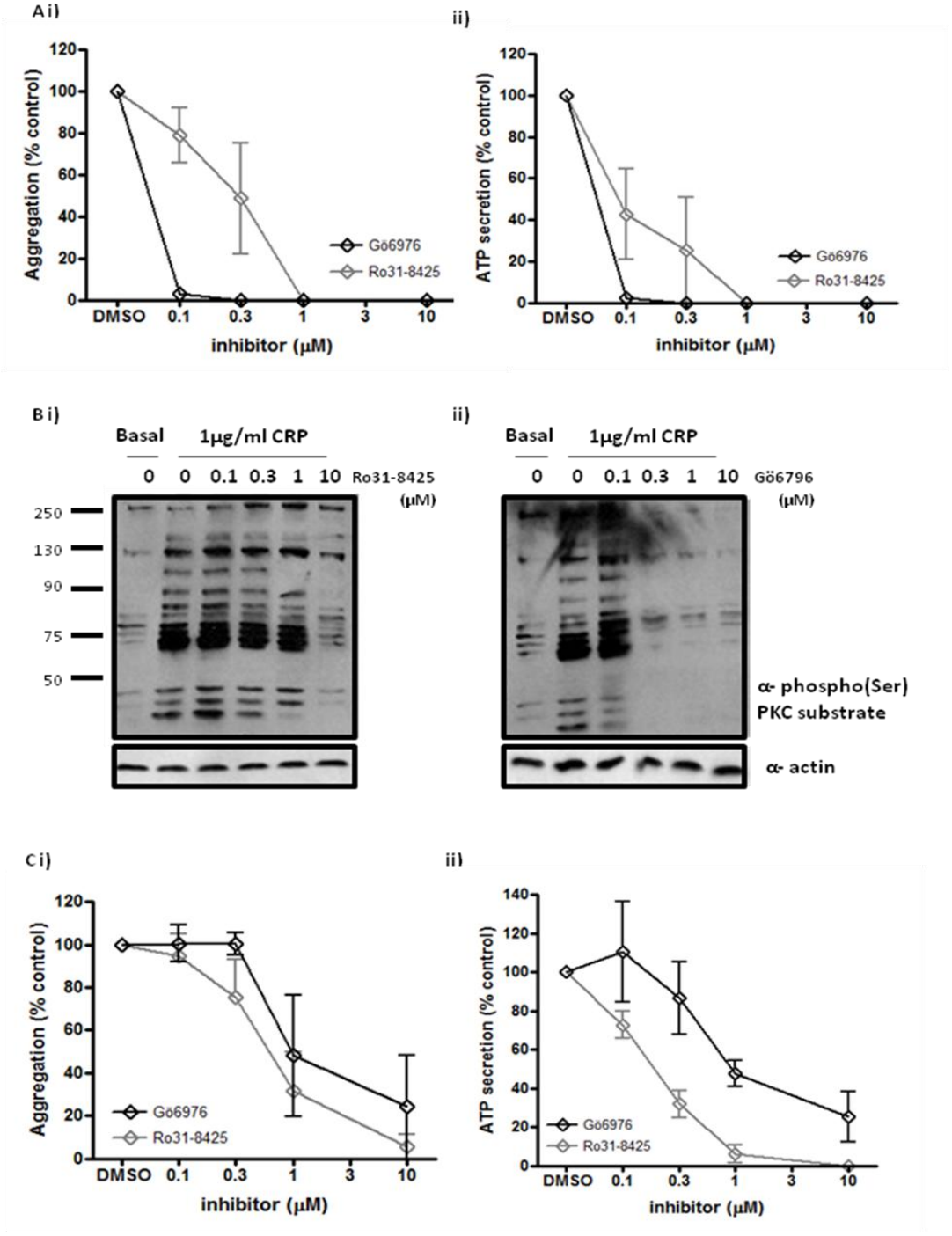
Yanaga, F., A. Poole, et al. (1995). "Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fc gamma-IIA receptor." Biochem J **311 (Pt 2)**: 471-478.

Yoshioka, A., R. Shirakawa, et al. (2001). "Identification of protein kinase Calpha as an essential, but not sufficient, cytosolic factor for Ca2+-induced alpha- and dense-core granule secretion in platelets." J Biol Chem **276**(42): 39379-39385.

Zanin-Zhorov, A., Y. Ding, et al. (2010). "Protein kinase C-theta mediates negative feedback on regulatory T cell function." Science **328**(5976): 372-376.

APPENDIX - I

Appendix -1 Effect of Gö6976 on human platelet activation. Washed human platelets were pre-treated with increasing concentrations Gö6976 or Ro31-8425 and stimulated with (A and B) CRP (1µg/ml) or (C) 0.1U/ml thrombin. (A and C) Dose response curves for each inhibitor are shown, i) aggregation and ii) dense granule secretion. Effects are expressed as percentages of control condition, mean ± SEM. (B) Effect of (i) Ro31-8425 and (ii) Gö6976 on PKC substrate phosphorylation detected using α-phospho(Ser)-PKC substrate antibody. Whole cell lysates resolved by SDS-PAGE and subjected to western blot analysis. Actin was used as a loading control. Representative blots shown. ($n \geq 3$).



APPENDIX - II

PUBLICATIONS ARISING FROM THIS THESIS

UNSWORTH A.J, SMITH H, GISSEN P, WATSON S.P, PEARS C.J, (2011).

"Submaximal Inhibition of Protein Kinase C Restores ADP-Induced Dense Granule Secretion in Platelets in the Presence of Ca^{2+}." J. Biol. Chem 286(24): 21073-21082.

(Appendix II)