The Role of Glucocorticoids in Platelet Function

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Abstract

Platelets are pivotal in regulating haemostasis, but can precipitate atherothrombosis associated to cardiovascular diseases. The synthetic glucocorticoid prednisolone is widely used as an anti-inflammatory and immunosuppressive drug. Previously it was shown that prednisolone inhibited platelet aggregation and adhesion under conditions of flow, although the mechanisms remained unclear. In the present study we examined the mechanisms responsible for the inhibitory effects of prednisolone on thrombin-mediated platelet activation. Prednisolone caused concentration-dependent inhibition of thrombin-induced platelet aggregation. The inhibition of aggregation by prednisolone was rapid suggesting a non-genomic mode of action of the glucocorticoid on platelets. Prednisolone also targeted the protease-activated receptors PAR1 and PAR4 that mediate platelet activation following thrombin stimulation. Thrombin triggers two distinct signalling cascades in platelets resulting in the phosphorylation of myosin light chains (MLC). One of these pathways is calcium-dependent, while the other is RhoA/ROCK-dependent. In order to understand the molecular mechanism underpinning the inhibitory effects of prednisolone, we examined the RhoA/ROCK pathway. Stimulation of platelets with thrombin led to the RhoA/RhoA kinase (ROCK)-dependent phosphorylation of MLC. Pre-treatment of platelets with prednisolone caused a concentration-dependent inhibition of MLC-ser^{19} phosphorylation. The inhibition was rapid and transient. Consistent with this observation, prednisolone also reduced the inhibitory phosphorylation of the myosin light chain phosphatase (MLCP) at two key residues thr^{696} and thr^{853}. In all cases the effects of prednisolone were inhibited by the glucocorticoid receptor antagonist RU486. Finally, prednisolone also inhibited RhoA activation in platelets following thrombin stimulation. Thus, prednisolone inhibited platelet activation by targeting RhoA/ROCK-mediated signalling events following thrombin stimulation. Modulation of the RhoA activity represents one of the non-genomic effects of this synthetic glucocorticoid on platelets and these might have important clinical implications in the treatment of cardiovascular diseases.

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Abbreviations

11β-HSD	11β-hydroxysteroid dehydrogenase
$\alpha_{IIb}\beta_3$	Integrin alpha IIb beta 3
$\alpha_2\beta_1$	Integrin alpha 2 beta 1
AC	Adenylate cyclase
ACD	Acid citrate dextrose
ADP	Adenosine 5'-diphosphate
AF-1	Activation function-1
ALL	Acute lymphoblastic leukemia
AP-1	Activation protein-1
APS	Ammonium persulfate
ATP	Adenosine 5'-triphosphate
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) Bis N,N-methylenebis-acrylamide
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CD34	Cluster of differentiation 34
CD41	Cluster of differentiation 41
cGMP	Cyclic guanosine monophosphate
CLP	Common leukocyte progenitor
CMP	Common myeloid progenitor
COX-1	Cyclooxygenase-1
CRP	Collagen related peptide
CVD	Cardiovascular disease
DAG	1, 2 diacylglycerol
DBD	DNA binding domain
DMS	Demarcation membrane system
DMSO	Dimethyl sulfoxide
DTS	Dense tubular system
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethyleneglycoltetraacetic acid
ERK	Extracellular signal-regulated kinase
FcR γ-chain	Fc receptor gamma chain
FITC	Fluorescein isothiocyanate
FVa	Activated factor V
Gads	GRB2-related adaptor protein
GAP	GTPase-activating protein
GATA-1	GATA-binding protein 1
GDI	GTPase-dissociation inhibitors
GDP	Guanosine diphosphate
GP	Glycoprotein
GPIb-V-IX	Glycoprotein Ib-V-IX receptor complex
GPCR	G protein coupled receptor
GC	Glucocorticoid
GCR	Glucocorticoid receptor
GPVI	Glycoprotein VI
GRE	Glucocorticoid response element
GTP	Guanine 5'-triphsphate
HAB	Haemangioblast
HEPES	N-(2-Hydroxyethyl) piperayine-N'-(2ethanesulfonic acid)
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell
HSP	Heat shock protein
IgG	Immunoglobulin G
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IP_3	Inositol 1,5,4-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun amino-terminal kinase
LAT	Linker for activation of T cells
LBD	Ligand binding domain
LIMP-II	Lysosomal integral membrane protein-II
LPA	Lysophosphatidic acid

MAPK	Mitogen activated protein kinases
MCR	Mineralocorticoid receptor
MEP	Megakaryocyte-erythrocyte progenitor
Mk	Megakaryocyte
MKP	Megakaryocyte progenitor
ML-7	1-(5-Iodonaphthalene-1-sulfonyl)homopiperazine
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MYPT1	Myosin phosphatase targeting subunit 1
NF-κB	Nuclear factor κB
NLS	Nuclear localization signal
NO	Nitric oxide
NR3C1	Nuclear receptor subfamily 3 group C member 1
NTD	N- (amino-) terminal domain
OCS	Open canalicular system
PAR1	Protease-activated receptor-1
PAR4	Protease-activated receptor-4
PBMC	Peripheral blood mononuclear cells
PC	Phosphatidylcholine
PDE	Phosphodiesterase
PE	Phycoerythrin
PGE_1	Prostaglandin E_1
PGI_2	Prostacyclin
PI3K	Phosphatidylinositol 3-kinase
PI-4,5-P_2	Phosphoinositide 4,5 bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA_2	Phospholipase A_2
PLCβ	Phospholipase C beta
PLCγ2	Phospholipase C gamma 2
PMSF	Phenyl methyl sulphonyl fluride
PPP	Platelet poor plasma

PRP	Platelet rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand 1
PVDF	Polyvinylidene fluoride
RBC	Red blood cell
RGDS	Arginine-glycine-aspartic acid-serine
ROCK	Rho kinase
RhoGEF	Rho guanine nucleotide exchange factor
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
SH2	Src homology 2
SLP-76	Src homology 2 domain-containing leukocyte protein of 76kDa
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
Syk	Spleen tyrosine kinase
TBS-T	Tris-buffered saline-tween
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF-α	Tumor necrosis factor α
TPO	Thrombopoietin
TSP-1	Thrombospondin-1
TXA2	Thromboxane A2
VASP	Vasodilator-stimulated phosphoprotein
vWF	von Willebrand factor
WCL	Whole cell lysate
Y27632	(R)-(+)-*trans*-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide

Amino acid abbreviations

A	**Ala**	Alanine
C	**Cys**	Cysteine
D	**Asp**	Aspartic acid
E	**Glu**	Glutamic acid
F	**Phe**	Phenylalanine
G	**Gly**	Glycine
H	**His**	Histidine
I	**Ile**	Isoleucine
K	**Lys**	Lysine
L	**Leu**	Leucine
M	**Met**	Methionine
N	**Asn**	Asparagine
P	**Pro**	Proline
Q	**Gln**	Glutamine
R	**Arg**	Arginine
S	**Ser**	Serine
T	**Thr**	Threonine
V	**Val**	Valine
W	**Trp**	Tryptophan
Y	**Tyr**	Tyrosine

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Authors declaration

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Chapter 1

Introduction

1.1 General introduction

Haemostasis is a vital physiological process that operates to maintain the equilibrium and proper functioning of the circulatory system. The principle objectives of haemostasis are to ensure the integrity of blood vessels and preserve the blood reservoir of the body. Several elements work in coordination to achieve these objectives, including the blood vessel wall, a range of plasma proteins that constitute the coagulation cascade and blood platelets. Platelets participate in the formation of a primary haemostatic plug that seals the site of vascular injury to prevent further blood loss. The vascular endothelium, which lines the blood vessels, serves as a barrier between the circulating blood and the underlying tissues and releases chemicals into the blood to maintain its fluid state. Upon damage to the endothelium the blood interacts with extracellular matrix, which contains proteins that act to capture and activate platelets. The subsequent activation of the coagulation cascade leads to the formation of fibrin. The fibrin network surrounds and permeates the platelets leading to stabilisation of the platelet plug and later contracts to allow wound healing by bringing together the damage points.

When a vascular damage occurs as a result of physical injury platelets act as benefactors by participating in blood clot and initiating the healing process. However, pathological vascular damage associated with the rupture of atherosclerotic plaques leads to unregulated platelet activation and arterial thrombosis, which is the underlying pathology that causes a majority of cardiovascular associated disorders (CVDs). Hence, the study of platelets and their function is imperative for the understanding of thrombotic disorders in the field of cardiovascular research.

1.2 Platelets

Platelets are small anucleate blood cells, which are of paramount importance in haemostasis and also contribute to immune response, inflammation and cancer. In the following sections the important features of platelets including their structure and how they are intimately associated with haemostasis will be discussed.

1.2.1 Platelet biogenesis

Platelets originate from progenitor cells called megakaryocytes (Mks). These are the largest among the various hematopoietic cells, with cell diameters ranging from 20-60 µm. Mks are generated as a consequence of sequential differentiation and specific lineage commitment of pluripotent haematopoietic stem cells (HSCs) through the intermediate bipotent haematopoietic progenitors called megakaryocyte-erythrocyte progenitors (MEPs) (Figure 1.1 A). Mk development, commonly referred to as megakaryopoiesis, occurs primarily in the adult bone marrow and to a much lesser degree in the spleen. Although Mks were shown to terminally differentiate to yield platelets, the exact mechanism of platelet release from Mks had been a subject of wide speculations for years. One theory suggested that there remained specific areas within the Mk cytoplasm, which during the course of Mk maturation, fragmented to release platelets (Tavassoli, 1980). Other investigations pointed towards the phenomenon of platelet release through the intermediate step of proplatelet formation (Tablin et al., 1990). Over the course of years, the second theory became the most accepted due to detailed characterisation. Proplatelets have been visualised as long beaded cytoplasmic projections of variable thickness and appear to be branched at several points (Figure 1.1 B). The process of platelet release from Mks was shown to occur within the bloodstream (Behnke and Forer, 1998) as well as other sites such as the bone marrow sinusoids (Lichtman et al., 1978; Tavassoli, 1980) and the lung capillary bed (Zucker-Franklin and Philipp, 2000).

As Mks progress through the life cycle, they undergo extensive cellular transformation and maturation in order to be prepared for generating platelets. One of the striking features of a maturing Mk is the occurrence of endomitosis,

where the nucleus undergoes several rounds of prematurely terminated mitosis, thus resulting in a polyploid nucleus (Nagata et al., 1997; Vitrat et al., 1998). A typical Mk can display at least 16N nucleus (Therman et al., 1983). Endomitosis is followed by a number of maturation events occurring in the Mk cytoplasm such as an increase in the cytoplasm volume and accumulation of platelet-specific proteins and organelles as well as the demarcation membrane system (DMS). DMS is a collection of Mk cytoplasmic membranes and consists of flattened cisternae and tubules (Behnke, 1968; Shaklai and Tavassoli, 1978; Yamada, 1957). Although DMS was initially thought to be involved in delineating specific regions in the Mk cytoplasm for platelet formation (Shaklai and Tavassoli, 1978; Yamada, 1957), subsequent studies confirmed its role as membrane reserve for subsequent proplatelet formation and platelet release (Radley and Haller, 1982). DMS has been shown to express phosphoinositide 4,5 bisphosphate (PI-4,5-P_2), a principal component of plasma membranes and this corroborates the hypothesis that DTS primarily exists as a supplier of membranes required for platelet formation (Schulze et al., 2006). Mk development and platelet production is stimulated by the cytokine thrombopoietin (TPO), which appears to be crucial during the early stages of Mk maturation, especially endomitosis and functions primarily to increase platelet numbers (Gurney et al., 1994; Kaushansky, 1995; Kaushansky and Drachman, 2002).

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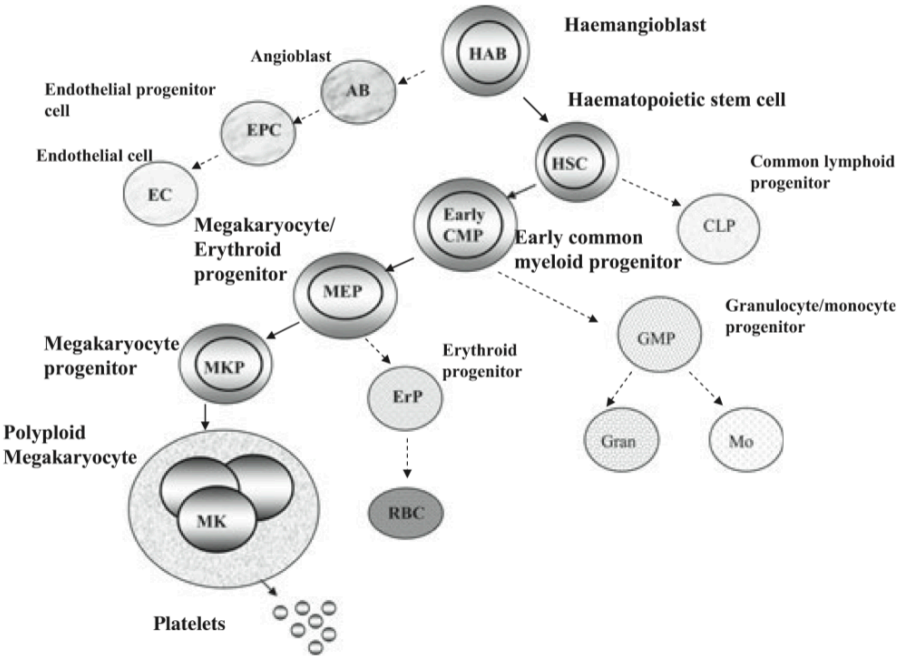

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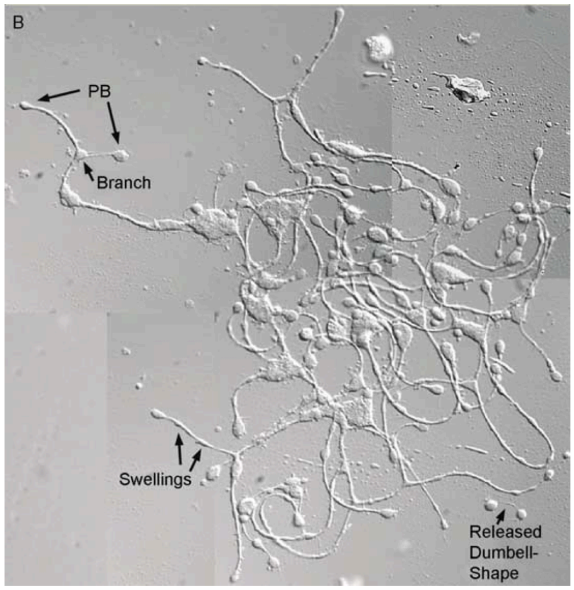

Figure 1.1 The development of platelets. (A) Megakaryocytes are developed from the haemangioblast (HAB), which gives rise to both vascular and hematopoietic stem cells (HSC). HSCs can give rise to early progenitor cells of all the haematopoietic lineages, including the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). The megakaryocyte progenitor (MKP) originates from the common megakaryocyte-erythroid progenitor (MEP). The MEP retains some characteristics of the HSC, including the expression of CD34, c-Mpl thrombopoietin receptor, erythropoietin receptor, CD41, or glycoprotein IIb or integrin receptor α_{IIb}, and is regulated by GATA-1. Solid arrows indicate the MK-platelet axis, while dashed arrows point to the other pathways. RBC indicates red blood cells, Gran granulocytes and Mo monocyte. Taken from Deutsch and Tomer, 2006. (B) Proplatelet formation from Mks and platelet budding from the proplatelet tips. Taken from Hartwig and Italiano, 2003.

1.2.2 Platelet structure

When platelets circulate in blood, they appear in the form of round discs of 2-5 µm in diameter. Initial structural studies of platelets based on light and phase contrast microscopy revealed a simple appearance of platelets where granules were shown to be dispersed in a clear fluid that was enclosed by a plasma membrane. More appropriate terms to define platelet regions came into prevalence since 1970s. Thus, 'peripheral zone' was used to describe the membrane region, 'sol-gel zone' for the internal matrix and 'organelle zone' for the various organelles and particles dispersed within the matrix (White, 1971). The structural components of platelets are all involved in their transition from round discs to a spherical conformation with an enlarged surface area that aids in further thrombus formation and clotting in the event of a vascular trauma.

1.2.2.1 Platelet membrane

The platelet surface is composed of a few layers. The outermost layer is an exterior coat commonly known as the glycocalyx, The glycocalyx is rich in acidic mucopolysaccharides (Behnke, 1967; White, 1970) and is robust enough to be retained on the platelet throughout the entire hemostatic process (White, 1970). The glycocalyx harbours a number of glycoproteins (GPs) such as the GP I/V/IX complex, GPVI and integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, which serve essential roles in platelet adhesion and aggregation. Beneath the glycocalyx lies a unit membrane. Similar to other cells the platelet membrane is a phospholipid bilayer comprising phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (Chap et al., 1979, 1977; Perret et al., 1979). An important feature of the platelet membrane is the presence of multiple invaginations, which form a convoluted network of channels through the cytoplasm called the open canalicular system (OCS) (Behnke, 1968, 1967). This increases the platelet surface available for interaction with plasma and enhances absorption and release of substances (Escolar et al., 1989; White and Krumwiede, 1987). Moreover, the OCS provides extra membrane required for platelet spreading (Behnke, 1969; White, 2007). In addition to the OCS, platelets also possess a separate channel

network called the dense tubular system (DTS). It is a remnant of megakaryocyte smooth endoplasmic reticulum (Behnke, 1968; White, 1972) and serves as a calcium storage site (White and Gerrard, 1976). Release of calcium from this store into the cytoplasm drives a number of platelet activation responses. The DTS is also the site for the generation of platelet prostaglandins and thromboxane A_2 (TXA_2) (Gerrard et al., 1976), a major contributor to platelet recruitment at sites of thrombus formation. Just beneath the unit membrane lies a submembrane area, which contributes to platelet activation by facilitating interaction of specific proteins present within this region with the cytoplasmic domains of the transmembrane receptors embedded in the glycocalyx (White, 2007). The submembrane region consists of a regular arrangement of filaments that resemble the filamentous system of microtubules (White, 1969).

1.2.2.2 Platelet cytoskeleton

The platelet cytoskeleton provides a structural framework that not only serves as an important determinant to maintain the resting platelet discoid shape but also contributes to the platelet contractile function and shape change upon platelet activation (White, 1968). Different filamentous structures are present within the platelet cytoskeleton. The first type is known as microtubules, which appears along the entire circumference of the platelet immediately beneath the peripheral zone (Behnke, 1965). Microtubules provide a structural support to the resting platelet and are responsible for maintaining its discoid shape. Upon activation, they are however displaced towards the platelet centre where they tightly encircle the organelles that also cluster at the centre (White, 2007). The second type of filamentous structure is the actin filament (F-actin), which is the result of polymerisation of several globular actin (G-actin) monomers. A part of the F-actin associates with the submembrane region thereby defining the membrane contractile region, while the rest provides cytoskeletal framework for the platelet cytoplasm. In resting platelets, the F-actin cytoskeleton acts as the matrix on which the organelles are dispersed and kept apart from each other (Escolar et al., 1986). During activation, it plays a critical role in driving platelet shape change by

compressing the microtubule coils, promoting the movement of platelet granules towards the centre and allowing granule secretion.

1.2.2.3 Platelet organelles

Platelets contain a number of organelles including mitochondria and lysosomes. In addition platelet contain specialist granules including alpha (α) granules and dense (δ) granules (Figure 1.2). The contents of these granules are released into the surrounding environment upon platelet activation. The most abundant of the platelet granules are the α-granules, of which there can be between 40-80 per platelet. These granules contain an exhaustive list of proteins important for haemostasis and other accessory functions of platelets including inflammation and wound healing. The list includes adhesive proteins synthesised by megakaryocytes, plasma-derived proteins, chemokines, growth factors, fibrinolytic molecules, immunoglobulins, protease inhibitors and coagulation factors (Harrison and Martin Cramer, 1993; McNicol and Israels, 1999) (Table 1.1). The core of some α-granules may contain proteins with binding sites for heavy metals for which they appear denser compared to the rest of the granular region. The second type of granules in the platelet cytosol is the δ-granule, which are much smaller and fewer than α-granules, with just about 4-8 per platelet. As the name suggests, these granules appear dense owing to their high electron opacity and thus can be easily distinguished from the α-granules. The δ-granules are the storehouse of various molecules important in the process of haemostasis such as adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), serotonin, Ca^{+2} and pyrophosphate (McNicol and Israels, 1999).

Besides granules, an important organelle found in the platelet cytosol is lysosome, though the numbers are few. It contains an array of hydrolytic enzymes to degrade various biomolecules or cellular particles and proteases like cathepsins, which are secreted upon platelet activation (Ciferri et al., 2000). Another organelle found in the platelet is mitochondria, which is relatively fewer in number and responsible for energy metabolism and is sufficient to support platelet energy requirements. Platelet cytosol is rich in glycogen, which appears as either individual granules or a

mass of glycogen granules randomly distributed throughout the cytosol. Some golgi-derived smooth and coated vesicles could also be seen in platelets, although their identification could be difficult as they are sparse in numbers (White, 2007).

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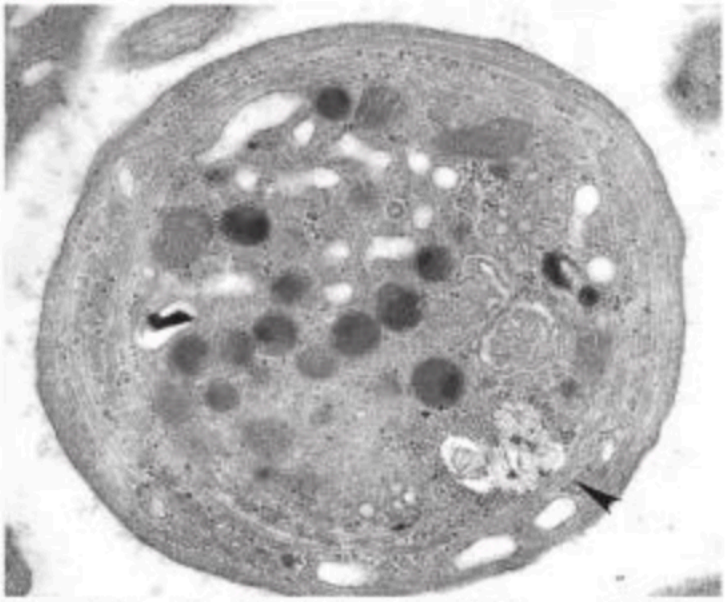

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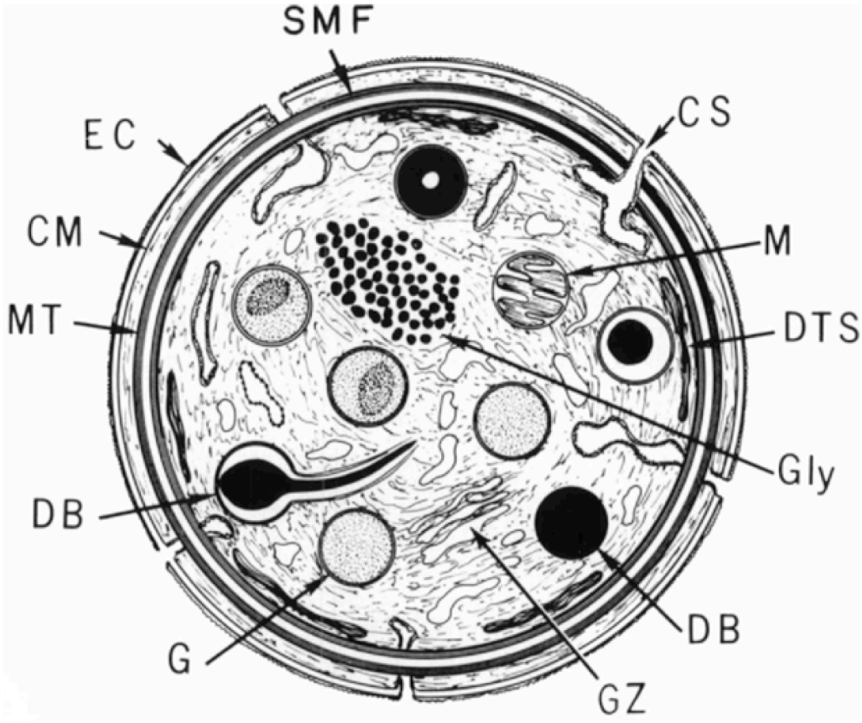


Figure 1.2 Ultrastructure of a platelet. (A) Structure of a platelet as viewed under a scanning electron microscope (SEM). The outer membrane region consists of an outermost glycocalyx, a phospholipid bilayer and a submembrane region. Beneath this is the platelet cytoskeleton composed of microtubules and actin filaments. The innermost core region consists of platelet organelles and granules. (B) A diagrammatic representation of a platelet showing the various components of platelets. EC denotes exterior coat (glycocalyx), CM unit membrane, SMF submembrane filaments, MT microtubules, G granules, DB dense bodies, GZ golgi zone, Gly glycogen particles, DTS dense tubular system, M mitochondria and CS open canalicular system. Taken from White, 1971.

α-granules	δ-granules	Lysosomes
vWF	ADP	Cathepsin D
P-selectin	ATP	Cathepsin E
Thrombospondin	Serotonin	Acid Phosphatase
Albumin	Pyrophosphate	β-D- Glucosidase
Fibrinogen	Ca^{+2}	β-D- and α-D-Galactosidase
Fibronectin		β-D- and α-L-Fucosidase
Vitronectin		β-D-Glucuronidase
Platelet factor 4 (PF4)		α-D-Mannosidase
Platelet derived growth factor (PDGF)		Carboxypeptidase A
Vascular endothelial growth factor (VEGF)		Carboxypeptidase B
Insulin growth factor (IGF)		Proline carboxypeptidase
Transforming growth factor beta (TGFβ)		
Plasminogen activator inhibitor-1 (PAI-1)		
IgG		
IgM		
IgA		
C1-inhibitor		
α_2-antitrypsin		
Platelet derived collagenase inhibitor		
Coagulation factor V		
Coagulation factor VIII		

Table 1.1 Constituents of platelet granules. Table lists the various constituents of platelet α- and δ- granules and lysosomes (Ciferri et al., 2000; McNicol and Israels, 1999).

1.2.2.4 Platelet receptors

The surface of platelets harbours several receptors that participate in the various stages of platelet plug formation. Of these glycoproteins GPVI and GPIb/V/IX and integrins $\alpha_2\beta_1$ participate in platelet adhesion, while the integrin $\alpha_{IIb}\beta_3$ is crucial for platelet aggregation. Soluble platelet agonists activate platelet through receptors that are coupled to G-proteins. These receptors have an extracellular N-terminal domain, a seven transmembrane domain and an intracellular C-terminal domain (Hamm, 1998). Agonists involved in the extension phase interact with the extracellular domain of the G-protein coupled receptors (GPCRs) on the platelet surface and expose new binding sites on this domain. This induces a conformational change at the N-terminus of the GPCR, which is transmitted through the transmembrane domains to the G-proteins associated with the GPCR on the inner surface of the platelet membrane and leads to their activation (Hamm, 1998). G-proteins are heterotrimeric units comprised of a single α, β and γ subunit. The α-subunit contains a guanine-nucleotide binding site, which is occupied by GDP in the inactive state and GTP in the active state of the receptor. The propeller-like β-subunit is tightly associated to the γ-subunit (Hamm, 1998). Upon receptor activation, G_α is partly hydrolysed from $G_{\beta\gamma}$ and this reveals binding sites on both these subunits which are targeted by downstream effectors (Ford et al., 1998; Hamm, 2001, 1998). Fatty acylation of G_α and prenylation of $G_{\beta\gamma}$ allows the receptor to associate to the plasma membrane. Hydrolysis of GTP by the intrinsic GTPase activity of G_α helps to reform the original heterotrimer.

In mammals, there are 22 genes that encode G_α, 5 genes encoding G_β and 8 genes encoding G_γ. There are 4 different sub-types of G_α that take part in the regulation of platelet function, albeit through distinct mechanisms. Human platelets express one $G_{\alpha s}$, four $G_{\alpha i}$ ($G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$ and $G_{\alpha z}$), three $G_{\alpha q}$ ($G_{\alpha q}$, $G_{\alpha 11}$ and $G_{\alpha 16}$) and two $G_{\alpha 12}$ isoforms ($G_{\alpha 12}$ and $G_{\alpha 13}$). With the exception of $G_{\alpha s}$, which stimulates adenylyl cyclase activity to inhibit platelet function, all the G_α subunits are involved in platelet activation. $G_{\alpha i}$ isoforms inhibit adenylyl cyclase activity, which results in low levels of cyclic adenosine monophosphate (cAMP) in the presence of prostanoids secreted by endothelial cells. $G_{\alpha q}$ isoforms activate the β isoform of phospholipase C

(PLCβ), which can hydrolyse PI-4,5-P_2 to generate second messengers such as 1,4,5-inositol triphosphate (IP_3) that raises cytosolic calcium (Ca^{+2}) concentration and 1,2-diacylglycerol (DAG) that activates protein kinase C (PKC). $G_{\alpha 12/13}$ isoforms participate in the regulation of the actin cytoskeleton reorganisation and platelet shape change by Rho signalling (Woulfe et al., 2004).

1.2.3 Platelet plug formation

In the event of a vascular injury the circulating blood is exposed to proteins embedded underneath the endothelial layer. This leads to platelet activation and marks the onset of platelet plug formation, which appears to proceed in three distinct phases: initiation, extension and perpetuation. The end result of the plug formation is the formation of a thick insoluble fibrin clot from soluble plasma protein called fibrinogen, which poses a strong barrier against severe blood loss (Figure 1.3).

During a blood vessel injury, the endothelial layer is damaged exposing the platelets to extracellular matrix proteins such as collagen and von Willebrand factor (VWF). This leads to the tethering and attachment of platelets on collagen, which is followed by platelet activation to form a monolayer on the collagen surface. The attachment of platelets to collagen under high shear forces, found inside arteries, is assisted by VWF, a large multimeric protein secreted by the endothelial cells (Woulfe et al., 2004). The activated platelets adhered to collagen release their granular contents, which contains ADP. In addition, platelet activation leads to the generation of thrombin (generated from plasma prothrombin via activation of the coagulation cascade) and thromboxane A_2 (TxA_2; produced from arachidonate by the cyclooxygenase pathway in platelets). These soluble platelet agonists participate in further stimulation of platelet activation through the stimulation of specific G-protein coupled receptors (GPCRs). The accumulation of these physiological agonists leads to their increased local concentration, which in turn stimulate surrounding circulating platelets and attract them towards the platelet monolayer. This results in recruitment of additional platelets to the growing plug (Woulfe et al., 2004). For the extension phase to proceed, activation of the platelet

fibrinogen receptor $\alpha_{IIb}\beta_3$ must take place so that fibrinogen can bind to activated platelets and bridge adjacent platelets in the growing platelet plug. The process of activation of integrin $\alpha_{IIb}\beta_3$ is referred to as inside-out signalling. The very last events of plug formation stabilise the platelet plug to prevent its premature disaggregation until wound healing takes place. The key players in this phase are some molecules, which generate intracellular signals only after platelets come into direct, sustained contact with each other (Woulfe et al., 2004).

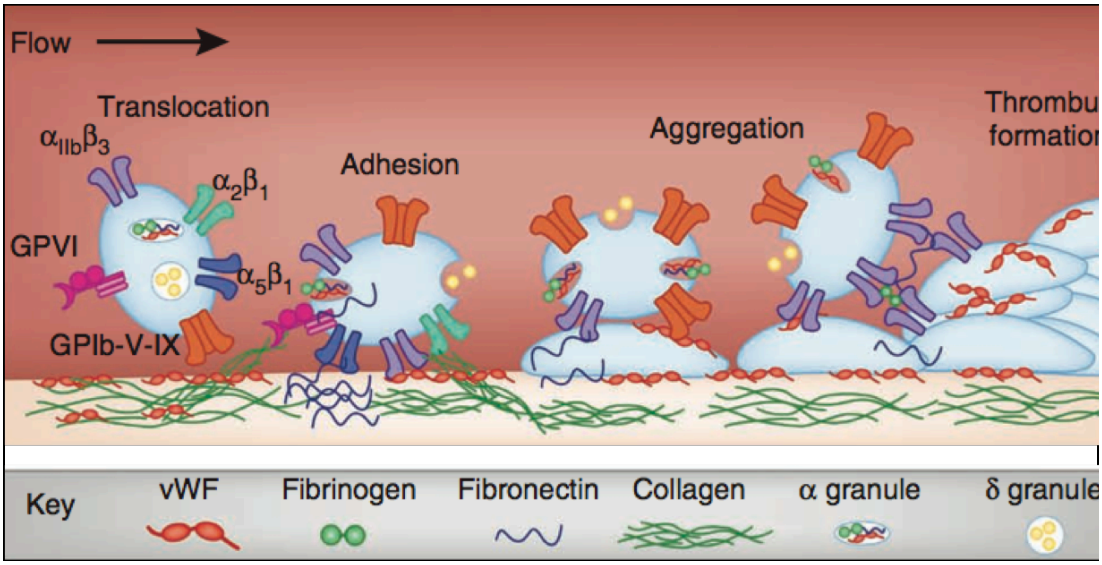


Figure 1.3 Stages of platelet plug formation. Following a vessel injury, platelets come in contact with subendothelial matrix proteins and adhere to them. Under conditions of high shear initial tethering of platelets to the surface of immobilized platelets involves a combination of interactions involving the platelet receptors GPIb/V/IX, GPVI and integrin $\alpha_2\beta_1$ with matrix proteins vWF and collagen. As a result of these interactions, the forward movement of platelets is slowed down followed by platelet rolling, translocation and initial attachment on these protein surfaces. These interactions generate activation stimuli, which result in integrin $\alpha_{IIb}\beta_3$ activation that promotes stable platelet adhesion by interaction with fibrinogen and fibronectin embedded in the matrix. Adherent platelets are activated, spread fully to form a monolayer on the damaged endothelium and release soluble agonists. Additional circulating platelets are recruited to this platelet monolayer and result in the formation of stable platelet aggregates. This is achieved through the interaction of activated platelet integrin $\alpha_{IIb}\beta_3$ with fibrinogen thereby bridging together adjacent platelets. Taken from Jackson, 2011.

1.2.4 Signalling mechanisms involved in platelet activation and thrombus formation

1.2.4.1 Signalling involved in platelet adhesion

In the event of a vascular damage when the endothelium lining blood vessels gets denuded, platelets circulating in the flowing blood are exposed directly to the protein constituents of the vessel wall. Of these proteins, the two most important ones, which are directly involved in platelet adhesion, are vWF and collagen. Platelet interaction with these proteins is greatly dependent on the type of blood vessel and the shear force generated from the flow of blood in these vessels (Varga-Szabo et al., 2008). In small arteries and arterioles where blood flows under higher shear stress and a greater velocity, platelet attachment to the exposed subendothelium is facilitated by interaction with the multimeric protein vWF. It is present in the circulating blood as well as secreted from endothelial cells and α-granules of platelets. Soluble vWF does not interact with platelets, but acquires the property when it binds to collagen under high shear force. vWF interacts with collagen types I and III through the A3 domain (Cruz et al., 1995; Lankhof et al., 1996) and with collagen type VI through the A1 domain (Denis et al., 1993; Hoylaerts et al., 1997). The principal platelet receptors that participate in interactions with vWF are the glycoprotein (GP) Ib/V/IX complex and the integrin $\alpha IIb\beta_3$. GPIb/V/IX complex is composed of four different subunits GPIbα, GPIbβ, GPV and GPIX, of which the two GPIb subunits are linked with a disulfide bridge and GPV and GPIX are covalently linked to GPIb (Modderman et al., 1992). This receptor complex on platelets interacts with the A1 domain of vWF through residues in the amino-terminus of the GPIbα subunit (Handa et al., 1986). This interaction is very rapid in onset and labile. Thus although platelets are not completely arrested on the surface, their forward movement is slowed down thereby facilitating the initial tethering of platelets. Moreover, the slow surface translocation allows platelets to become exposed to other adhesive matrix proteins on the damaged vessel for longer. Meanwhile, platelet integrins become activated and facilitate platelet interaction with other matrix proteins resulting in stable adhesion (Savage et al., 1998, 1996).

Collagen constitutes about 40% of the total proteins of the vessel wall, where it is found in various types mainly types I, III, IV, V and VI. The two most important collagen receptors on platelets are integrin $\alpha_2\beta_1$ (Nieuwenhuis et al., 1985; Santoro, 1986) and GPVI (Gibbins et al., 1997). These receptors engage in stable platelet adhesion to the damaged vessel wall and platelet activation in a two-step, two-site model as proposed by Santoro et al., 1991. The participation of the receptors is shear-dependent. At high shear vWF mediates platelet rolling, which slows them down allowing low-affinity interaction of platelets with GPVI. This interaction provides enough signal strength for the activation of integrin $\alpha_2\beta_1$, eventually exposed on the platelet surface, through which platelets firmly adhere to collagen with high affinity. At low shear, GPVI can perform this function without the need for vWF. Interaction between the platelet receptor GPVI with collagen generates intracellular signalling cascades that lead to platelet activation, secretion and aggregation. Since GPVI can activate integrin $\alpha_2\beta_1$, it is believed to play a key role in platelet adhesion and aggregation to fibrillar and soluble collagen (Nieswandt et al., 2001). Moreover, platelet activation mediated by either GPVI or other G-protein coupled receptors (GPCRs) is also believed to switch integrin $\alpha_2\beta_1$ from a low-affinity state to a high-affinity state (Jung and Moroi, 2000, 1998). Thus, both these platelet collagen receptors seem to cooperate and strengthen each other's effects (Varga-Szabo et al., 2008).

GPVI is a member of the immunoglobulin (Ig) superfamily (Clemetson et al., 1999) and associated noncovalently to the F_c receptor (F_cR) γ-chain (Gibbins et al., 1997). The F_cR γ-chain contains an immunoreceptor tyrosine-based activation motif (ITAM), which serves as a substrate for phosphorylation by tyrosine kinases and activation of the receptor. When collagen binds to platelets on GPVI, it causes clustering of the GPVI receptor and its associated γ-chain. This leads to the phosphorylation of the F_cR γ-chain at residues within the ITAM (Gibbins et al., 1996, 1997) by Src-family tyrosine kinases Fyn and Lyn (Briddon and Watson, 1999; Quek et al., 2000), which are constitutively associated to GPVI via their Src-homology 3 (SH3) domains (Ezumi et al., 1998; Suzuki-Inoue et al., 2002). This generates a phosphotyrosine motif that is recognised by the tyrosine kinase Syk, which in turn

binds to this motif via its tandem SH2 domains (Shiue et al., 1995) and gets activated through autophosphorylation (Gibbins et al., 1996; Gibbins, 2004). Syk then phosphorylates a number of adaptor molecules including LAT (linker for activator of T-cells), SLP-76 (Gross et al., 1999a, 1999b), Gads and Grb2, that provide a framework on which several other signalling molecules can assemble. Of these molecules, Phospholipase Cγ2 (PLCγ2) is phosphorylated by Syk or Tec family of kinases Bruton tyrosine kinase (Btk) (Quek et al., 1998) mainly at tyrosine^{753} and tyrosine^{759} (Suzuki-Inoue et al., 2004). Activated PLCγ2 can then hydrolyse membrane phospholipids like PI-4,5-P_2 to form second messengers IP_3 and DAG. IP_3 then stimulates intracellular mobilisation of Ca^{+2} from the dense tubular system across platelet plasma membrane and thus the intracellular Ca^{+2} concentration within the adherent platelets is raised. DAG activates PKC. These two events are very important for complete platelet activation, secretion and aggregation (Gibbins, 2004; Rivera et al., 2009).

The essential roles of GPVI, F_cR γ-chain and Syk has been demonstrated in collagen-mediated stimulatory signalling in platelets through knock-out mouse models. Platelet response to collagen stimulation has been shown to be defective in mice lacking GPVI (Kato et al., 2003) and in patients expressing low amounts of or completely lacking GPVI (Arai et al., 1995; Moroi et al., 1989). Platelet secretion and aggregation in response to collagen were also inhibited in mice lacking either F_cR γ-chain or Syk, although thrombin-mediated responses were unaffected (Poole et al., 1997). Similarly, PLCγ2 phosphorylation and downstream signalling events have also been shown to be greatly impaired in mice lacking the adaptor molecules like SLP-76 and LAT, with greater dependency observed on SLP-76 (Gross et al., 1999a; Judd et al., 2002). Absence of PLCγ2 leads to defects in platelet aggregation (Suzuki-Inoue et al., 2003).

Collagen also supports thrombin generation indirectly, since platelets upon interaction with collagen expose negative charged phospholipids such as phosphatidylserine on their surface. This provides catalytic surface for the further assembly of the components of coagulation cascades and thrombin generation (Farndale et al., 2004).

1.2.4.2 Signalling involved in platelet shape change

The earliest platelet activation response followed by platelet attachment on thrombogenic surfaces or interaction with soluble agonists is shape change. It is initiated with the transition of the platelet discoid shape to a spherical one and followed by formation of finger-like projections called filopodia and web-like structures called lamellipodia to fill the spaces in between filopodia (Figure 1.4 A). These events ultimately give rise to a spread platelet with a greater surface area to accommodate the recruitment of additional platelets for thrombus formation. Shape change is an outcome of the reorganisation of the actin-based platelet cytoskeleton and formation of new actin filaments by polymerisation. Cross-linking of actin filaments during polymerisation is achieved by its interaction with the contractile protein myosin IIA in an ATP-dependent manner (Figure 1.4 Bii). Myosin IIA consists of three pairs of peptide chains namely the heavy chain (230 KDa), two essential light chains, ELC (17 KDa) and two regulatory light chains, RLC (20 KDa). The structure of myosin is composed of two globular head domains, a neck domain and a rod domain (Figure 1.4 Bi). The head domain contains binding sites for both ATP and actin. The head and rod domains are connected via the neck domain, where ELCs and RLCs interact with the heavy chains. This domain acts as a lever arm to maximise the globular head movement. The rod domain consists of the heavy chain dimers. ELCs stabilise the heavy chain structure, while RLC regulate the activity of myosin (Vicente-Manzanares et al., 2009). Interaction of actin with the myosin globular head facilitates an increased ATPase activity of myosin in a Mg^{+2}-dependent manner, which is regulated by phosphorylation of the myosin regulatory light chains (Adelstein et al., 1973; Adelstein and Anne Conti, 1975; Sellers et al., 1981). Agonist stimulation of platelets has been shown to cause an increase in myosin light chain (MLC) phosphorylation followed by an increase in association of myosin with the actin cytoskeleton (Fox and Phillips, 1982). MLC phosphorylation is shown to slightly precede platelet shape change in response to various agonists and hence thought to be necessary for this functional response (Daniel et al., 1984).

Phosphorylation of MLC is controlled by two enzymes, Ca^{+2}/calmodulin-dependent myosin light chain kinase (MLCK) and Ca^{2+}-independent myosin light chain

phosphatase (MLCP) (Hathaway and Adelstein, 1979). When platelets are stimulated by various agonists through the activation of $G_{\alpha q}$-coupled receptors, an increase in intracellular Ca^{+2} levels activates MLCK, which phosphorylates MLC on a specific residue, ser^{19}. To ensure that phosphorylation is maintained for subsequent shape change events, a $G_{\alpha 12/13}$/RhoA/ROCK-mediated signalling pathway causes simultaneous inhibition of MLCP (Klages et al., 1999). Agonist stimulation leads to co-activation of $G_{\alpha 12/13}$-coupled receptors, which is followed by activation of RhoA, a small GTPase. RhoA is associated to GDP in its inactive form and GTP in its active form. RhoA activation induces activation of the Rho kinase (ROCK) enzyme, which phosphorylates MLCP on two key inhibitory residues, Thr^{696} and Thr^{853} within its targeting subunit (MYPT1) followed by MLCP inactivation (Feng et al., 1999; Kimura et al., 1996; Lincoln, 2007; Nakai et al., 1997; Suzuki et al., 1999). Hence MLCP can no longer dephosphorylate MLC under such conditions. Thus MLC phosphorylation is achieved through the coordinated events of MLCK activation and MLCP inactivation following co-stimulation of $G_{\alpha q}$ and $G_{\alpha 13}$ pathways in response to agonists such as thrombin and TxA_2 (Bauer et al., 1999; Benjamin Z. S. Paul et al., 1999). In case of stimulation with agonists like collagen or ADP, MLC phosphorylation occurs only as a result of MLCK activation due to a rise in Ca^{+2} levels following activation of PLCγ or PLCβ. Thus various agonists stimulate diverse signalling pathways, which ultimately culminate in a common functional response of shape change.

RhoA activation is regulated by a number of proteins as follows:

- Rho-guanine nucleotide exchange factors (RhoGEFs) that catalyse removal of GDP and binding to GTP to RhoA to promote RhoA activation,
- GTPase activating proteins (GAPs) that promote the GTPase activity of RhoA thereby accelerating hydrolysis of GTP to GDP inactivating RhoA, and
- GTPase dissociation inhibitors that modulate RhoA functions by RhoA sequestration to specific intracellular locations and regulation of GTPase enzymatic activity and RhoA expression (Aslan and McCarty, 2013; Ren, 1999).

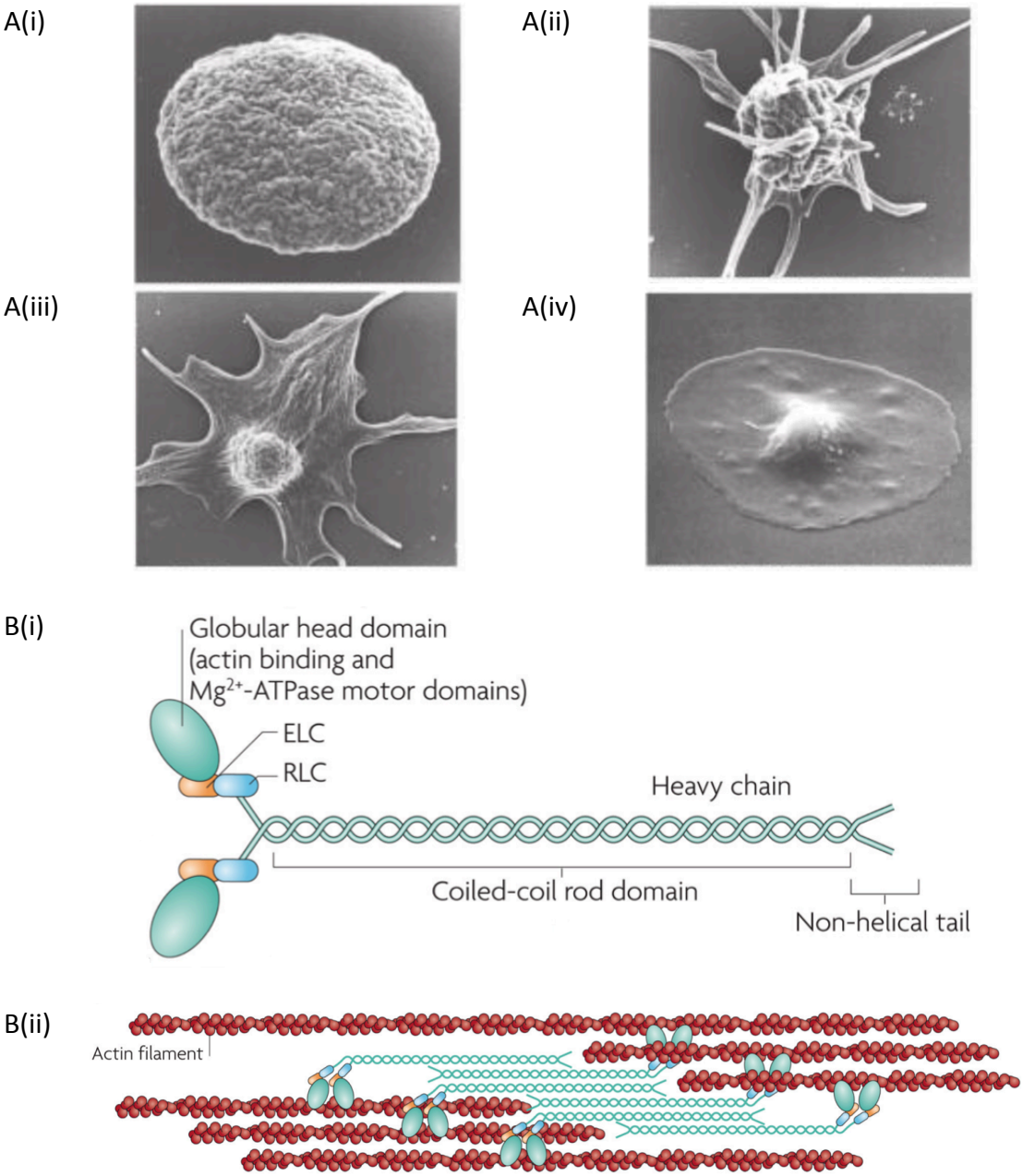


Figure 1.4 Platelet shape change and spreading. (A) Upon activation, platelet undergoes transition of its (i) discoid shape, (ii) extends several filopodia on its surface, (iii) forms lamellipodia in between the filopodia and finally (iv) converts to a fully spread platelet. (Bi) The subunit and domain structure of non-muscle myosin II (NM II), a critical regulator of platelet shape change. The globular head domain contains the actin-binding regions and the enzymatic Mg2+-ATPase motor domains. The essential light chains (ELCs) and the regulatory light chains (RLCs) bind to the heavy chains at the lever arms that link the head and rod domains. (Bii) NM II molecules assemble into bipolar filaments through interactions between their rod domains. These filaments bind to actin through their head domains and the ATPase activity of the head enables a conformational change that moves actin filaments in an anti-parallel manner. Taken from Vicente-Manzanares et al., 2009.

1.2.4.3 Signalling involved in platelet secretion and generation of soluble mediators

Once a platelet monolayer is formed at sites of vascular damage and platelet activation is initiated, amplification of platelet activation occurs with the aid of several soluble mediators such as ADP, TxA_2, thrombin and epinephrine that are accumulated at these sites. Mediators such as ADP and serotonin are secreted from δ-granules of activated platelets. Actin cytoskeletal rearrangements and microtubule reorganisation initiated upon platelet activation promote the centralised movement of platelet granules (Stenberg et al., 1984; White, 1968). The granules coalesce with each other and ultimately fuse with the membrane of the open canalicular system (OCS) or plasma membrane when their contents are discharged into the external environment (Stenberg et al., 1984). The process of granular fusion is regulated by specific lipids and proteins. A family of proteins called soluble NEM-sensitive attachment protein receptors or SNAREs are key to the secretion process. Distinct members of this family are expressed on granules, OCS and plasma membranes. Interactions between these proteins facilitate the movement of these structures towards each other and allow their fusion. Additionally, specific chaperone proteins bind to the SNARE proteins and regulate fusion of granules with membranes. (Flaumenhaft, 2003)

1.2.4.3.1 Signalling induced by thrombin

Thrombin is a serine-protease that is generated at sites of vascular injury from prothrombin by the activation of coagulation cascades. It plays a key role in the stabilisation of platelet thrombi through the generation of an insoluble fibrin clot following cleavage of the plasma protein fibrinogen (Wolberg, 2007). Once generated on the phosphatidylserine (PS) exposing surface of activated platelets, thrombin acts as a very potent platelet agonist mediating robust platelet activation. Thrombin-induced platelet activation is mediated by specialised GPCRs called protease activated receptors (PARs). Four such receptors (PAR-1, PAR-2, PAR-3 and PAR-4) have been identified, out of which PAR-2 is cleaved by other serine proteases like trypsin and tryptase, but not thrombin (O'Brien et al., 2001).

Thrombin activates platelets by cleaving the N-terminal sequences of these receptors and exposing previously masked sequences, which then act as tethered ligands to activate these receptors (Vu et al., 1991). Among the three remaining PARs, PAR-1 and PAR-4 are responsible for mediating platelet activation in human following thrombin stimulation (Kahn et al., 1999, 1998). PAR-1 is the major thrombin receptor and is required for rapid and robust platelet responses under low thrombin concentration (Kahn et al., 1999). PAR-4, on the other hand, mediates platelet activation only at high thrombin concentrations (Kahn et al., 1999). PAR-4 activation requires about 10 to 100-fold higher dose of thrombin owing to the lack of hirudin-like sequences in PAR-4 that aid in thrombin-mediated receptor cleavage (Brass, 2003; Ishii et al., 1995; Xu et al., 1998). Despite its slower onset compared to PAR-1, it has been shown PAR-4 mediated platelet signalling is sustained longer (Covic et al., 2000; Shapiro et al., 2000). Both these receptors are coupled to $G_{\alpha 12/13}$ that induces RhoA activation (Offermanns et al., 1994) and eventually triggers shape change and $G_{\alpha q}$ that activates PLCβ to generate second messengers IP_3 that promotes intracellular Ca^{+2} mobilisation and DAG that activates PKC (Offermanns, 2006; Offermanns et al., 1994) (Figure 1.5). Platelets from mice deficient in $G_{\alpha q}$ exhibit defects in platelet activation particularly aggregation, IP_3 production and mobilisation of intracellular Ca^{+2} levels in response to stimulation with thrombin, ADP, TxA_2 analogue and collagen in addition to an increased tail bleeding time compared to wild-type littermates (Offermanns et al., 1997). Similarly, $G_{\alpha 13}$-deficient platelets show defects in platelet shape change, RhoA activation, aggregation and formation of stable thrombi under high shear (Moers et al., 2003). In murine platelets PAR-3 and PAR-4 together participate in mediating platelet activation in response to thrombin stimulation (Kahn et al., 1998). PAR-3 itself however does not generate activation signals; it rather assists in the cleavage and activation of PAR-4 by thrombin (Nakanishi-Matsui et al., 2000).

1.2.4.3.2 Signalling induced by adenosine 5'-diphosphate (ADP)

ADP is secreted from the δ-granules of activated platelets. Upon release, ADP stimulates platelet shape change, TxA2 formation, increase in cytosolic Ca^{+2} concentrations, protein phosphorylation, inhibition of cAMP formation, aggregation

and secretion. Human platelets express three purinergic receptors that transmit the activation stimulus initiated by ADP (Daniel et al., 1998). Of these, two are G-protein coupled receptors (GPCRs) and are denoted as $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ is associated to $G_{\alpha q}$ and, upon ADP binding, mediates platelet shape change and PLCβ activation to generate IP_3 and DAG, which stimulate intracellular calcium mobilisation and PKC activation (Jin et al., 1998; Savi et al., 1998) (Figure 1.5). Mice lacking $P2Y_1$ have impaired ADP-mediated platelet responses such as change shape and aggregation as well as increased tail bleeding times and resistance to thromboembolism (Fabre et al., 1999; Léon et al., 2001, 1999). $P2Y_{12}$ is associated to $G_{\alpha i2}$ (Foster et al., 2001; Zhang et al., 2001) and is crucial for lowering cAMP formation through the inhibition of adenylyl cyclase, which contributes to the potentiation of ADP-mediated platelet activation and thrombus formation (Dorsam and Kunapuli, 2004) (Figure 1.5). Deletion of $P2Y_{12}$ is associated with defects in platelet adhesion, activation and thrombus growth and stability (André et al., 2003). Similar defects in ADP-mediated platelet responses are observed when each of these receptors are blocked by selective receptor antagonists (Jin et al., 1998; Jin and Kunapuli, 1998). Concomitant signalling from both the ADP purinergic receptors is essential to achieve complete ADP-mediated platelet activation (Jin and Kunapuli, 1998). The third purinergic receptor on platelets denoted as $P2X_1$ is a calcium channel, which promotes ADP-mediated Ca^{+2} influx in platelets, but has no role in ADP-induced platelet shape change and aggregation (Daniel et al., 1998; Jin and Kunapuli, 1998; Savi et al., 1998). $P2X_1$ is however believed to act synergistically to promote P2Y receptor activation (Vial et al., 2002).

1.2.4.3.3 Signalling induced by thromboxane A_2 (TxA_2)

Platelet activation results in release of arachidonic acid through phospholipase A_2 (PLA_2)-dependent hydrolysis of membrane phosphatidylcholine (PC). Arachidonic acid is converted to TxA_2 through the sequential modification by cyclooxygenase-1 (COX-1) and thromboxane synthase. It serves as a powerful platelet agonist and reinforces platelet activation. It stimulates platelets by binding to its receptors, TxA_2 receptors α and β (TPα and TPβ) (Takahara et al., 1990). These are two splice variants of the same gene and differ in their cytoplasmic tails (Narumiya et al.,

1992). These receptors are shown to couple to $G_{\alpha q}$ and $G_{\alpha 12/13}$ family of G-proteins (Figure 1.5). Thus, TxA_2 stimulation results in RhoA activation and platelet shape change as well as PLCβ activation, increase in cytosolic Ca^{+2} concentrations and PKC activation (Djellas et al., 1999; Knezevic et al., 1993; Offermanns et al., 1994; Benjamin Z S Paul et al., 1999).

1.2.4.3.4 Signalling induced by epinephrine/adrenaline

The neurotransmitter epinephrine on its own is a relatively weak platelet activator. However, it acts to potentiate platelet activation induced by any other platelet agonist (Lanza et al., 1988, 1986). The effect of epinephrine on platelets is mediated by the α_{2A}-adrenergic receptors (Lanza et al., 1986), which are coupled to $G_{z\alpha}$ that can inhibit adenylyl cyclase (AC) and lower cyclic AMP levels (cAMP) (Wong et al., 1992) (Figure 1.5). Deletion of $G_{z\alpha}$ in mice was shown to reduce platelet aggregation in response to epinephrine due to impaired inhibition of cAMP (Yang et al., 2000).

1.2.4.4 Signalling involved in platelet aggregation

Monolayer of activated platelets formed at sites of vascular injury recruit additional platelets to develop larger thrombi though the process of secretion and platelet aggregation. This is achieved by establishing physical contacts between adjacent platelets through the involvement of specialised surface receptors called integrins and adhesive plasma proteins. The integrin indispensible for platelet aggregation is known as integrin $\alpha_{IIb}\beta_3$ or GPIIb-IIIa. It comprises of two α-chains linked non-covalently to a single β-chain and the structure is divided into an extracellular domain containing the N-terminal and majority of the remaining residues of the α and β subunits, a short transmembrane domain and a C-terminal cytoplasmic domain composed of 20 and 47 amino acids of α_{IIb} and β_3 subunits, respectively (Shattil et al., 1998).

Prior to platelet activation, integrin $\alpha_{IIb}\beta_3$ is maintained in a low-affinity state. Platelet adhesion and activation results in the conversion of the integrin to a high-affinity state that can interact with a broad spectrum of adhesive proteins such as vWF, fibrinogen and fibronectin through their Arg-Gly-Asp (RGD) recognition

sequence with sites in the globular head domain of the integrin (Shattil and Newman, 2004; Weisel et al., 1992). The process of transition of integrin from a low-affinity state to a high-affinity state is referred to as “inside-out signalling” (Figure 1.6). This is the ultimate point at which all the distinct activation pathways initiated by different agonists downstream of various platelet GPs and GPCRs converge (Dorsam et al., 2002; Kasirer-Friede et al., 2004; Nieswandt et al., 2002; Quinton et al., 2002). These distinct pathways transmit signals to the cytoplasmic tails of the resting integrin $\alpha_{IIb}\beta_3$, which is then propagated to its extracellular domains. A number of proteins are known to interact with one or both cytoplasmic tails to promote integrin activation, of which Talin-1, Kindlin-3, Rap1b and Rap1-GTP- interacting adaptor molecule (RIAM) have been characterised for functional significance (Nieswandt et al., 2009). Divalent fibrinogen molecules and vWF multimers engage activated integrins on adjacent platelets thereby forming bridges between them and causing aggregation (Shattil et al., 1998). Subsequently, integrin $\alpha_{IIb}\beta_3$ transmits separate sets of activation signals inside the platelet that sustain and strengthen aggregates and promote clot retraction, a process known as outside-in signalling.

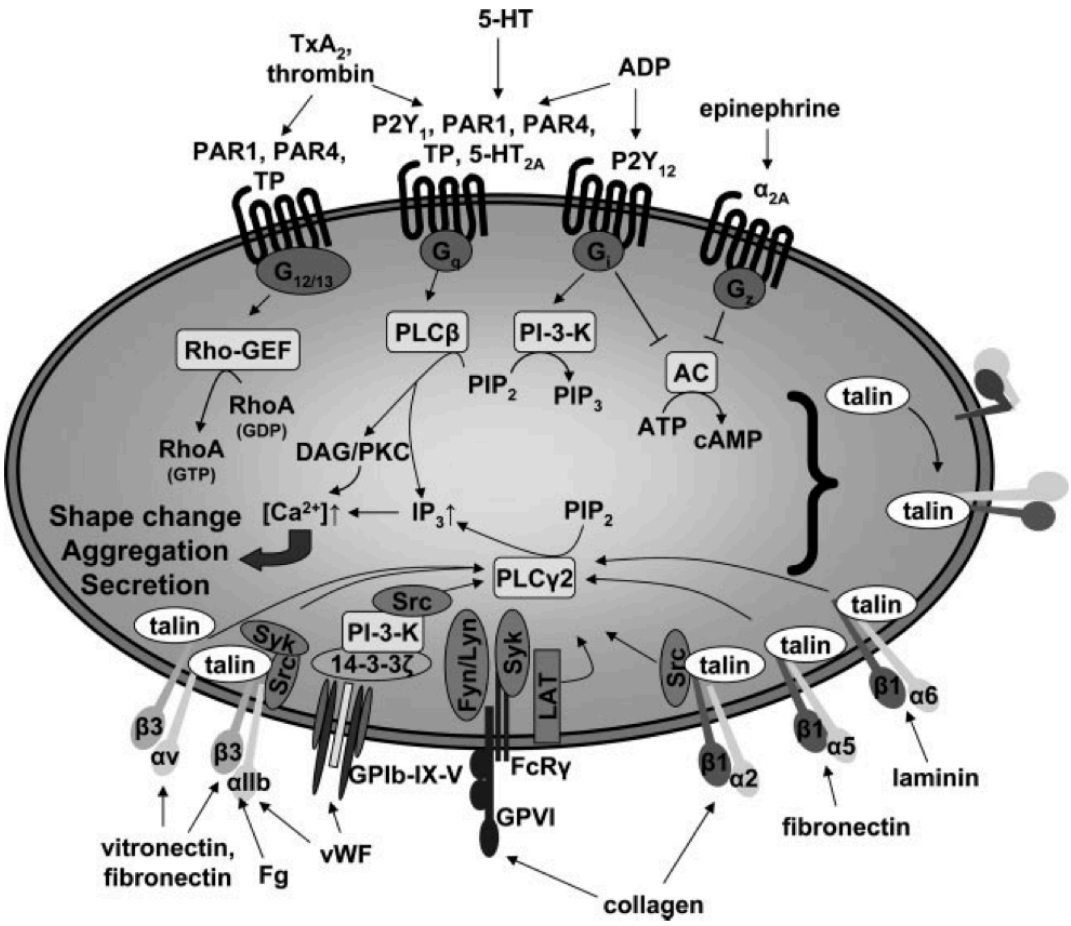


Figure 1.5 Signaling mechanisms activated by physiological agonists causing integrin activation. Various agonists induce signaling pathways, which cooperate with each other to maximally activate platelets. GPVI GPIb/IX/V and integrin $\alpha_{IIb}\beta_3$ ligation activates PLCγ2 leading to Ca^{+2} mobilisation and PKC activation. Stimulation of GPCRs trigger pathways involving $G_{\alpha q}$, $G_{\alpha i/z}$, and $G_{\alpha 12/13}$, which induce PLCβ activation, inhibition of adenylyl cyclase and RhoA activation, respectively. Taken from Varga-Szabo et al., 2008.

1.2.4.5 Signalling involved in stabilisation of platelet aggregates

Platelet aggregation results in direct interactions between platelets in the platelet plug, which generate another wave of signalling known as contact-dependent signalling. The principal aims of these signalling events is to stabilise the platelet plug by sustaining platelet-platelet interactions, extend platelet aggregation and regulate clot retraction for sufficient time until wound healing occurs. This phase of signalling events comprise mainly of outside-in signalling through the platelet integrin $\alpha_{IIb}\beta_3$ and also signalling generated from the interaction between Eph kinases and ephrins (Woulfe et al., 2004).

The binding of fibrinogen to the platelet integrin $\alpha_{IIb}\beta_3$ initiates outside-in signalling in the platelet plug (Figure 1.6). The engagement of $\alpha_{IIb}\beta_3$ by its ligand stimulates the microclustering of the integrin (Loftus and Albrecht, 1984). This leads to the activation of the Src family of tyrosine kinases constitutively associated to the β_3 cytoplasmic tail, which thereby recruits and activates Syk tyrosine kinases (Arias-Salgado et al., 2003; Hato et al., 1998; Obergfell et al., 2002). Src and Syk then activate a series of substrates including molecular adaptors SLP76, ADAP and c-Cbl and a Rac GTPase Vav, which are important for signalling events in the rearrangement of the actin cytoskeleton (Miranti et al., 1998; Obergfell et al., 2001; Shattil and Newman, 2004). As the outside-in signalling propagates additional proteins are recruited to the signalling complex. Of these β_3, PLCγ and α-actinin are particularly important as they later participate in processes like cytoskeletal rearrangements, clot stabilisation and retraction (Izaguirre et al., 1999; Jenkins et al., 1998; Wonerow et al., 2003).

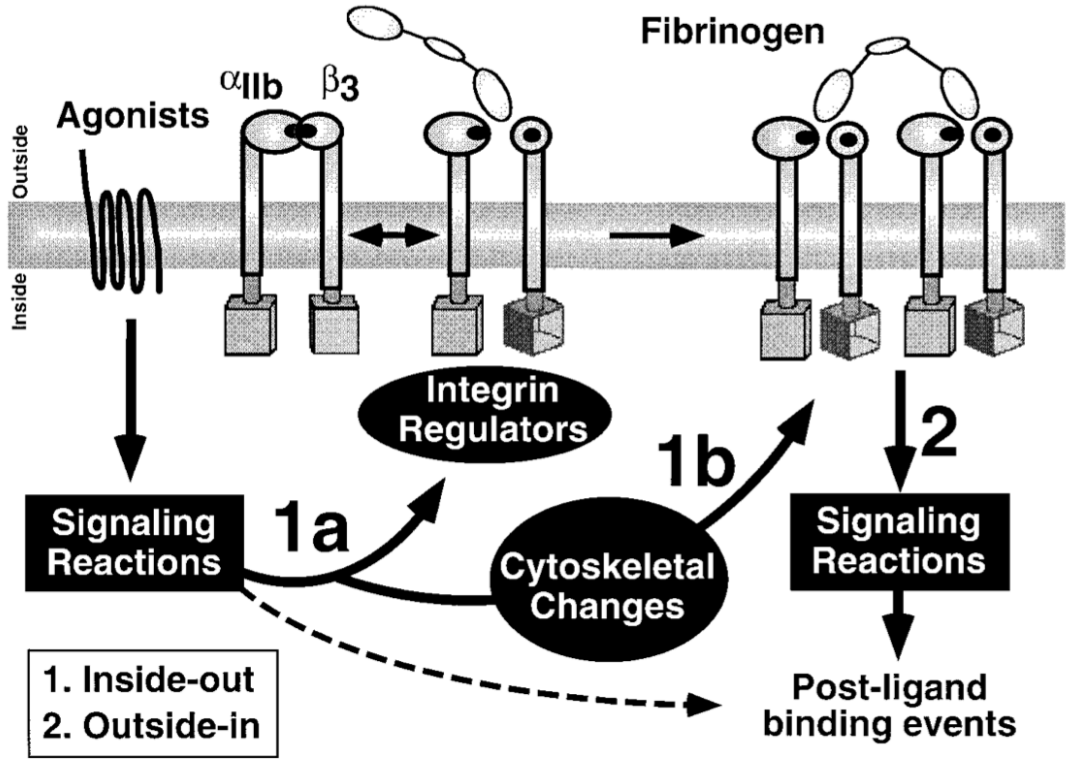


Figure 1.6 Inside-out and outside-in signalling. Agonist stimulation of platelets generate activation signals at the platelet membrane, which are transmitted to the platelet cytosol and promote cytoskeletal rearrangements. These stimuli are then carried forward to the cytoplasmic regions of the integrin $\alpha_{IIb}\beta_3$ and converts its to a high-affinity ligand binding state. The process of integrin activation is known as inside-out signaling (arrows 1a and 1b), which increases its affinity and avidity for ligands such as fibrinogen by unmasking fibrinogen binding sites in its extracellular domains. Following fibrinogen binding, a number of activation signals are generated and these are known as outside-in signaling. These events promote stabilisation of the platelet plug and reinforcement of the fibrin clot as well as the subsequent steps of clot retraction. Taken from Shattil et al., 1998.

1.2.5 Negative regulation of platelets by cyclic nucleotides

To ensure that platelet activated is controlled, potent inhibitory signals must be generated in order to prevent excessive activation and encourage the platelets to revert back to their quiescent state after activation. The endothelium contributes to the negative regulation of platelet activation through the release of vasoactive chemicals such as nitric oxide (NO) and prostaglandin I_2 (PGI_2 or prostacyclin). These endothelial-derived agents are potent inhibitors of all aspects of platelet function and are through to play a principle role in the regulation of haemostasis. In addition, the endothelium expresses certain surface molecules that contribute towards inhibition of platelet responses.

1.2.5.1 Platelet inhibition by NO

NO is a free radical gas released from the endothelium by enzymatic conversion of L-arginine by nitric oxide synthase (NOS). Thus it can easily diffuse into platelets, where it activates its intracellular receptor soluble guanylyl cyclase (sGC). Once activated, sGC catalyses the conversion of GTP to cyclic guanosine monophosphate (cGMP). The resulting high intracellular concentration of cGMP activates protein kinase G (PKG), which then phosphorylates a number of protein substrates associated with platelet inhibition (Gambaryan et al., 2004; Li et al., 2003) (Figure 1.7). cGMP formation is regulated by phosphodiesterase (PDE) enzymes that hydrolyse cGMP into inactive forms. cGMP stimulates the activity of both PDE2 and PDE5 and can be hydrolysed by both these PDE isoforms (Schwarz et al., 2001). cGMP-mediated signalling inhibits platelet function by suppressing a number of events responsible for platelet activation such as adhesion (Oberprieler et al., 2007; Radomski et al., 1987), rise in intracellular Ca^{+2} levels, cytoskeletal rearrangements, integrin activation, granule secretion and platelet aggregation.

An important substrate for PKG is the platelet cytoskeletal-associated vasodilator stimulator phosphoprotein (VASP) (Butt et al., 1994; Halbrügge et al., 1990) at Ser^{239} (Smolenski et al., 1998). PKG-mediated VASP phosphorylation has been associated with reduced integrin activation and lower agonist-induced platelet aggregation (Aszódi et al., 1999). HSP27 phosphorylation mediated by PKG is shown

to reduce actin polymerisation and platelet aggregation (Butt et al., 2001). PKG has also been implicated in phosphorylation of MLCK without any detectable effect on MLCK activity (Nishikawa et al., 1984). Other identified substrates of PKG are the IP_3 receptor (IP_3R) (Cavallini et al., 1996; El-Daher et al., 2000) and Rap1b (Reep and Lapetina, 1996).

Although NO-mediated inhibition of platelet activation is primarily cGMP-dependent, a number of studies have provided substantial evidence for a cGMP-independent mechanism (Gambaryan et al., 2004; Nikolaus G. Oberprieler et al., 2007; Nikolaus G Oberprieler et al., 2007). This is proposed to occur through S-nitrosylation and nitration of platelet proteins (Nikolaus G. Oberprieler et al., 2007).

1.2.5.2 Platelet inhibition by prostacyclin

PGI_2 belongs to the member of lipids known as eicosanoids. It is released from endothelial cells, acts as a vasodilator and seems to be the most potent among prostaglandins. PGI_2 binds to platelets on its surface G-protein coupled receptor called prostacyclin receptor (IP). This leads to receptor activation followed by activation of the membrane enzyme adenylyl cyclase (AC), which catalyses the conversion of ATP to cyclic adenosine monophosphate (cAMP) (Best et al., 1977). Increased production of cAMP subsequently activates the enzyme protein kinase A (PKA) (Figure 1.7). Mice lacking IP receptor are more susceptible to thrombotic events (Murata et al., 1997). PGI_2 has been shown to inhibit a number of platelet functions such as platelet adhesion (Weiss and Turitto, 1979), shape change, aggregation and secretion. Platelets acquire the characteristic of being functionally inhibited by cAMP during their biogenesis from Mks (den Dekker et al., 2002).

PKA phosphorylates a number of platelet proteins that are associated with platelet inhibition. Some of these proteins, such as VASP, IP_3R, MLCK, Rap1b, are common substrates for PKA and PKG. PKA-mediated VASP phosphorylation (Butt et al., 1994; Halbrügge et al., 1990) at Ser^{157} (Horstrup et al., 1994) correlates with the inhibition of integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation (Aszódi et al., 1999; Horstrup et al., 1994) as well as negative regulation of actin dynamics (Harbeck et al., 2000). IP_3R phosphorylation mediated by PKA has been shown to inhibit its Ca^{+2}-releasing

function (Cavallini et al., 1996; El-Daher et al., 2000). PKA-mediated phosphorylation of MLCK has been shown to inhibit MLCK activity, that can have an impact on actin-myosin interactions and subsequent platelet cytoskeletal rearrangements (Hathaway et al., 1981; Nishikawa et al., 1984). PKA-mediated Rap1b phosphorylation has been shown to reduce Rap1b activation in response to agonist stimulation (Franke et al., 1997). PKA also phosphorylates the β-chain of GPIb, which is responsible for platelet inhibition through suppression of collagen-induced actin polymerisation (Fox and Berndt, 1989).

Similar to cGMP, the levels of cAMP and subsequent PKA signalling is regulated by PDE activity. cGMP-stimulated PDE2 and cGMP-inhibited PDE3 are responsible for the hydrolysis of cAMP, which indicates that cGMP has a role in regulating cAMP levels. PDE3 activity is however also enhanced by its phosphorylation mediated directly by PKA, which demonstrates the negative feedback regulation of cAMP to maintain basal levels (Schwarz et al., 2001). Substances like dipyridamole, which inhibits phosphodiesterases from metabolising cAMP, potentiates the effect of PGI_2.

1.2.5.3 Platelet inhibition by surface molecules on endothelial cells (ECs)

The endothelium and platelets express a surface adhesion molecule called platelet endothelial cell adhesion molecule-1 (PECAM-1) or CD31 that can suppress platelet activation. PECAM-1 is phosphorylated at sites within specific sequences known as immunoreceptor tyrosine inhibition motifs (ITIMs), similar to the stimulatory ITAM sequences, in response to various agonists (Cicmil et al., 2000). This phosphorylation is accompanied by association of specific phosphatases (SHP-1 and SHP-2), which can dephosphorylate key regulators of platelet activation and hence can blunt platelet activation. It is suggested that PECAM-1 inhibits platelet activation in a negative-feedback mechanism in response to agonist stimulation (Rathore et al., 2003). PECAM-1 appears to downregulate platelet activation and signalling mechanisms in response to collagen, collagen-related peptide (CRP), thrombin and oxidised low-density lipoprotein (oxLDL) as well as GPVI- and GPIb-mediated thrombus formation under flow conditions (Cicmil et al., 2002, 2000;

Jones et al., 2001; Patil et al., 2001; Rathore et al., 2003; Relou et al., 2003). Another barrier posed to platelet activation by a proportion of ECs is through the expression of CD39, which can hydrolyse small amounts of ADP released from damaged red cells and activated platelets, thereby preventing further platelet activation (Brass, 2003; Marcus et al., 1997).

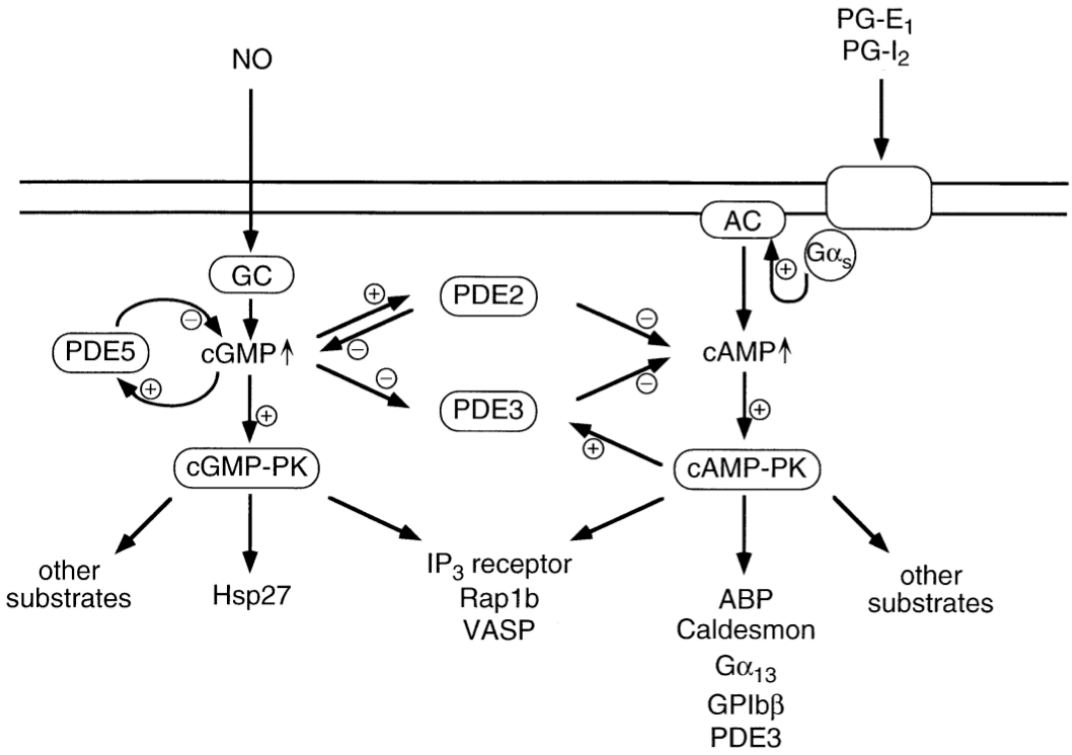


Figure 1.7 Negative regulation of platelet by cyclic nucleotide signalling. Platelet inhibition is achieved by the physiological inhibitors nitric oxide (NO) and prostanoids (prostacyclin PGI_2 and prostaglandin PGE_1). NO diffuses through the plasma membrane and interacts with the soluble guanylyl cyclase (sGC) enzyme to enhance intracellular cyclic GMP (cGMP) levels that activates protein kinase G (PKG). The prostanoids however interact with a platelet transmembrane receptor, which is coupled to the $G_{\alpha s}$ protein. Upon interaction of this receptor with the prostanoids, $G_{\alpha s}$ is activated and it activates adenylyl cyclase (AC) to elevate intracellular cyclic AMP (cAMP) levels that activates protein kinase A (PKA). These kinases phosphorylate a number of platelet substrates, which are associated with platelet inhibition. Levels of these cyclic nucleotides are regulated by the activity of phosphodiesterase (PDE) enzymes and hence these also are critical regulators of platelet activity. Adapted from Schwarz et al., 2001.

1.3 Glucocorticoids

Glucocorticoids (GCs) are a class of steroid hormones that are produced and secreted from the adrenal cortex lying above the kidneys. The principal physiological GC is cortisol (Wang, 2005). The plasma GC level is largely controlled by the action of the hypothalamic-pituitary-adrenal (HPA) axis (Vegiopoulos and Herzig, 2007). Besides, only a tiny proportion of plasma cortisol is in the free form and hence active, as most of it is bound to corticosteroid-binding globulin (CBG) and some to albumin. GCs participate in a range of physiological activities in the body and thus have important roles in basal and stress-related homeostasis, development, metabolism, neurobiology, programmed cell death and many more (Macfarlane et al., 2008). A large number of synthetic GCs have been designed which are structurally similar to cortisol and mimic its effects. Pharmacologically, GCs are administered as anti-inflammatory, immunosuppressive and anti-cancer drugs (Wikstrom, 2003). Although having a high therapeutic efficacy, GCs have potential limitations in the form of a plethora of metabolic side effects (Vegiopoulos and Herzig, 2007), when administered in higher doses or for a prolonged period. The biological effects of GCs are mediated via a classical intracellular protein called the glucocorticoid receptor (GCR) (Vegiopoulos and Herzig, 2007; Wang, 2005).

The physiological action of GCs are regulated by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) that catalyses the interconversion between cortisol (or corticosterone in rodents) and its inert 11-keto metabolite cortisone (or 11-dehydrocorticosterone in rodents) (Macfarlane et al., 2008; Wang, 2005). Two isoforms of the enzyme are known to exist. The type I enzyme 11β-HSD1 is known to convert the inert 11-keto forms to active GCs such as cortisol or corticosterone (Krozowski et al., 1999). 11β-HSD1 is expressed in high amounts in the liver, where the ratio of active cortisol to inactive cortisone is high suggesting cortisol production and the activity of this enzyme within this tissue is thought to enhance the autocrine/paracrine effects of GCs (Krozowski et al., 1999). Among other tissues where significant expression of 11β-HSD1 has been detected include the adrenal

cortex, the brain specifically in the hippocampus and the cortex regions (Krozowski et al., 1999).

The type II enzyme 11β-HSD2 catalyses the conversion of the active GCs to the inert 11-keto compounds (Krozowski et al., 1999), which exhibit low affinity for the GCR and the mineralocorticoid receptor (MCR) (Funder et al., 1988), another closely-related family of nuclear receptors. 11β-HSD2 expression occurs mainly in mineralocorticoid (MC) target tissues such as the renal cortex, rectal and sigmoid colon, salivary glands and sweat glands (van Uum et al., 1998), where this enzyme plays a crucial role by preventing an unwarranted access of cortisol and promoting access of aldosterone (a physiological MCR ligand) to the MCR, which would otherwise bind cortisol with equal affinity. Lack of this enzyme causes an overstimulation of the MCR by both aldosterone and cortisol leading to sodium retention and severe hypertension (Krozowski et al., 1999).

Both these isoforms have been detected in the vasculature with 11β-HSD1 detected within the vascular smooth muscle cells and 11β-HSD2 within the vascular endothelial cells (Hadoke et al., 2009, 2006). Other reports have presented opposite findings with the expression of 11β-HSD1 within the vascular endothelial cells (Brem et al., 1998; Luo et al., 2013) and 11β-HSD2 within the vascular smooth muscle cells (Krozowski et al., 1995; Smith et al., 1996). Within the vascular endothelial cells where the presence of excess GCs have been shown to regulate the contractile response of adjacent vascular smooth muscle cells by suppressing their vasorelaxation through decreased production of vasodilators (Brem et al., 1998). 11β-HSD2 has also been detected in the vasculature in both the vascular endothelial cells and smooth muscle cells (Brem et al., 1998). MCR activation in the vasculature has been found to be pro-atherogenic and pro-inflammatory, thereby presenting an approach of MCR antagonism to ameliorate cardiovascular disease. However, it has been shown that 11β-HSD2 knockout mice developed atherosclerotic lesions at an accelerated rate compared through the activation of the non-renal MCR by mainly GCs thereby arising enhanced inflammatory process within those lesions, which indeed highlights the atheroprotective effects of MCR (Deuchar et al., 2011).

GC excess has been associated with the development of the metabolic syndrome (Wang, 2005) characterised by obesity, insulin resistance, dyslipidemia and hypertension, all of which are considered risk factors for CVD. An 11β-HSD1 inhibitor, compound 544, has been shown to improve multiple aspects of metabolic syndrome as well as prevent atherosclerotic plaque progression in ApoE-/- mice accompanied by a reduction in plasma lipids (Hermanowski-Vosatka et al., 2005). In a separate study, another 11β-HSD1 inhibitor, compound L-750 was shown to significantly reduce plaque progression by decreasing accumulation of aortic total cholesterol, free cholesterol and cholesterol ester without directly affecting plasma triglycerides and total cholesterol. The vascular cells, particularly the macrophages within the atherosclerotic plaques and smooth muscle cells, were shown to exhibit a dampened inflammatory state in response to 11β-HSD1 inhibition (Luo et al., 2013). (Luo et al., 2013) presented compelling evidence that the inhibition of 11β-HSD1 has beneficial effects on the vascular inflammation, which were in agreement with older concepts that GCs regulate a number of cellular mechanisms to allow an efficient response within the host organism under a number of stress conditions such as starvation, infection and injury. Another study determined that the lack of 11β-HSD1 overcomes the anti-angiogenic effect of endogenous GCs and hence suggested a potential therapeutic approach to ameliorate healing of ischaemic or injured tissues (Small et al., 2005). To establish a coordinated approach towards the multiple aspects of GC function in host defense mechanisms, (Sapolsky et al., 2000) proposed a biphasic response of GCs comprising of both permissive and suppressive functions. According to this hypothesis, physiological amounts of GCs promote permissive functions on host defense mechanism by acting through both GCR and MCR, while high amounts of GCs under conditions of stress act as suppressors on host defense by acting through the GCR. (Christy et al., 2003) (Dover et al., 2007) (Hammer and Stewart, 2006) (Hatakeyama et al., 2001) (Young et al., 2003) (Walker et al., 1991) (Hadoke et al., 2009) (Hadoke et al., 2013) (Chapman et al., 2009) (Chapman et al., 2006) (Hadoke et al., 2006) (Funder et al., 1988) (Walker, 2007) (Wamil and Seckl, 2007) (Bray et al., 1999) (Osmond and Dorrance, 2009) (Thieringer et al., 2001) (Wake and Walker, 2004) (Seckl and Walker, 2004) (Seckl, 2004) (Tomlinson and Stewart, 2005) (Espíndola-Antunes and Kater, 2007)

Both cortisol and cortisone are metabolised in the liver into 5α- and 5β-tetrahydrocortisol (5α- and 5β-THF) and 5β-tetrahydrocortisone (5β-THE) and eliminated through urinary excretion.

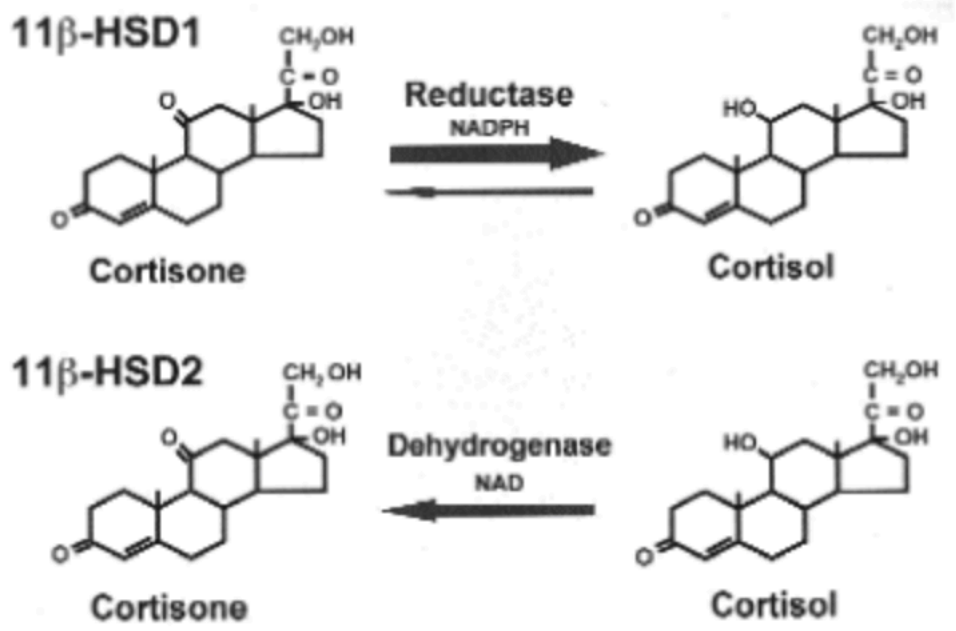


Figure 1.8 11β-HSD isoforms and their role in the interconversion of the physiologically active GC, cortisol and its inactive keto-compound, cortisone. Both these isoforms are expressed within the vasculature and regulate the formation of atherosclerotic lesions. Adapted from (Espíndola-Antunes and Kater, 2007).

1.3.1 Glucocorticoid receptor (GCR)

GCs exert their action through the glucocorticoid receptor (GCR), which belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors. Other members of this receptor family include mineralocorticoid receptor (MCR) or aldosterone receptor, estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), peroxisome proliferator-activated receptor (PPAR), retinoid X receptor (RXR), vitamin D receptor (VDR) and thyroid hormone receptor (TR) among others. GCR is the first member of the subfamily 3 and group C and thus alternatively designated as NR3C1. The other closely related member of this subfamily is the MCR (NR3C2), which shares a high similarity with GCR.

1.3.1.1 GCR gene organisation

Human GCR (hGCR) protein is the product of the hGCR gene, also known as NR3C1, is located on chromosome 5q–q32 (Francke and Foellmer, 1989) and contains 10 exons denoted as exons 1-8, 9α and 9β (Encío and Detera-Wadleigh, 1991) (Figure 1.9). Exon 1 entirely contains untranslated sequences and hence defines the 5'-untranslated region (UTR). Between exons 2 and exon 9 lies the coding region, while the terminal sequences in exon 9 define the 3'-UTR. Cloning of hGCR cDNA in 1985 revealed the presence of two isoforms of the hGCR protein with different C-terminal amino acid sequences and distinct phenotypes (Hollenberg et al., 1985). Subsequent genomic analyses revealed that the two isoforms were products of the same gene and resulted from hGCRα and hGCRβ mRNA variants, which were generated by alternative splicing of the hGCR primary transcript. The two hGCR transcripts were shown to contain identical sequences encoded by exons 1-8, while their distinct C-terminal sequences and 3'-UTR were encoded by the mutually exclusive exons 9α and 9β (Encío and Detera-Wadleigh, 1991). The hGCR gene contains three different promoters, which have given rise to five hGCR transcripts with different 5'-UTR first exons. The exons are denoted as exons 1A, 1B and 1C, of which exon 1A itself has three distinct variants based on different splice donor sites (1A1, 1A2 and 1A3). These hGCR transcripts exhibit distinct tissue-specific expression (Breslin et al., 2001).

1.3.1.2 GCR protein structure

The hGCR protein has a modular structure composed of an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (Figure 1.9). A flexible hinge region is present between the DBD and the LBD. The NTD contains a strong transcriptional activation function (AF-1 or τ1) that facilitates the recruitment of and interaction with various co-factors and components of the basal transcriptional machinery. The DBD is the most conserved region across members of the nuclear receptor superfamily. It contains two zinc-finger motifs, which participate in the recognition as well as interaction with target genes at specific sequences known as glucocorticoid responsive elements (GREs) (Kadmiel and Cidlowski, 2013; Oakley and Cidlowski, 2011). The LBD consists of 11 α-helices and 4 small β-strands that fold into a three-layer helical domain and form a cavity for binding of GCs (Bledsoe et al., 2002). LBD also contains a second activation function (AF-2 or τ2), which facilitates interaction with co-factors in a ligand-dependent manner. Two nuclear localisation signals (NL1 and NL2) are present within the DBD-hinge junction and LBD, respectively (Kadmiel and Cidlowski, 2013; Oakley and Cidlowski, 2011).

1.3.1.3 GCR isoforms

Several GCR isoforms arising from alternative splicing as well as translation initiation have been reported, which are discussed below.

1.3.1.3.1 GCR isoforms produced by alternative splicing

As mentioned earlier, two different GCR transcripts generated by alternative splicing of the last two exons 9α and 9β were initially identified and characterised (Encío and Detera-Wadleigh, 1991; Hollenberg et al., 1985). Amino acid sequence of the proteins translated from these transcripts revealed identical amino acid sequences 1-727, after which hGCRα had 50 additional amino acids, while hGCRβ contained 15 additional non-homologous amino acids (Hollenberg et al., 1985) (Figure 1.9). GCRα and GCRβ mRNA are expressed ubiquitously with differential levels of each detected in a tissue-specific manner. GCRα mRNA is however

expressed several fold higher than GCRβ mRNA in a majority of tissues. Eosinophils and PBMCs were shown to contain lower levels of GCRα and higher levels of GCRβ when compared to the brain cortex (Pujols et al., 2002). Both hGCRα and hGCRβ proteins are expressed in a variety of human tissues and cell-lines (Oakley et al., 1996). However, it is the hGCRα protein that functions as the predominant active ligand-dependent transcription factor, while hGCRβ is transcriptionally inactive, does not bind GCs and exerts a dominant-negative effect on hGCRα-mediated transactivation of target genes (Bamberger et al., 1995; Oakley et al., 1999, 1996). Overexpression of hGCRβ has been noted in several diseases such as asthma, ulcerative colitis, nasal polyposis, acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) and the observed reduction in the relative expression of hGCRα:hGCRβ proteins in these patients has been linked to generalised and tissue-specific GC resistance (Hamid et al., 1999; Honda et al., 2000; Lewis-Tuffin and Cidlowski, 2006; Sousa et al., 2000).

The inability of hGCRβ to bind GCs is due to its structural difference with hGCRα. The conformation of the final (AF-2) helix in the hGCRα isoform domain structure is crucial for ligand binding upon interaction with other helices as well as ligand-dependent transactivation. This helix is completely absent in the hGCRβ isoform, which contains unique C-terminal amino acids. Hence this isoform neither can bind GCs nor can directly be involved in transactivation (Lewis-Tuffin and Cidlowski, 2006).

In addition to these two predominant isoforms, several other isoforms generated by alternative splicing of the hGCR gene have been reported (Figure 1.9). GCRγ isoform is generated from an alternative splice donor in the intron between exons 3 and 4 and contains an insertion of an arginine residue between the two zinc fingers of the DBD. Although it can bind DNA and GCs with similar efficacy as GCRα, it is incapable of inducing a transcriptional response from GC-responsive reporters. This splice variant has been linked to glucocorticoid-resistance in some forms of leukemia (Beger et al., 2003). Two non-hormone binding GCR transcripts missing portions of the LBD have also been reported. GCR-A is generated by alternative splicing linking the end of exon 4 to the beginning of exon 8 and hence does not

contain the N-terminal half of the LBD encoded by exons 5-7. In contrast, GCR-P is generated from a failure to splice the exon 7/8 boundary and hence lacks the C-terminal region of the LBD encoded by exons 8 and 9. GCR-P has been found to be the major splice variant in several glucocorticoid-resistant cancer cells (Kadmiel and Cidlowski, 2013; Oakley and Cidlowski, 2011).

1.3.1.3.2 GCR isoforms produced by alternative translation initiation

Sequence alignment of GCR identified 8 start codons in exon 2 conserved across species and each of these give rise to an individual protein (Lu and Cidlowski, 2005). Thus 8 GCRα proteins are known to exist and are denoted as GCRα-A, GCRα-B, GCRα-C1, GCRα-C2, GCRα-C3, GCRα-D1, GCRα-D2 and GCRα-D3, where GCRα-A represents the classical full-length protein (777 amino acids) originated from the first translational initiator codon (Figure 1.9). These isoforms display similar affinities for GCs as well as GREs on target genes (Nehmé et al., 2009), yet distinct subcellular localisation and nuclear-cytoplasmic shuttling patterns (Lu and Cidlowski, 2005), suggesting a possible role of the NTD in regulating the subcellular distribution of the receptor (Oakley and Cidlowski, 2011). They also differ in relative expression levels within tissues (Lu and Cidlowski, 2005) with GCRα-A and GCRα-B being the predominant isoforms in most human cell-lines examined (Oakley and Cidlowski, 2011). Owing to the differences in their NTD sequences, these isoforms differ in their ability to induce transcription of target genes (Lu and Cidlowski, 2005; Yudt and Cidlowski, 2001), which is thought to be due to their differential ability in recruiting RNA polymerase II and co-factors on target gene promoters (Lu et al., 2007). Hence the cellular response to GCs appears to be a reflection of the collection and relative levels of the GCR isoforms it expresses (Lu et al., 2007). Like GCRα, each of the other GCR splice variants (β, γ, A and P) are also thought to generate separate set of translational GCR isoforms (Oakley and Cidlowski, 2011). Other members of the nuclear receptor superfamily have also been found to generate multiple translational isoforms, which suggests the existence of multiple receptor isoforms to account for the diverse signalling responses of steroids (Oakley and Cidlowski, 2011).

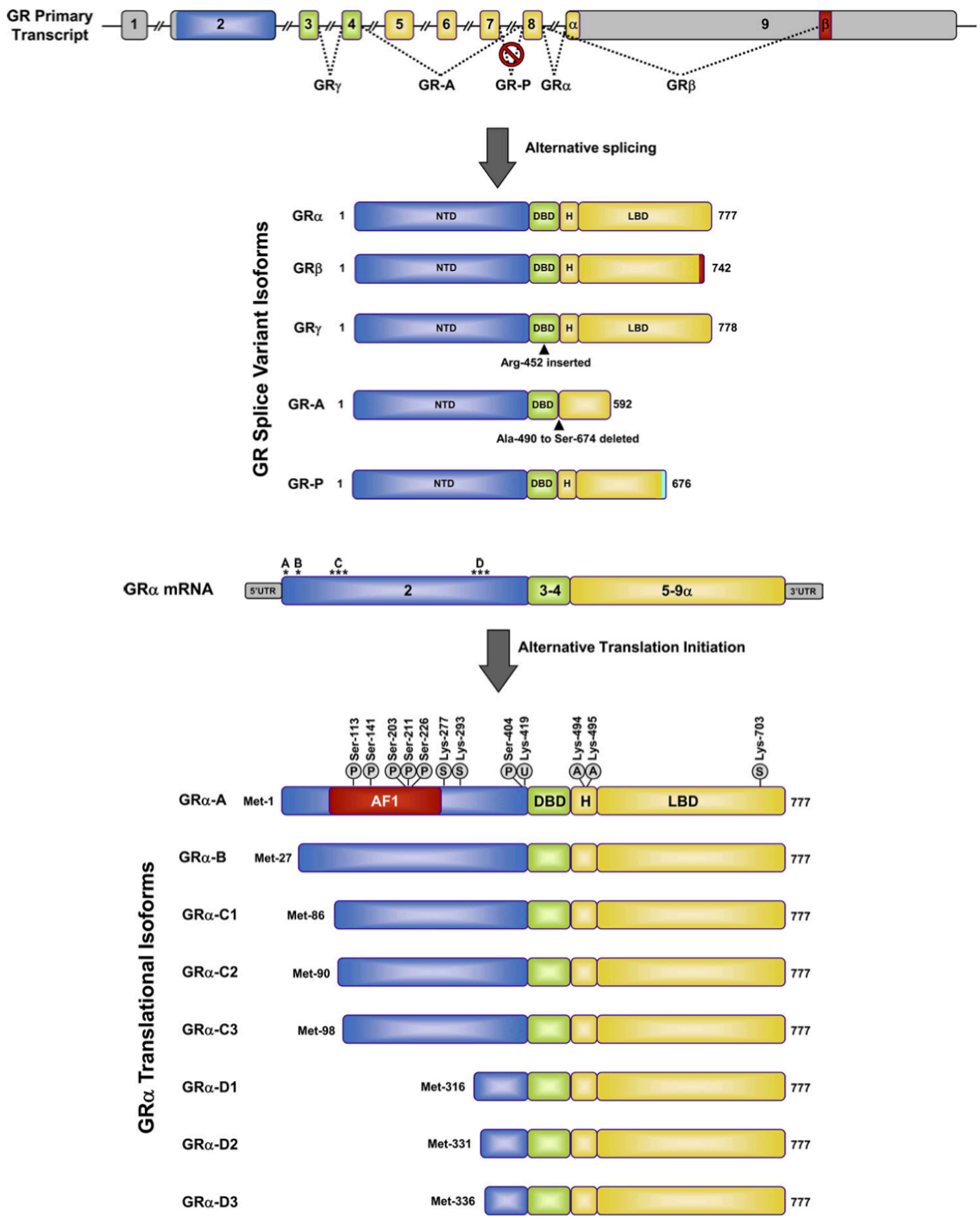


Figure 1.9 A single GCR (NR3C1) gene gives rise to multiple GCR mRNA splice variants as well as translational isoforms. The GCR gene contains nine exons, which are spliced alternatively to give rise to five different transcripts. The predominant transcript is GCRα containing exons 1-8 and 9α, while the other very similar GCRβ transcript contains exon 9β. Each of the different GCR mRNA transcripts gives rise to various translational proteins due to the presence of distinct start codons within exon 2. Thus these translational isoforms have different N-terminal domains (NTD). Taken from Oakley and Cidlowski, 2011.

1.3.1.4 Post-translational modifications of GCR

The translational isoforms of hGCR undergo various post-translational modifications that in turn regulate receptor activity, thereby enlarging the repertoire of receptors involved in glucocorticoid-mediated signalling. Phosphorylation is the first post-translational modification to be identified for GCR and so far the most studied. Other modifications include acetylation, SUMOylation and ubiquitination.

1.3.1.4.1 GCR phosphorylation

Several residues within the hGCR including ser^{113}, ser^{141}, ser^{203}, ser^{211}, ser^{226} and ser^{404} can be basally phosphorylated with differential level of phosphorylation noted across the residues. Most of these sites are conserved in rats and mice. The receptor undergoes hyperphosphorylation following interaction with GC ligands with phosphorylation levels being determined by the nature of the GC (Avenant et al., 2010; Chen et al., 2008; Wang et al., 2002). Similarly, GC-induced hyperphosphorylation of GCR has been noted on homologous residues in rodents (Bodwell et al., 1995; Hoeck and Groner, 1990; Ortí et al., 1989). A number of cellular kinases are shown to be involved in mediating site-specific phosphorylation of GCR, of which the most notable ones are mitogen activated protein kinases (MAPKs) and cyclin-dependent kinases (Cdks). For example, human cyclin A-dependent Cdk2 has been shown to phosphorylate rat GCR at ser^{224} and ser^{232}, which are homologous to human ser^{203} and ser^{211} respectively, while human cyclin E/Cdk2 phosphorylates rat ser^{224} only (Krstic et al., 1997). In humans, studies on p38 MAPK-mediated ser^{211} phosphorylation (Miller et al., 2005), extracellular regulated kinases (ERK)-mediated ser^{203} phosphorylation (Takabe et al., 2008), c-jun N-terminal kinases (JNKs)-mediated ser^{226} phosphorylation (Itoh et al., 2002) and GSK3β-mediated Ser^{404} phosphorylation (Galliher-Beckley et al., 2008) have been documented.

GCR phosphorylation is reported to modulate GCR-mediated cellular responses. Earlier studies on mouse GCR suggested that mutation of the key phosphorylation sites markedly reduced GC-mediated transactivation of a minimal promoter containing GREs in addition to the complete lack of GC-mediated autoregulation of

GCR mRNA and protein, but increased GCR protein stability (Webster et al., 1997). In humans, p38 MAPK-mediated phosphorylation at ser^{211} in response to GCs has been shown to be important for GC-mediated apoptosis in lymphoid cells (Miller et al., 2007, 2005). Moreover, GC-induced GCR phosphorylation on ser^{211} can encourage nuclear localisation of GCR (Wang et al., 2002) and is also indispensible for maximal GCR-mediated transactivation of its target genes (Blind and Garabedian, 2008; Chen et al., 2008; Wang and Garabedian, 2003). Conversely, ser^{226} phosphorylation of GCR mediated by JNKs has been shown to blunt GCR transactivation thus suggesting ser^{226}-mediated antagonism on GCR-mediated transactivation (Chen et al., 2008; Rogatsky et al., 1998a). Moreover, JNK-mediated ser^{226} phosphorylation promotes nuclear export of GCR after GC withdrawal, thereby hindering its nuclear retention (Itoh et al., 2002). Phosphorylation of GCR at ser^{404} mediated by GSK3β has been associated with increased GCR nuclear export, decreased GCR protein stability, reduced GCR-mediated transcriptional regulation of target genes as well as regulation of a distinct set of genes (Galliher-Beckley et al., 2008). Interestingly, interaction of the non-phosphorylated form of GCR with the tumor susceptibility gene TSG101 can increase GCR protein stability through the obstruction of its degradation by proteasomes and extension of its half-life. This is complemented by another important observation that dual phosphorylation of GCR at ser^{203} and ser^{211} promotes GCR degradation, as mutation of these sites displayed greater stability than their wild-type counterparts (Ismaili et al., 2005).

Although the above phosphorylation events are reported to occur following GC stimulation, hyperphosphorylation of GCR at ser^{134} independent of GC administration has been demonstrated under conditions of cellular stress including glucose starvation, oxidative stress, UV irradiation and osmotic shock. This is followed by an increased association of GCR with the 14-3-3 zeta signalling proteins, thereby resulting in a redirection of transcriptional responses of GC-regulated genes (Galliher-Beckley et al., 2011).

1.3.1.4.2 Other GCR post-translational modifications

GCR is acetylated on lys^{494} and lys^{495} upon GC-binding and this modification induces GC-insensitivity through impaired repression of nuclear factor kappa B (NF-κB). GCR deacetylation, primarily by histone deacetylase 2 (HDAC2), has been shown to restore GC-sensitivity by allowing GCR interaction with NF-κB thereby promoting the repression of NF-κB-mediated expression of proinflammatory genes (Ito et al., 2006). GCR can be ubiquitinated at a lys residue within the conserved PEST motif in its NTD between amino acids 407-426. This is necessary for GC-dependent GCR downregulation and protein turnover by proteasomal degradation (Wallace and Cidlowski, 2001). Proteasomal degradation of GCR can lead to defects in the transcriptional response of GC-responsive genes (Deroo et al., 2002; Wallace and Cidlowski, 2001). GCR is also modified by various small ubiquitin-related modifiers (SUMO) peptides, especially SUMO-1, at lys^{277} and lys^{293} within its NTD and lys^{703} within the LBD, although the latter appears to be a poor *in-vivo* SUMO-1 acceptor (Tian et al., 2002). SUMOylation of GCR is associated to decreased protein stability and has been shown to regulate GCR-mediated gene transactivation and transrepression in a promoter-specific context (Davies et al., 2008; Holmstrom et al., 2008; Le Drean et al., 2002; Tian et al., 2002). A very recent study demonstrated that SUMOylation regulate chromatin occupancy of GCR thereby influencing its target gene selection as well as modulating the anti-proliferative effect of GCs by affecting the activity of GCR on target genes important for cellular growth (Paakinaho et al., 2014).

Tyrosine nitration of GCR has also been reported in human PBMCs and monocytes using a synthetic nitro-derivative of prednisolone (NCX-1015) and this modification has been linked to enhanced GCR activation and anti-inflammatory action of this steroid (Paul-Clark et al., 2003).

1.3.2 Mechanisms of glucocorticoid action

GCs can exert their effects in both a genomic and non-genomic manner. The genomic effects of GC action are a delayed response, require interaction with specific hormones and depend on protein synthesis. In contrast, the non-genomic mode of GC action is very rapid and does not alter in response to inhibitors of mRNA and protein synthesis (Falkenstein et al., 2000).

1.3.2.1 Genomic effects of glucocorticoids

Most of the long-lasting effects of GCs that support its anti-inflammatory and immunosuppressive roles are mediated through a genomic mode. These require interactions of the GCR with DNA following altered expression patterns and responses of GC-regulated genes (Figure 1.10). However, some of the genomic effects can also arise due to the impact of GCs on target mRNA stability, protein translation and post-translational modifications of the effector proteins (Stellato, 2004). Thus, most of the genomic effects are sensitive to inhibitors of transcription and translation as well as antagonists of the classical cytoplasmic GCR (Falkenstein et al., 2000).

Under basal conditions GCR is kept sequestered in the cytoplasm by a multiprotein complex consisting of heat shock proteins (hsp) such as hsp90, hsp70, hsp56, hsp40, immunophilins such as FK506 binding proteins FKBP51 and FKBP52, co-chaperones such as p60, p23, Src kinase and some kinases of the mitogen activated protein kinases (MAPKs) signalling system (Alangari, 2010; Stahn and Buttgereit, 2008; Wikstrom, 2003). The ATP-dependent binding of hsp90 to unligated GCR is important in order to maintain the LBD in a high-affinity ligand binding conformation possibly by keeping the hydrophobic ligand-binding pocket open and accessible to ligands (Bresnick et al., 1989; Pratt and Toft, 1997). Moreover, hsp90 appears to be involved in GCR-mediated signal transduction, as reduced levels of hsp90 are associated with a reduced activation of GC-bound GCR (Picard et al., 1990). p23 binds to hsp90 in a ATP-independent manner and stabilises the interaction of hsp90 with unligated GCR to prevent their disassembly and inactivation (Dittmar et al., 1997). Owing to their lipophilic nature, GCs can diffuse

through the plasma membrane and bind to the cGCR with high affinity. Upon ligand binding, GCR becomes activated and undergoes a conformational change and dissociates from the basal multiprotein complex. Subsequently, ligand-bound GCR is engaged in nuclear translocation upon unmasking of the nuclear localisation signals on GCR (Akner et al., 1994; Bamberger et al., 1996). Upon entering the nucleus, it dimerises and binds to the major groove of DNA at consensus sequences known as glucocorticoid responsive elements (GREs) on the promoter regions of its target genes. GCR can form homodimers (GCR-GCR) or heterodimers with the mineralocorticoid receptor (GCR-MCR), another closely related member of the steroid receptor superfamily (Savory et al., 2001). Dimerisation plays a significant role in the determination of transactivation potential of GCR, as mutations in the dimerisation interface brings upon significant loss in GCR transactivation (Bledsoe et al., 2002).

1.3.2.1.1 GCR target gene transactivation by direct DNA binding

GREs mostly have an imperfect palindromic structure with the consensus sequence of 5'-GGTACAnnnTGTYCY-3' (Truss and Beato, 1993). GCR binding to GREs on some of its target genes causes gene transactivation or enhanced transcription (Figure 1.10) of some anti-inflammatory genes such as annexin-1 or lipocortin-1 (LC-1), interleukin-10 (IL-10) and inhibitor of nuclear factor kappa B (iκB), CD163, delta sleep inducing peptide (DSIP), interleukin-1 receptor II (IL-1RII) and receptor for Fc fragment of IgA (FCAR) as well as other genes involved in chemotaxis, phagocytosis and antioxidation. (Alangari, 2010; Ehrchen et al., 2007; Goulding et al., 1990; Roviezzo et al., 2002). The AF-1 (τ-1) region of GCR can recruit components of the basal transcriptional machinery such as TFIID to the TATA-box of target gene promoters, thereby directly facilitating transactivation (Ford et al., 1997). Alternatively, GCR can either interact with several transcriptional coactivators that serve to recruit the basal transcriptional machinery to the target gene promoters or recruit chromatin-remodelling cofactors such as, which can alter the DNA nucleosomal structure to facilitate gene expression (Revollo and Cidlowski, 2009). Some of the well-known coactivators are SWI/SNF complex, CBP/p300, p160, P/CAF and TRIP/DRIP/ARC complexes (Glass and Rosenfeld, 2000). Among the chromatin-

remodelling cofactors, histone acetyltransferase (HAT) and Spt-Ada-Gcn5 acetyltransferase (SAGA) are well characterised (Wallberg et al., 1999).

1.3.2.1.2 GCR gene transactivation by protein-protein interaction

GCR is also known to physically interact with the STAT family of transcription factors involved in mediating cytokine signalling through activation of Janus kinases (JAK) (Figure 1.10). GCR interaction with STAT-1, STAT-3 and STAT-5 synergises transcription of STAT-regulated genes (Aittomäki et al., 2000; Stocklin et al., 1996; Takeda et al., 1998; Tronche et al., 2004; Zhang et al., 1997).

1.3.2.1.3 GCR gene transrepression by direct DNA binding

Some GCR-regulated gene promoters contain sites at which GCR is recruited, but GCR binding silences the gene expression by either blocking the recruitment of transcriptional machinery or competing with coactivators required for transcription of the gene (Figure 1.10). Thus, these GR recognition sites are known as negative GREs (nGREs) (Barnes, 2001; Gupta and Lalchhandama, 2002). Osteocalcin and prolactin genes contain such nGREs overlapping with sequences for transcription factors (TFs) and thus after GC administration, when GC-GCR complexes occupy these nGREs, transcription of these genes are blocked as TFs cannot be recruited on their promoters (Meyer et al., 1997; Morrison and Eisman, 1993; Sakai et al., 1988). FasL is another gene, which contains an nGRE overlapping with a NF-κB-binding sequence. Ligated GCR occupancy on this promoter blocks the binding of NF-κB required for its transcription (Novac et al., 2006). Some other genes containing nGREs and negatively regulated by GCR include proopiomelanocortin, human corticotropin-releasing hormone (CRH), neuronal serotonin receptor, glutathione S-transferase and insulin (Necela and Cidlowski, 2004; Revollo and Cidlowski, 2009).

1.3.2.1.4 GCR gene transrepression by protein-protein interaction

GCR negatively regulates the expression of several pro-inflammatory genes independent of direct DNA binding. In such instances, GCR has been shown to physically interact with several transcriptional coactivators thereby preventing them from enhancing transcription of such genes (Figure 1.10). The two very

common candidates of this mode of GCR-mediated transrepression are the transcription factors activation protein-1 (AP-1), NF-κB and Smad3 (Necela and Cidlowski, 2004; Revollo and Cidlowski, 2009).

AP-1 is a mediator of cytokine signalling (TNF-α and IL-1β) and promotes the expression of several proinflammatory genes. It is composed of homo- or hetero-dimers of the basic leucine zipper transcription factors Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra1 and Fra2) and can be activated by JNK MAPKs. GCR interacts with the c-Jun subunit of AP-1, thereby hindering AP-1 mediated gene transcription. The GCR can also suppress activation of JNK and its TF targets ATF-2 and Elk-1 and subsequent induction of c-Jun and c-Fos resulting in the reduction of AP-1 mediated gene transcription (Caelles et al., 1997). Alternatively, GCR has been shown to physically interact with JNK leading to its inactivation and the subsequent inhibition of c-Jun transactivation (Bruna et al., 2003). Genes repressed by GCR through repression of AP-1 are collagenase and other matrix metalloproteinases (Jonat et al., 1990; Revollo and Cidlowski, 2009; Yang-Yen et al., 1990).

NF-κB, a homo- or hetero-dimer of Rel family of proteins (p50, p52, p65, Rel-B and c-Rel), is implicated in the transcription of several pro-inflammatory genes. GCR is known to physically interact with p65, thereby sequestering NF-κB and preventing it from binding to its target genes (Nelson et al., 2003; Revollo and Cidlowski, 2009; van der Velden, 1998). It may also inhibit assembly of the NF-κB complex by inducing expression of the inhibitor of NF-κB (IκBα), which inactivates NF-κB and traps it within cytoplasmic complexes (Auphan et al., 1995). Moreover, GCR can also mediate NF-κB transrepression by either interfering with the interaction between p65 and the basal transcriptional machinery or limiting coactivator availability (De Bosscher et al., 2000; Revollo and Cidlowski, 2009). In a recent report, GCR was shown to repress NF-κB activity through the involvement of the tumor suppressor protein p53 (Murphy et al., 2011).

1.3.2.1.5 Post-transcriptional gene regulation by GCR

It has become increasingly recognised that GCR can modulate several kinase signalling pathways activated by proinflammatory stimuli and thus can regulate a

number of post-transcriptional and translational events. Some of these examples will be discussed here briefly. GCs have been shown to inhibit JNK pathway resulting in an increased VEGF mRNA turnover and reduced LPS-induced TNF-α translation. GCs also can accelerate COX-2 mRNA decay through inhibition of p38 MAPKs. However, although GCs are known to inhibit the ERK pathway through the induction of MKP-1 as well as target mRNA stability of some ERK-regulated genes such as GM-CSF, no direct association between these events is apparent (Stellato, 2004).

GCs can also target several translation initiation factors, ribosomal genes and post-translational modification. For instance, GC can inhibit iNOS expression by reducing its protein translation and increasing its degradation. They also reportedly increase degradation of the rat acetlycholinesterase and GLUT-2. Besides, GCs regulate maturation of the murine mammary tumor virus (MMTV) protein in rat by perturbing the membrane compartmentalisation, processing and protein phosphorylation (Stellato, 2004).

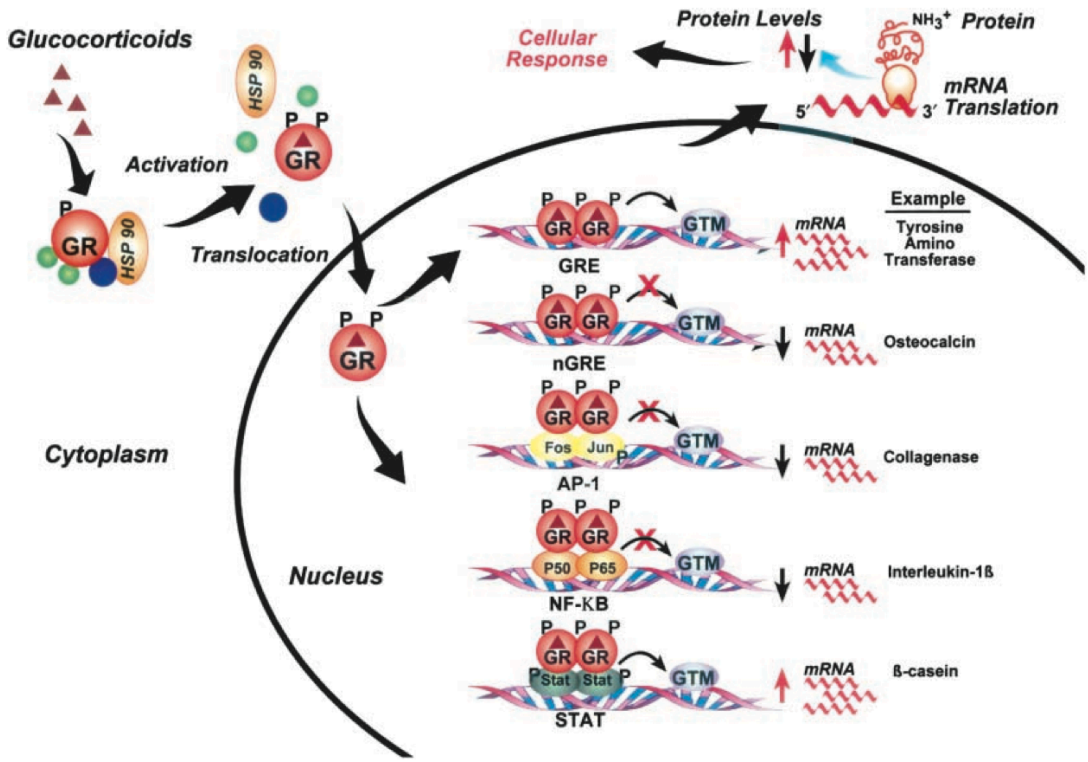


Figure 1.10 Basic mechanisms of glucocorticoid receptor (GR) action. GCR protein primarily resides in the cytoplasm forming a complex with several chaperones including hsp90, co-chaperones and immunophilins. On binding to GCs, the receptor is activated, dissociates from its basal complex and translocates into the nucleus. GCR then acts as a transcription factor (TF) and regulates the expression of various genes by several modes. GCR can bind as a dimer to either glucocorticoid response elements (GREs) in its target genes to activate gene transcription or to negative GREs (nGREs) to inhibit gene transcription. GCR can also physically interact with other TFs such as the c-Jun subunit of the AP-1 complex to inhibit AP-1-mediated gene expression or the p65 subunit of NF-κB to inhibit NF-κB-mediated gene expression. Finally, GCR can also physically interact with members of the STAT family (STAT1, STAT5, and STAT3) to synergise with STAT-regulated gene transactivation. GTM denotes general transcriptional machinery and P denotes a phosphate group. Taken from Necela and Cidlowski, 2004.

1.3.2.2 Non-genomic effects of glucocorticoids

There have been an increasing number of reports on the rapid actions of GCs. These effects are independent of GCR-mediated transcription and translation of target genes and are referred to as non-genomic effects of GCs (Figure 1.11). They can also be categorised into specific and non-specific effects. Specific non-genomic effects occur within a few minutes of GC administration and are carried out by either the cytoplasmic GCR (Croxtall et al., 2000; Hafezi-Moghadam et al., 2002; Löwenberg et al., 2005) or a specialised membrane-bound form of the receptor (Bartholome et al., 2004; Gametchu, 1987; Pérez et al., 2013) through the activation of cellular signalling pathways and/or generation of various second messenger systems. Non-specific non-genomic effects occur within seconds and are carried out independent of GCR by physicochemical interactions of GCR with the cell membrane (Buttgereit et al., 1997; Buttgereit and Scheffold, 2002). The non-genomic effects of GCs exhibit a distinct pharmacological profile when compared to the genomic effects in that they are insensitive to the inhibitors of transcription and translation. These effects have been also noted in anucleate cells such as platelets, erythrocytes and spermatozoa, where GCs cannot exert genomic effects. These effects have also been stimulated with GC-analogs, which cannot cross the plasma membrane and reach the intracellular compartment, e.g. bovine serum albumin (BSA)-conjugated GCs (Stellato, 2004).

1.3.2.2.1 Non-genomic actions of GCs mediated by cytoplasmic GCR

Some studies have reported rapid actions of GCs, which are independent of transcription or translation but are mediated by the cytoplasmic GCR (cGCR), as these were abolished in the presence of GCR antagonists. GCs are known to exert acute cardio-protective effects, for example hydrocortisone reduced the size of myocardial infarcts in dogs when administered after 30 minutes of vessel occlusion (Libby et al., 1973).

The synthetic GC, dexamethasone has been widely used in experimental studies because of its pharmacological importance and has been shown to exert of number of rapid effects on different cell types. It induces a rapid, non-transcriptional

activation of eNOS followed by NO-dependent vasorelaxation and an *in-vivo* reduction in vascular inflammation and myocardial infarct size following ischemia and reperfusion (I/R) injury (Hafezi-Moghadam et al., 2002). These effects of dexamethasone were mediated by the cGCR through the activation of the phosphatidylinositol 3-kinase (PI3K) signalling pathway resulting in the downstream activation of protein kinase Akt and glycogen synthase kinase 3 (GSK3) (Hafezi-Moghadam et al., 2002). Another study using a cerebral ischemic model demonstrated that dexamethasone increased cerebral blood flow and reduced cerebral infarct size by a cGCR-dependent, non-transcriptional activation of eNOS through a PI3K/Akt mechanism (Limbourg et al., 2002). A similar mechanism of PI3K/Akt-mediated activation of eNOS had been reported earlier (Dimmeler et al., 1999). Dexamethasone can inhibit phosphorylation and activation of Lck and Fyn kinases in T-cells both *in-vitro* and *in-vivo* through a GCR-dependent mechanism. Suppression of these earliest events of T-cell activation brought about reduced activation of downstream signalling pathways such as Akt, PKC and MAPKs (Löwenberg et al., 2005). Dexamethasone also caused rapid GCR-mediated, non-transcriptional inhibition of insulin signalling by targeting phosphorylation and activation of downstream effectors of the insulin receptor (Löwenberg et al., 2006). Dexamethasone promoted rapid GCR-mediated serine phosphorylation of annexin-1 (ANXA-1) and its membrane translocation in the cells of human anterior pituitary gland in a transcription-independent manner involving activation of the PI3K, PKC and MAPK signalling pathways (Solito et al., 2003). This observation confirmed earlier findings where dexamethasone was shown to inhibit cytosolic phospholipase A_2 ($cPLA_2$) activity and subsequent arachidonic acid (AA) release via the involvement of annexin-1 or lipocotin-1 (LC-1). In this particular report, dexamethasone was again shown to rapidly enhance the phosphorylation of LC-1 and its membrane translocation in a GCR-mediated manner independent of transcription. Enhanced membrane localisation of LC-1 promoted its recruitment on the epidermal growth factor receptor (EGF-R) by disrupting the interaction of EGF-R with the adaptor protein Grb-2 that is important for $cPLA_2$ activity and subsequent AA release in a MAPK-dependent manner (Croxtall et al., 2000).

1.3.2.2.2 Non-genomic actions of GCs mediated by membrane GCR

Numerous instances of the existence of membrane receptors that participates in mediating rapid GC effects have been documented. Synaptic membranes of the amphibian (*Taricha granulosa*) brain were shown to contain high affinity binding sites for corticosterone and the male reproductive behaviour in these organisms were rapidly suppressed following corticosterone administration. Thus, it was suggested that brain membranes could harbour a receptor responsible for mediating the rapid effects of GCs (Orchinik et al., 1991). In these organisms the membrane receptors also mediated the rapid modulation of the electrophysiological excitability and sensory responsiveness of hindbrain neurons in response to corticosterone (Rose et al., 1993). GCR has also been identified in plasma membranes of mouse and human lymphoid cells and human leukemic cells by various immunodetection methods. These membrane GCRs exhibit higher molecular weights than cytoplasmic GCRs and are linked to GC-induced lymphocytolysis (Gametchua et al., 1999; Gametchu, 1987; Gametchu et al., 1993). mGCRs have also been identified in plasma membrane of rat kidney and mammalian skeletal muscle fibres where they have been shown to mediate the rapid, non-genomic effects of GCs (Ibarrola et al., 1991; Pérez et al., 2013). Human monocytes, B cells and peripheral blood mononuclear cells (PBMCs) also express mGCRs. Their levels increase after *in-vitro* stimulation with lipopolysaccharides (LPS) as well as in patients with rheumatoid arthritis and systemic lupus erythmatosus (Bartholome et al., 2004; Spies et al., 2006). Thus membrane GCRs are believed to mediate non-genomic actions of GCs in various cell types.

1.3.2.2.3 Non-specific non-genomic actions of GCs

GCs molecules are highly lipophilic in nature and hence can easily diffuse through the plasma membrane. When administered in high concentrations, they interfere with several transmembrane receptors or membrane ion channels, which elicit various cellular responses. In resting rat thymocytes and those induced by the mitogen Concavalin A (ConA), methylprednisolone was shown to suppress respiratory rate and Ca^{+2} and N^{+} cation cycling by preventing the cation influx and

uptake across the plasma membrane due to altered physicochemical properties of the membrane and activities of membrane-associated proteins or ion-channels, which led to the reduction in cytosolic free Ca^{+2} concentrations (Buttgereit et al., 1997, 1993). It was also shown that methylprednisolone reduced oxidative phosphorylation, increased mitochondrial leak, thereby reducing ATP availability and inhibited nucleic acid synthesis, although ATP production and turnover as well as protein synthesis was not affected (Buttgereit et al., 1997, 1994).

Subsequent experiments were performed with normal physiological concentrations of GCs to establish the *in*-vivo relevance of these non-specific non-genomic effects. When primary human bronchial epithelial cells and related cell-lines were treated with dexamethasone (0.1-1 µM), a rapid reduction in basal and ATP-induced intracellular Ca^{+2} concentration was observed followed by a reduction in Ca^{+2}-dependent ATP-induced Cl^{-} secretion by the cells. The effect of dexamethasone was mediated by stimulation of the Ca^{+2}-ATPase pump through the adenylyl-cyclase/protein kinase A (AC)/PKA signalling pathways and was independent of cellular translation. This anti-secretory role of dexamethasone might add another dimension to the therapeutics of airway diseases, specially in asthma, where mucus overproduction is associated with increased expression of Ca^{+2}-dependent Cl^{-} channels (Urbach et al., 2002).

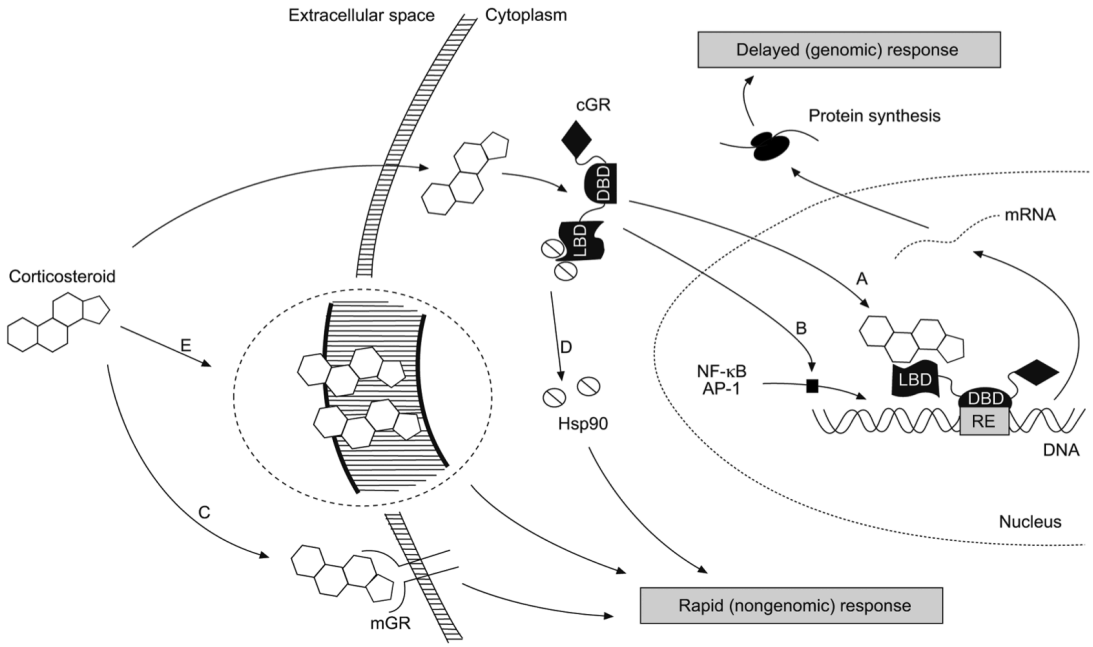


Figure 1.11 Non-genomic mode of glucocorticoid action. Non-genomic actions are mediated by membrane-bound receptors or mGR (C), cytosolic receptors or cGR (D) or interaction with cell membrane (E). These actions are characterized by their rapid onset and lack of dependence on transcription and translation. On the other hand, genomic actions are mediated through a) direct DNA binding (transactivation) or b) transcription factor inactivation (transrepression). Taken from Alangari, 2010.

1.3.3 Regulation of GCR

1.3.3.1 Regulation of GCR expression by GC

In GCR-expressing HeLa S3 cells, dexamethasone administration was shown to reduce nuclear and cytoplasmic GCR numbers without affecting GC binding (Cidlowski and Cidlowski, 1981). Dexamethasone was also shown to reduce GCR mRNA levels considerably in two other cell-lines, human IM-9 lymphocytes and rat pancreatic acinar AR42J cells by reducing GCR gene transcription (Rosewicz et al., 1988). In HeLa cells and cells where GCR were expressed through transfections, GC administration was again shown to downregulate the amount of GCR mRNA and protein through reduction of the GC-binding capacity of GCR (Burnstein et al., 1991). Additionally, dexamethasone administration reduced GCRα expression in a time-dependent manner and increased GCRβ expression in a concentration- and time-dependent manner in a human monocytic cell-line known as THP-1 (Bo et al., 2006).

GCR expression is also regulated by GCs in patients with autoimmune inflammatory diseases. When whole cell extracts of lymphocytes were analysed by immunoblotting, patients with rheumatoid arthritis (RA) on low-dose prednisolone treatment demonstrated reduced GCR density compared to untreated patients (Neeck et al., 2002). Similarly membrane GCR on monocytes in patients with systemic lupus erythmatosus (SLE) exhibited a decreased expression following prednisolone treatment (Spies et al., 2006).

1.3.3.2 Regulation of GCR by other transcription factors and signalling pathways

Regulation of target gene expression by GCR via modulation of several TFs and signalling pathways has been discussed earlier in section 1.3.2.1. GCR activity itself can be regulated by a majority of these TFs and signalling pathways. Both the c-Jun and c-Fos subunits of AP-1 have been shown to interact with GCR and antagonise GCR activity (Jonat et al., 1990; Schüle et al., 1990; Yang-Yen et al., 1990). Similarly IL-2 and interferon-α (IFN-α) are shown to inhibit GCR activity via the activation of STAT5 (Biola et al., 2001; Pace et al., 2011; Stocklin et al., 1996). In contrast, GCR

activity has been shown to be upregulated by other signalling pathways. Induction of STAT-3 signalling by IL-6 has been shown to act as a co-activator in GCR-mediated transcriptional responses (Zhang et al., 1997). Activation of the cAMP/PKA signalling abolishes IFN-α-mediated GCR repression by inhibition of the STAT-5 signalling (Pace et al., 2011).

1.3.4 Glucocorticoid resistance

A proportion of asthma patients and a vast majority of patients suffering from chronic obstructive pulmonary diseases (COPD) show insensitivity to GC treatment, commonly referred to as glucocorticoid resistance, which necessitates the administration of higher drug doses to manage their clinical condition (Adcock, 2000). However, this can give rise to a different set of complications emerging from the side effects of these high GC doses without actual clinical benefit. A number of mechanisms have been identified that explain the occurrence of this challenging condition. Some of these have already been discussed in section 1.3.4.5 detailing the regulation of GCR. Some other mechanisms, which have been observed in patients insensitive to GC treatment, will be discussed below.

Genes encoding cytokines IL-2 and IL-4 have been found to be upregulated in GC-resistant asthma patients compared to GC-sensitive patients. Moreover the GC-resistant group had altered regulation of IL-4, IL-5 and IFN-α mRNA levels after GC treatment (Leung et al., 1995). A combination of IL-2 and IL-4 has been shown to reduce binding affinity of GCR in T-cells of the GC-resistant group (Sher et al., 1994). GCs exhibit a reduced ability to inhibit antigen-induced release of the cytokine GM-CSF in the PBMCs of this group. In some GC-resistant patients, reduced GCR levels are also reported (Sher et al., 1994). An increase in p38 activation and a reduction of MKP-1 expression is observed in the macrophages of some patients with severe asthma (Bhavsar et al., 2008). Reduced nuclear translocation of GCR mediated by p38 MAPK is found in patients exhibiting GC-resistant severe asthma (Irusen et al., 2002). GC resistance in these patients can be reversed in the presence of p38 inhibitors by improving GCR nuclear translocation as well as reduce ser^{226} phosphorylation (Irusen et al., 2002; Mercado et al., 2012). Thus, p38 might be

employed as a biomarker to identify the response of GCs in severe asthma patients, thereby improving the clinical management of this condition.

Increased AP-1 activation due to either enhanced JNK activity or higher c-Fos levels and defective GCR signalling has been noted in bronchial biopsies of some GC-resistant patients (Adcock et al., 1995). Another important biomarker of GC resistance is HDAC-2, a repressor of activated inflammatory genes. Its expression is reduced in alveolar macrophages, airways and peripheral lungs of patients with COPD and refractory asthma. GC insensitivity in these patients are reversed by HDAC-2 overexpression (Barnes, 2011).

GC resistance has been frequently linked with the GCRβ isoform. In GC-resistant patients with asthma, ulcerative colitis, rheumatoid arthritis and inflammatory bowel diseases (IBD), increased expression of GCRβ has been observed (Hamid et al., 1999; Honda et al., 2000; Kozaci et al., 2007; Orii et al., 2002; Sousa et al., 2000). Since GCRβ has been shown to antagonise transactivation potential of the predominant GCRα isoform, increased GCRβ levels could be a mode of blunting GCRα activity in these patients, thereby contributing to GC resistance (Bamberger et al., 1995; Oakley et al., 1999). Pro-inflammatory cytokines such as TNF-α and IL-1 can increase GCRβ levels over the major GCRα isoform and this observation correlates with GC-resistance (Webster et al., 2001). GCRβ overexpression has also been shown to reduce HDAC-2 expression in GC-resistant asthma patients (Li et al., 2010). In mice GC resistance has also been established in conditions of tumor necrosis factor α (TNF-α) challenge via downregulation of GCR protein and suppression of GCR transactivation (Van Bogaert et al., 2011).

Several polymorphisms have been reported in the GCR gene (NR3C1), which give rise to modified GCR transcripts that can determine individual responsiveness to GCs. For example, *Bcl*I polymorphism has been associated with hypersensitivity to GC treatment, while the N363S polymorphism improves GC sensitivity. On the contrary the ER22/E23EK polymorphism has been associated with reduced GC responsiveness. Some GCRβ polymorphisms have also been reported, which also are associated with GCR insensitivity. However, as the occurrence of these

polymorphisms are rare, their impact might not be significant (Kadmiel and Cidlowski, 2013).

1.3.5 Glucocorticoids and platelets

Human platelets are known to express several nuclear receptors and activation of these receptors with selective ligands modulated platelet activation (Ali et al., 2005; Khetawat et al., 2000; Moraes et al., 2007). Thus platelets have been deemed as an apt model for the study of the non-genomic mode of steroid hormones (Bishop-Bailey, 2010). Platelets are believed to be crucial in mediating inflammatory responses as they act as a storehouse of several proinflammatory mediators, which are released at sites of vascular damage (Weyrich and Zimmerman, 2004; Zimmerman et al., 1996). Since GCs are reputed for their anti-inflammatory properties in immune cells (Barnes, 1998), there is considerable potential for these molecules to target platelet functions too. However, initial studies suggested that administration of conventional clinical concentrations of prednisone in healthy volunteers could neither delay hemostatic events as measured by bleeding times nor inhibit platelet aggregation in response to collagen, adenosine diphosphate and adrenalin (Thong et al., 1978). Similarly, administration of a more widely employed synthetic GC dexamethasone could not affect collagen-induced platelet aggregation and thromboxane generation in healthy volunteers (Rosenkrantz et al., 1985). Thus, GCs were thought to be generally ineffective in modulating platelet functional responses, barring one study where high concentrations of hydrocortisone, a derivative of the physiological GC cortisol, inhibited platelet aggregation in response to ADP and thrombin stimulation (Jørgensen and Stoffersen, 1981). This inhibitory effect could have resulted from the alteration of platelet membrane characteristics (as indicated in section 1.3.2.2.3), rather than directly affecting platelet activation. As platelets were shown to express various nuclear receptors, the scope of GCR expression in platelets was also explored years later. Human platelets were thereby shown to express GCR in a complex with mineralocorticoid receptor (MCR) (Moraes et al., 2005). This receptor was shown to modulate platelet function in ligand-specific manner, as interaction with one ligand (prednisolone) inhibited platelet aggregation and TxB_2 generation, while others (dexamethasone,

hydrocortisone and aldosterone) seemed to have no such effect (Moraes et al., 2005). The observation that inhibition of normal platelet functions mediated by prednisolone was reversed by the GR-antagonist mifepristone (RU486) further reflected some selectivity in prednisolone action and importance of the ligand-receptor complex (Moraes et al., 2005). Thus, the finding of an inhibitory action of GC on human platelets provided an exciting opportunity in the field of platelet regulation, as this could create a promising therapeutic regime against CVDs that are specifically triggered by inappropriate platelet function, e.g. atherosclerosis.

1.4 Aims of the project

As GCs have been shown to be therapeutically promising in the treatment of inflammatory disorders and CVDs, it will be invaluable to specifically study the role of GCs in modulating platelet function, which is a critical determinant of both the above-mentioned clinical conditions. Although previous studies have shown considerable evidence that platelet functions can be inhibited by the synthetic GC prednisolone (Moraes et al., 2005), the mechanisms behind these inhibitory effects have not yet been explored. It is indeed imperative to understand the mode of action of prednisolone in platelets at the molecular level in order to judge potential usefulness as a platelet inhibitor in the clinic. Keeping this in mind, the following aims were drawn for this project:

- Characterisation of the inhibitory effects of prednisolone on platelet functions induced by a variety of physiological stimuli in order to determine the potency of prednisolone as an inhibitor.

- Validation of the expression of GCR in human washed platelets by a combination of biochemical techniques.

- Determination of the involvement of GCR in mediating the effects of prednisolone through the use of a classical GCR antagonist.

- Identification of the molecular mechanisms underlying the inhibitory effects of prednisolone on platelet activation induced by thrombin.

Chapter 2

Methods and materials

2.1 Chemicals and reagents

Acrylamide and detergent compatible protein assay kit were purchased from Bio-Rad (Hemel Hempstead, UK). 1,2-Bis(2- aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) and (R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y27632) were purchased from Calbiochem (Nottingham, UK). PAR1 receptor agonist (SFLLRN) and PAR4 receptor agonist (AYPGKF) were purchased from Cambridge Bioscience (Cambridge, UK). Collagen horm reagent was purchased from Nycomed (Zurich, Switzerland). Biotinylated protein ladder was purchased from Cell Signaling (Hitchen, UK). Paraformaldehyde was from Electron Microscopy Sciences (USA). Thrombin, adenosine 5'-diphosphate, S-nitrosoglutathione, prednisolone, mifepristone, A3P5P, MRS2395, apyrase, indomethacin, ethyleneglycoltetraacetic acid, human fibrinogen, FITC-phalloidin, triton X-100, DMSO, bovine serum albumin, protease inhibitor cocktail, phosphatase inhibitor cocktail, protein A/G conjugated sepharose beads, ammonium persulfate, N,N,N^{I},N^{I}-tetramethylethylenediamine, developer and fixer solutions, saponin, propidium iodide, trypan blue, Virkon and the general chemicals used to prepare the various buffers used throughout the study were purchased from Sigma-Aldrich (Poole, UK). The RhoA activation assay kit was from Cytoskeleton (Cambridge, UK). Cell culture reagents such as DMEM medium and RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin were purchased from Invitrogen (Paisley, UK). Immunomagnetic sheep anti-rat IgG beads were from BD Biosciences (USA). Murine stem cell factor and thrombopoietin were from PeproTech (Hamburg, Germany).

2.2 Antibodies

Rabbit polyclonal anti-human glucocorticoid receptor (GCR) and anti-Syk antibodies were purchased from Santa Cruz (Wembley, UK). Mouse monoclonal anti-human

GCR antibody, anti-rabbit IgG conjugated to Alexa647 and anti-mouse CD41 antibody conjugated to FITC were purchased from BD Biosciences (Oxford, UK). Anti-mouse B220 and anti-mouse CD16/32 antibodies were purchased from BD Biosciences (USA). Anti-mouse GR1 and anti-mouse CD11b antibodies were purchased from eBioscience (USA). Anti-phospho-GCR-ser^{211}, anti-phospho-MLC-ser^{19}, anti-phospho-MYPT1-thr^{696} and anti-phospho-MYPT1-thr^{853} antibodies were purchased from Cell Signaling (Hitchen, UK). Anti-phospho-GCR-ser^{203} and anti-phospho-GCR-ser^{226} antibodies were purchased from Abcam Plc (Cambridge, UK). Anti-RhoA antibody was purchased from Cytoskeleton (Cambridge, UK). Anti-β-tubulin, normal mouse IgG isotype control and normal rabbit IgG isotype control antibodies were purchased from Upstate (Watford, UK).

2.3 Human blood collection

Ethical permission for the use of human blood in this project was granted by the ethics committee at the Postgraduate Medical institute (Hull York Medical School, Hull, UK). Blood was drawn, after informed consent, from healthy adult volunteers not taking any drug affecting platelet function, such as aspirin, within 14 days prior to blood donation. Donors were either males or females that we used at random from a larger pool of volunteers of age group between 20 and 50. No exclusion criteria for smokers and/or alcohol users were applied during our study. Blood was drawn by venepuncture using 21G butterfly needle into the anticoagulant acid citrate dextrose (ACD) buffer (113.8mM D-glucose, 29.9mM Tri-sodium citrate, 72.6mM NaCl, 2.9mM citric acid, pH 6.5) 5:1 (v/v) for washed platelet studies or into tri-sodium citrate (109mM) 9:1 (v/v) for the use of PRP. On every blood collection, the first 2 ml of blood was taken without anticoagulant and discarded in order to avoid initial platelet activation during venepuncture. Platelet rich plasma (PRP) was obtained by centrifugation of the blood at 200 g at 20°C for 20 minutes. PRP was carefully pipetted into a clean tube taking care not to disturb the buffy coat. After addition of citric acid (0.3 M) at a ratio of 1:50 (v/v), PRP was centrifuged at 800 g at 20°C for 12 minutes to obtain the platelet pellet. Citric acid is added to lower the pH of PRP and prevent platelet activation during further centrifugation steps. The supernatant referred to as the platelet poor plasma (PPP) was

centrifuged again to obtain residual platelets, which could not be recovered in the previous centrifugation step. The platelet pellet was resuspended in wash buffer (36mM citric acid, 10mM EDTA, 5mM D-glucose, 5mM KCl, 90mM NaCl, pH 6.5) and centrifuged for the final time at 800 g at 20°C for 12 minutes. The supernatant was removed and the platelet pellet was resuspended in modified Tyrode's buffer (150mM NaCl, 5mM HEPES, 0.55mM NaH_2PO_4, 7mM $NaHCO_3$, 2.7mM KCl, 0.5mM $MgCl_2$, 5.6mM D-glucose, pH 7.4). Washed platelets thus obtained were used within 3 to 4 hours. All the buffers used during washed platelet preparation were filtered through 0.2 µm Whatman filters.

2.4 Manual platelet count

Washed platelets were diluted in ammonium oxalate (1%, w/v) solution 1:100 (v/v). 10 µl of diluted platelet solution was then pipetted on to an improved Neubauer haemocytometer on both the chambers ensuring that the chambers were covered completely with the solution. The platelet solution was allowed to settle for 10 minutes ensuring that the haemocytometer was placed horizontally on the bench. Platelets were counted under an inverted light microscope using the ×40 objective and were recognised by their round shape. After counting both the chambers, platelet count per ml was determined by accounting for the volume of the solution, which was counted and the dilution factor as per the following equation:

Platelet count/ml= average of platelet number in both the chambers × 25000 × dilution factor

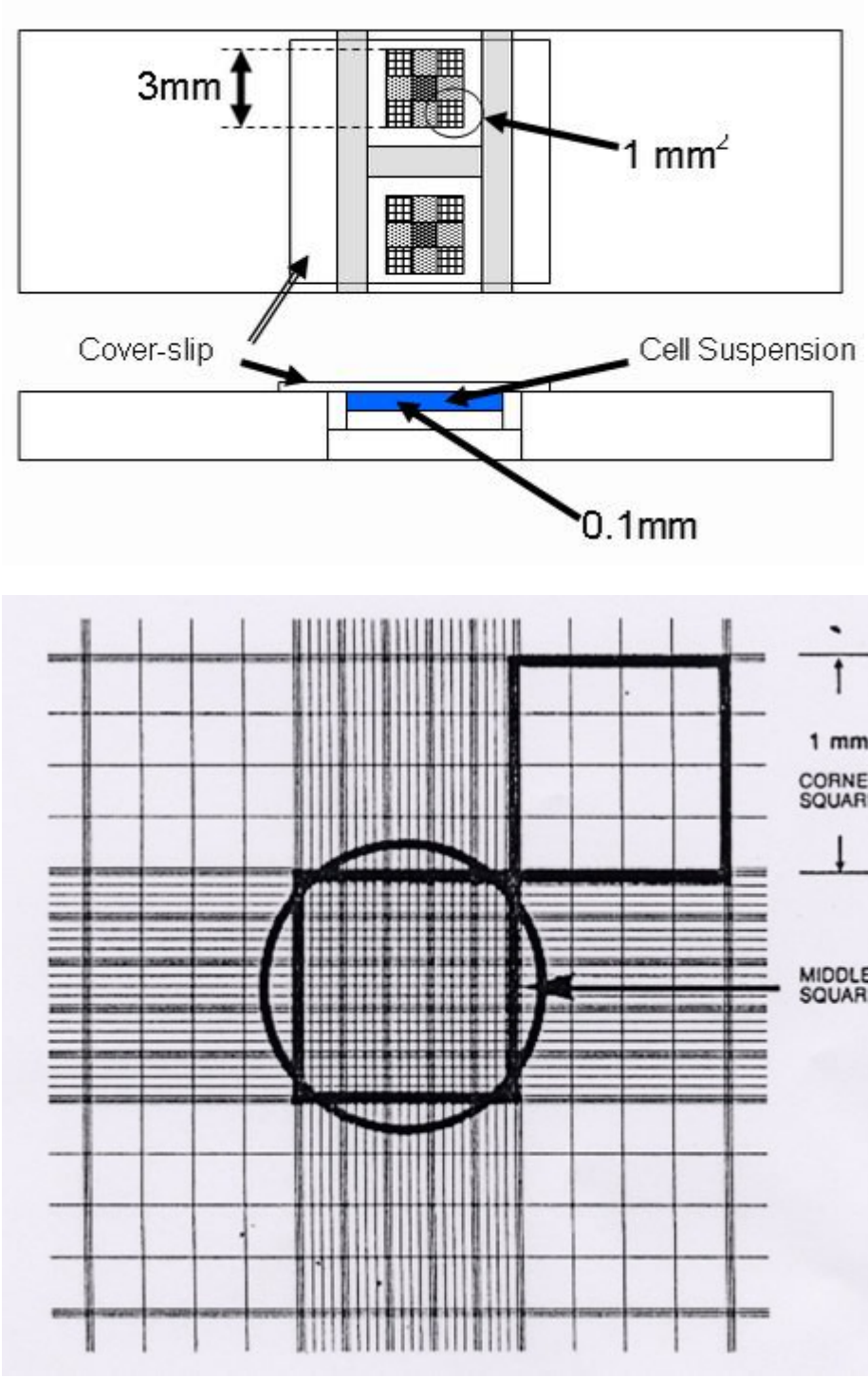


Figure 2.1: Representation of a typical Neubauer haemocytometer for counting platelets and other cells. Platelets in the central square are counted.

2.5 Light transmission aggregometry (LTA)

2.5.1 Principle of LTA

Light transmission aggregometry is a powerful technique, which was initially introduced by Born (1962). It is used to mimic the final outcome of platelet activation culminating into clot formation *in vivo* called platelet aggregation in an *in vitro* setting. It is also routinely employed as a platelet function test. This technique utilises the principle of light transmission passing through a turbid solution, which is then detected by a photocell. Washed platelets or PRP is pipetted in a glass cuvette which is then placed at 37°C under stirring conditions between a light source and a photocell inside an optical aggregometer. Upon agonist stimulation, platelets change their shape and the integrin $\alpha_{IIb}\beta_3$ receptors on the platelet surface undergo conformational change from a low-affinity state to a high-affinity state. This enables the integrin to bind fibrinogen released from the alpha granules of activated platelets. Fibrinogen can bind to two integrin molecules simultaneously, thus it acts as a bridge connecting two activated platelets. Thus several fibrinogen molecules can link several activated platelets forming microaggregates, which can then form macroaggregates. As these platelet aggregates start to form, the optical density of the platelet suspension is reduced, thus allowing more light to pass through it. Thus transmittance of light gradually increases from 0 towards 100% depending on the intensity of platelet aggregation, which is directly proportional to the concentration of agonist added to stimulate platelets under stirring conditions. The photocell detects the amount of light transmission through the platelet suspension and this is represented in the form of a trace, which is recorded through the computer software. There is however an initial decrease in light transmission through the platelet solution for a few seconds upon addition of the agonist when platelets undergo shape change, which is represented by a small initial bulge in the aggregation trace. Depending on the potency of the agonist, platelets can undergo three types of aggregation. Primary reversible aggregation results from a weaker agonist where platelets can revert back to their resting state. With stronger agonists platelets can undergo either biphasic aggregation, which is irreversible in

nature or a complete secondary aggregation that results due to granule secretion (Figure 2.2).

2.5.2 Methodology for LTA

In this study, washed platelets were used to determine platelet aggregation in response to various agonists using a Chronolog aggregometer (Figure 2.2). Aggregation was expressed as % increase in light transmission through a stimulated platelet suspension in comparison to the light transmission through a non-stimulated sample. For all experiments, modified Tyrode's buffer (500 µl) was used as the standard non-stimulated sample representing 0% aggregation. Before recording the light transmission through every test sample, the aggregometer was calibrated with this standard. Washed platelets (250 µl) adjusted to a count of 2.5×10^8/ml were pipetted into aggregation cuvettes and incubated at 37°C. Platelets were next incubated at the same temperature with stirring for 30 seconds to ensure that stirring did not cause any spontaneous platelet aggregation. Platelets were then stimulated with various agonists. Aggregation was monitored for 4 minutes upon which results were calculated manually and expressed as % aggregation. In conditions where effect of prednisolone on aggregation was to be determined, platelets were incubated with the appropriate concentration of prednisolone for the appropriate time prior to agonist stimulation. In selected samples, platelets were pre-incubated with the GCR antagonist RU486 (10 µM) for 5 minutes to reverse prednisolone-mediated effect on platelets prior to prednisolone treatment and agonist stimulation. For shape change experiments, platelets were pre-treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) to block the release of secondary mediators and aggregation before stimulation with an agonist.

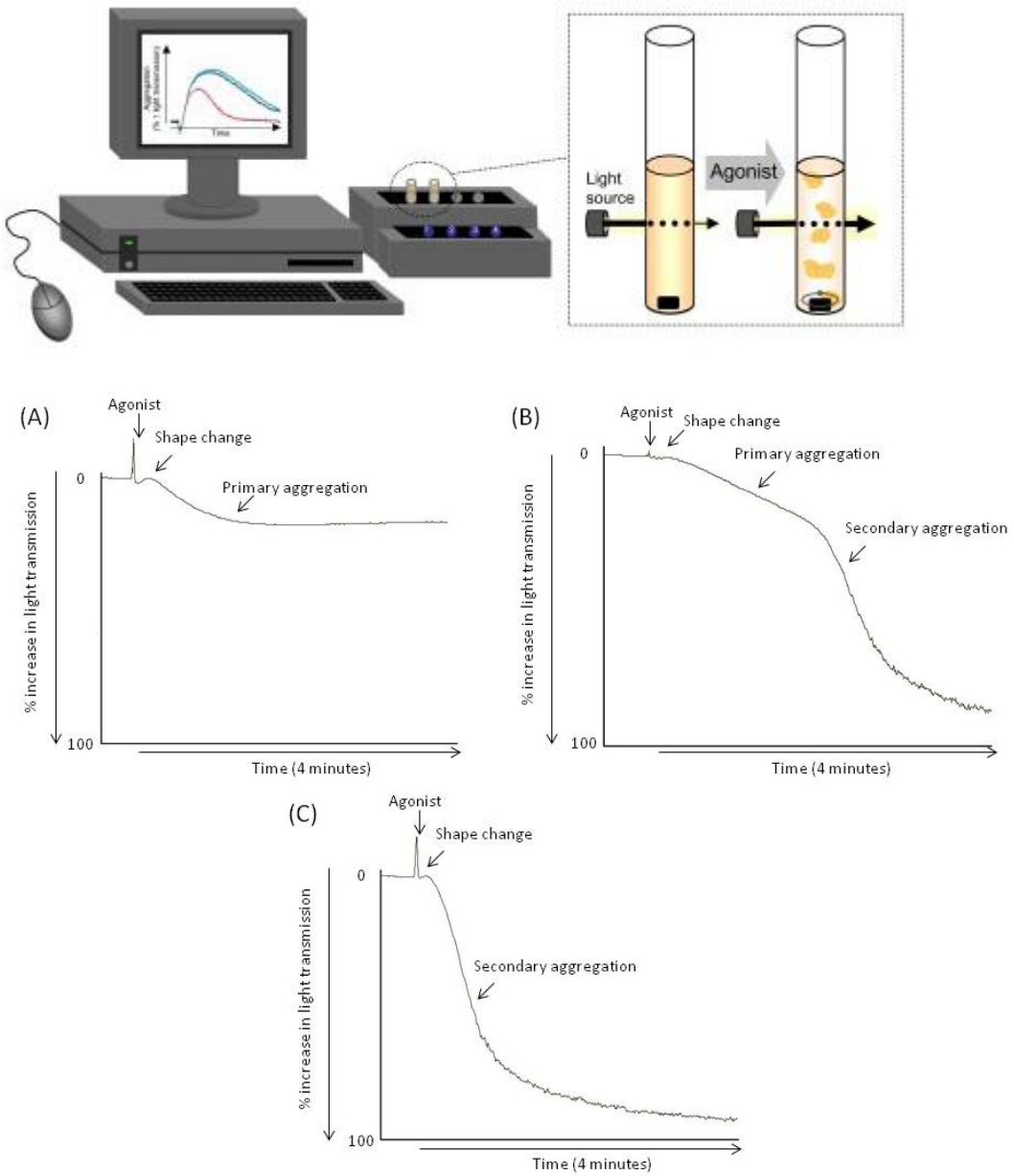


Figure 2.2: Representation of platelet aggregometry and aggregation traces. The top panel shows a typical aggregometer where a suspension of platelets is stimulated by the addition of an agonist under stirring conditions upon which platelet aggregation can be accurately determined by monitoring increase in light transmission through the platelet suspension. The bottom panels show representative aggregation traces. Panel (A) shows a primary or reversible aggregation trace induced by a mild agonist, while panel (B) shows a biphasic trace stimulated by a moderate agonist, which is sufficient to induce secondary or irreversible aggregation. Panel (C) shows a complete secondary aggregation response induced by a strong agonist.

2.6 Static adhesion of platelets to thrombogenic surfaces

Platelet adhesion to prothrombogenic surfaces is a powerful tool to analyse very early events of hemostasis and thrombosis involving the interaction between platelets and adhesive surfaces. Platelets express several glycoprotein receptors on their surface, which can bind to various subendothelial adhesive matrix proteins, plasma adhesive proteins or those released from activated platelet granules. These interactions are important in initial tethering of platelets followed by a stable and irreversible adhesion to the site of the damaged vasculature, which ultimately results in thrombus formation. Platelet adhesion can be monitored under two different conditions: one performed under artificially manipulated blood flow conditions and the other under static conditions. The latter is particularly important to study the initial contact of platelets with the adhesive matrix of interest.

2.6.1 Static adhesion methodology

Glass slides were coated with 100 µg/ml fibrinogen (reconstituted from powder form in 0.9 % saline pre-warmed at 37°C) and incubated overnight at 4°C in a humified chamber prepared by soaking paper towels with distilled water in a petri dish. As a negative control, a slide was prepared on which human serum was allowed to immobilise. Non-adsorbed fibrinogen was washed away in PBS. The slides were blocked with human serum to minimise non-specific platelet adhesion. Washed platelets (5×10^7/ml) were added on to slides and incubated for an hour at 37°C. Unbound platelets were washed with PBS. Platelets bound to immobilised fibrinogen were fixed with 4% paraformaldehyde for 30 minutes at room temperature followed by a wash step. Platelets were then permeabilised using 0.3% Triton X-100 solution for 7 minutes at room temperature followed by two successive wash steps. Platelets were then stained with Phalloidin-FITC (10 µg/ml) for an hour at room temperature in the dark followed by a wash step. Slides containing adhered platelets stained with Phalloidin were then mounted with glass coverslips using Histomount solution. Slides were visualised under the Olympus fluorescent microscope using the ×60 objective under oil immersion. Images of at least 8 independent fields were captured and each condition was performed in

duplicate on the same slide. The images were viewed using the Cell-M software provided by the Olympus manufacturer and analysed using the NIH-Image J software to determine the number of platelets adhered as well as surface coverage of each platelet. Data was obtained as the mean ± SEM of the eight different fields.

2.6.2 Preparation of human serum

Serum is the form of blood plasma, which is devoid of proteins like fibrinogen, prothrombin and coagulation factors. It is thus composed of the remaining plasma proteins such as albumin, globulin, enzymes, hormones as well as other plasma nutrients. Since platelets can adhere to immobilised fibrinogen present in plasma, it is important to use human serum as a negative control in adhesion experiments in order to exclude the possibility of platelet adhesion to other plasma proteins.

Human anti-coagulated blood from healthy volunteers was centrifuged at 200 g at 22°C for 20 minutes to obtain PRP. The PRP was removed carefully with a Pasteur pipette in a clean falcon tube and centrifuged at 800 g at 22°C for 12 minutes. The supernatant referred to as PPP was then pipetted in another falcon tube to which 0.1 U/ml thrombin and $CaCl_2$ (1 mM) were added and mixed well. The tube was left on the bench undisturbed for 30 minutes to allow clot formation, which was visually verified by the formation of a solid mass on a side of the falcon tube. Subsequently, the clear liquid was removed, aliquoted in smaller volumes and stored at -20°C for future use.

2.7 Protein analysis through SDS-PAGE and Western blotting

2.7.1 Basic principles

2.7.1.1 Principle of SDS-PAGE

The term electrophoresis refers to the process of separating macro biomolecules in an electric field. Nucleic acids like DNA and RNA which have an inherent net negative charge on their surfaces owing to the phosphate groups in their backbone can be easily separated in an electric field where they can migrate towards the anode through the pores of a solid matrix or gel (typically agarose). Since the

smaller fragments can move further than the larger ones in the electric field, the nucleic acids can be separated based on their size. In contrast proteins varying in their overall charge but an overall negative charge can be created by treatment with an anionic detergent such as sodium dodecyl sulphate (SDS). SDS coats the amino acids with a negative charge which then repel each other relieving the protein of its folded conformation. Thus, SDS not only imparts a net negative charge to the protein but also denatures its complex structures. Besides SDS treatment, protein samples also need to be treated with a reducing agent such as 2-mercaptoethanol or dithiothreitol (DTT) to disrupt the disulfide bridges linking adjacent cysteine residues. Denatured and reduced proteins samples are then heated at very high temperatures to aid their denaturation. Since SDS binds to the amino acids, hence the polypeptide chains within a protein take up negative charge proportional to their mass. Thus the polypeptides acquire a nearly uniform charge-to-mass ratio allowing them to be separated in the gel relative to their molecular mass solely when embedded in a gel to which an electric field is applied. Here polypeptides move towards the anode with smaller ones moving faster and further than the larger ones. The gel most commonly used to separate proteins is prepared by co-polymerisation of acrylamide and bis-acrylamide. The polymerisation is the result of free radical formation (SO_4^-) from a chemical called ammonium persulfate (APS) catalysed by another chemical called N,N,N^{I},N^{I}-tetramethylethylenediamine (TEMED). These free radicals then in turn catalyse the formation of polymers from acrylamide and bis-acrylamide monomers. Bis-acrylamide cross-links with the acrylamide polymers and this causes the formation of a gel with pores or sieves whose characteristics depend on the concentrations of the monomers, ratio of acrylamide to bis-acrylamide and polymerisation conditions.

2.7.1.2 Discontinuous SDS-PAGE

SDS-PAGE can be performed under two different buffer systems: continuous and discontinuous. As the name suggests, continuous buffer system uses the same buffer in the gel and running buffer. In contrast, the discontinuous system uses different buffers in the gel and running buffer. This system was first introduced by U K Laemmli (Laemmli, 1970), where the gel itself was composed of two different

Tris-based buffers. The 'separating' or 'resolving' gel was made at a pH of 8.8 with a higher percentage of acrylamide giving smaller pores while the 'stacking' gel layered on top of the resolving gel was made at a lower pH (6.8) with lower percentage to allow larger pore size. The tris-based running buffer contained glycine and SDS. As the name 'stacking' suggests, the function of the stacking gel is to accumulate all the proteins loaded so that they can migrate together and enter the resolving gel at the same time. Such a system provides a better resolution and sharpness of protein bands and hence is the most commonly used system. The proteins thus separated can either be visualised by various staining protocols or detected by immunochemical methods.

2.7.1.3 Western blotting

Western blotting or immunoblotting is an immediate downstream application used to detect proteins resolved by SDS-PAGE and their qualitative or quantitative analyses. This technique was first introduced by Towbin *et. al.* (Towbin et al., 1979) and later slightly modified by W N Burnette who also suggested the name for this technique (Burnette, 1981). The technique utilises the principle of transferring proteins resolved on a polyacrylamide gel to a solid membrane so that the proteins can be immobilised on a solid support such as a membrane of nitrocellulose or PVDF (polyvinylidene difluoride). This technique is crucial for immunochemical detection of proteins separated from a complex mixture using antibodies raised against specific antigens using various detection methodologies. The detection method commonly used for identifying the protein(s) of interest is chemiluminescence, which utilises the principle of light generation as a result of an enzyme-driven reaction. A primary antibody is applied to the membrane, which should then recognise the protein of interest transferred or 'blotted' to the membrane as it is generated against a specific epitope on the protein. An enzyme-labelled secondary antibody is then applied to the membrane, which should then recognise the primary antibody bound to the protein of interest and adds to amplify the signal. A chemiluminescent substrate is added to the membrane in the presence of an oxidising agent. The enzyme coupled to the protein-antibody complex catalyses the oxidation of the chemiluminescent substrate, which emits

light that is subsequently developed with an X-ray photographic film. Luminol is the light-emitting product, hydrogen peroxide the oxidising agent and horseradish peroxidase, coupled to the secondary antibody, is the enzyme mostly employed in this detection method.

2.7.2 Sample preparation for SDS-PAGE

Washed platelets were prepared from healthy volunteers as indicated in section 1.1. Platelet counts were adjusted with modified Tyrode's buffer and samples prepared according to the objective of each experiment. For example, to examine protein expression platelet count was adjusted to 7×10^8/ml with modified Tyrode's buffer. Washed platelets were incubated for one minute at 37°C in an aggregometer, stirred for 30 seconds and lysed with an equal volume of standard 2X Laemmli buffer (4% SDS (w/v), 10% 2-mercaptoethanol (v/v), 20% glycerol (v/v), 50mM Tris base, trace bromophenol blue, pH 6.8) under stirring conditions. To investigate signalling mechanisms downstream of agonist stimulation platelet count was adjusted to 3×10^8/ml with modified Tyrode's buffer. Platelets were then incubated for 20 minutes with indomethacin (10 μM), apyrase (2 U/ml) and EGTA (1 mM) to study molecular events following platelet activation independent of inside-out signalling and platelet aggregation. Indomethacin inhibits the enzyme cyclooxygenase 1/2 (COX 1/2) which is responsible for thromboxane A_2 (TxA_2) generation from membrane phospholipids. Apyrase is an enzyme that removes the β-phosphate from ADP to generate AMP and inorganic phosphate. EGTA inhibits integrin activation by chelating Ca^{+2}. After a one-minute of incubation at 37°C in the aggregometer, platelets were stimulated with different concentrations of the agonists for the appropriate time under constant stirring before lysis with an equal volume of 2X Laemmli buffer. In conditions where effect of prednisolone on signalling was to be determined, platelets were incubated with the appropriate concentration of prednisolone for the appropriate time prior to agonist stimulation. In selected samples, platelets were pre-incubated with the GCR antagonist RU486 (10 μM) for 5 minutes to reverse prednisolone-mediated effect on platelets prior to prednisolone treatment and thrombin stimulation. Lysates were transferred to eppendorf tubes, kept on ice for 30 minutes for complete lysis of cells and stored at -20°C for later use or heated for 5 minutes at 100°C to ensure complete denaturation of proteins before loading on polyacrylamide gels.

2.7.3 Protein quantification

The method for measuring protein concentration in platelet lysates was based on a colorimetric assay commonly called Lowry assay (Lowry *et. al.*, 1951). It measures the intensity of a blue colour, at an absorbance of 750 nm, which is produced as a result of a two-step chemical reaction between proteins and an alkaline copper tartrate solution followed by the reduction of a Folin reagent by copper-treated proteins. Bovine serum albumin (BSA) was used as a standard during the assay to determine the protein concentration of the test sample.

A detergent compatible protein assay kit was used which contained reagents A (alkaline copper tartrate), B (Folin reagent) and S. An aliquot (30-50 µl) of washed platelets was lysed with an equal volume of lysis buffer (150mM NaCl, 10mM Tris base, 1mM EGTA, 1mM EDTA, 1% Igepal (v/v), pH 7.4), since the components of the protein assay kit are not compatible with 2-mercaptoethanol in sample Laemmli buffer. After 30 minutes of lysis on ice, 5 µl of the lysate was added to a well of a 96-well microplate. At the same time 5 µl each of known concentrations of the protein standard BSA (0.3, 0.6, 0.9, 1.2 and 1.5 µl) were aliquoted in separate wells of the microplate. 25 µl of A-S reagent mix (20 µl of reagent S in 1 ml of reagent A) was added to each of these wells. 200 µl of reagent B was added to the wells and incubated at room temperature for 15 minutes to allow the development of the blue colour due to the chemical reactions. The plate was then introduced in a plate reader and light absorbance at 750 nm was recorded. The protein concentration of the platelet lysate was then determined from the standard curve of the absorbance of the BSA standards.

2.7.4 SDS-PAGE methodology

The concentration of acrylamide used in the resolving gel was determined depending on the molecular weight of the protein to be analysed. A 10% acrylamide gel (Table 2.2 and Table 2.4) was poured to study a single protein whereas a 10-18% gradient gel (Table 2.1 and Table 2.4) was poured to study a complete protein profile. Dual-chambered gradient mixer connected to a peristaltic pump was used to pour gels uniformly. After pouring, a small volume of methanol

was layered on top of the resolving gel to ensure that the surface of the gel was level and also to inhibit oxygen in the surrounding environment from interfering with the polymerisation process. The gel was allowed to polymerise for at least an hour at room temperature. When the gel was polymerised, methanol was discarded and wiped thoroughly from the top of the resolving gel. A stacking gel containing 3% acrylamide (Table 2.3 and Table 2.4) was then prepared and poured over the resolving gel and a 1.5 mm comb containing wells was immediately inserted between the two plates in this stacking gel. It was then allowed to polymerise for at least 20 minutes at room temperature. After polymerisation of the stacking gel, the plates containing the polymerised gels were displaced from the stand and clamp and placed in the chamber inside a running tank. Running buffer (0.1% SDS (w/v), 25mM Tris base, 192mM glycine) was then poured both inside and outside the gel chamber to immerse the gels completely in buffer. The comb was removed from the stacking gel and the wells were washed with running buffer. Biotinylated protein ladder (10 µl) was loaded in the first well. This ladder contained a mix of different proteins with known molecular weights that are coupled to biotin, which can be later identified by Western blotting by using an anti-biotin antibody. The use of this ladder enables the approximate determination of molecular weight of proteins resolved on the gel. 20 µg of protein were then loaded in the remaining wells. In case of GCR detection, higher amounts of protein (50-100 µg) were loaded. Any empty well was loaded with an equal volume of 2X Laemmli buffer. The protein samples were then allowed to migrate at 120 volts for 80 minutes in case of detection of phospho-myosin light chain (MLC) or RhoA or 150 minutes for all other immunodetection. After the end of the run, power was switched off, running buffer was discarded and the plates containing the gels were taken out of the tank to proceed to the next step of protein transfer.

Reagents	10% gel	18% gel
Distilled water	1.418 ml	0.708 ml
Acrylamide	1.182 ml	1.961 ml
Buffer 1	0.886 ml	0.886 ml
APS (10%, w/v)	18 µl	18 µl
TEMED	2 µl	2 µl

Table 2.1 Constituents of one 10-18% gradient polyacrylamide resolving gel

Reagents	10% gel
Distilled water	1.418 ml
Acrylamide	1.182 ml
Buffer 1	0.886 ml
APS (10%, w/v)	18 µl
TEMED	2 µl

Table 2.2 Constituents of two 10% polyacrylamide resolving gels

Reagents	3% gel
Distilled water	4.87 ml
Acrylamide	0.75 ml
Buffer 2	1.87 ml
APS (10%, w/v)	75 µl
TEMED	10 µl

Table 2.3 Constituents of two 3% polyacrylamide stacking gels

Reagents	Buffer 1 (pH 8.8)	Buffer 2 (pH 6.8)
Tris-HCl	1.5 M	0.5 M
Sodium dodecyl sulphate (SDS)	0.4%	0.4%

Table 2.4 Composition of buffers 1 and 2

2.7.5 Western blotting methodology

2.7.5.1 Wet transfer of proteins

The traditional method of transferring proteins separated by SDS-PAGE from a polyacrylamide gel to a PVDF membrane is carried out in the presence of a buffer and hence referred to as wet blotting as described below. A 0.2 µm PVDF membrane was incubated in methanol for 1 minute, followed by a wash in distilled water for 10 minutes and another wash in transfer buffer or Towbin buffer (25mM Tris base, 192mM glycine, 20% methanol (v/v)) for 10 minutes. After SDS-PAGE, the gel(s) was separated from the glass plates and the resolving gel soaked in transfer buffer. A sandwich was then assembled by placing successive layers of sponge, filter papers, gel, PVDF membrane, filter papers and sponge in the transfer cassette. The transfer cassette was then placed in a running tank, which was filled with transfer buffer. The gel was placed towards the cathode and the membrane towards the anode. Transfer was then initiated at a constant voltage of 100 volts and continued for 2.5 hours. An ice pack was put in the tank during the run to prevent overheating and consequent damage to the membrane. After the run was complete, the cassette was taken out and the membrane carefully retrieved from the sandwich for further processing.

2.7.5.2 Semi-dry transfer of proteins

In some cases, semi-dry method of protein transfer was used. A Trans-Blot® Turbo™ blotting system and ready-to-use transfer packs (Bio-Rad) were used and protein transfer was carried out according to manufacturer's guidelines. The transfer packs contained the sandwich of filter papers and PVDF membrane (0.2 µm) soaked in manufacturer's buffer. Gel(s) were placed on the membrane and transfer was carried out within a dry cassette without the requirement of transfer buffer. Transfer was performed at a constant current of 1.3 A for a single gel using the mini pack and 2.5 A for two gels using the midi pack. In both cases the voltage during the run was not allowed to exceed 25 volts. The run was performed for 5 minutes for a protein less than 30 KDa, 10 minutes for a protein greater than 150 KDa and 7

minutes for proteins between 5 KDa and 150 KDa. After the run terminated, the membrane was retrieved for further immunochemical processing.

2.7.5.3 Membrane processing for immunoblotting

The PVDF membrane was blocked for 45 minutes in BSA (10% w/v) or skimmed milk (5% w/v) solutions prepared in TBS-Tween (0.1%) buffer (150mM NaCl, 20mM Tris base, 0.1% Tween-20 (v/v)). This was done to block non-specific antibody binding sites on the membrane and reduce high background signals. The membrane was then incubated overnight at 4°C with agitation in primary antibody that was diluted in a solution of BSA or milk (2% w/v) prepared in TBS-Tween (as indicated in Table 2.5). The membrane was washed twice in TBS-Tween (15 minutes per wash) following which it was incubated for an hour at room temperature with agitation in HRP-conjugated anti-rabbit or anti-mouse secondary antibody (both 1:10000) and anti-biotin (1:2000; to recognise the biotinylated protein ladder) prepared in TBS-Tween, unless otherwise indicated. The membrane was then washed four times in TBS-Tween (15 minutes per wash) to remove excess or unbound antibody. The membrane was then incubated in ECL solution containing equal volumes of ECL 1 (250mM luminol, 90mM p-coumaric acid, 100mM Tris base) and ECL 2 (100mM Tris base, 30% hydrogen peroxide (v/v)) for 90 seconds and the light generated from the reaction between luminol and hydrogen peroxide catalysed by HRP was captured on an X-ray film and visualised with developer and fixer solutions. The X-ray films were exposed for time periods between 10 seconds to 15 minutes according to the intensity of the signal.

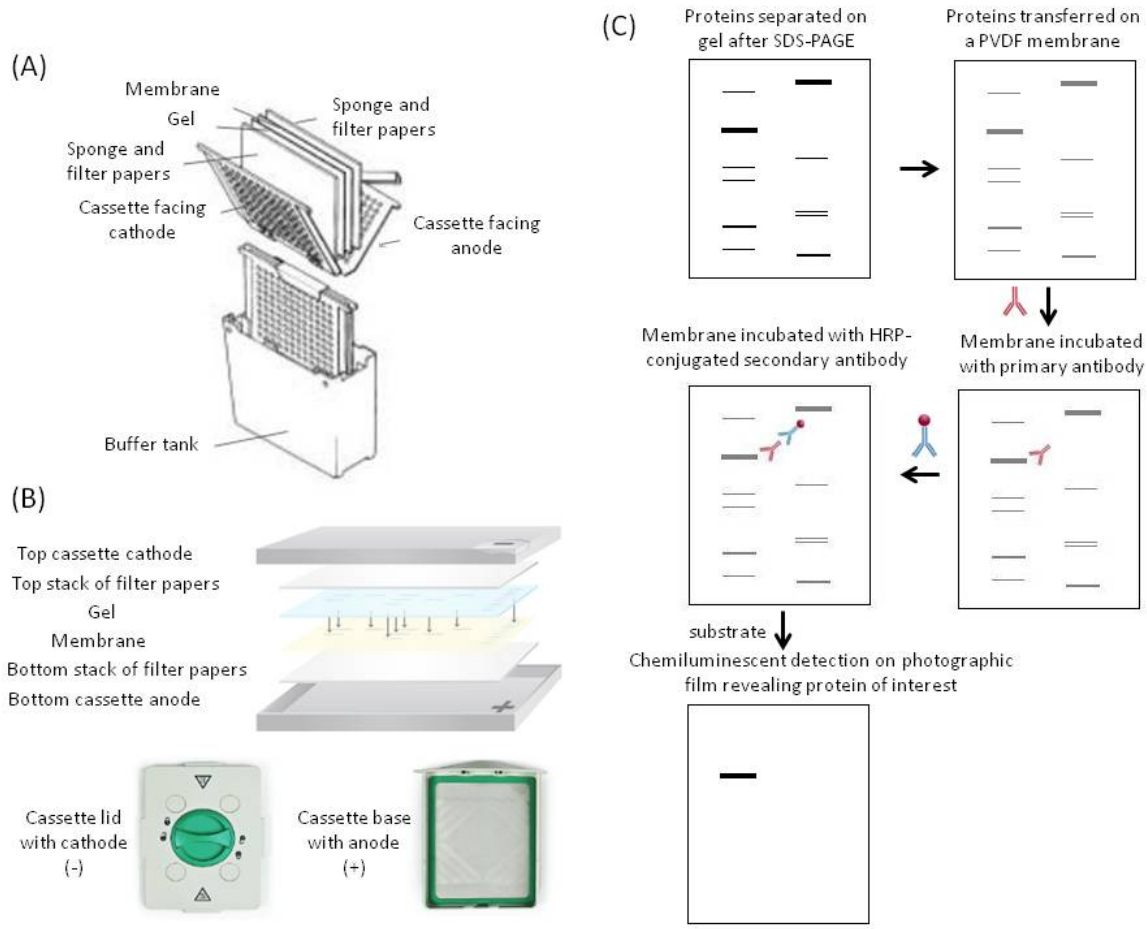


Figure 2.3: Representation of traditional wet transfer of proteins (panel A), semi-dry protein transfer (panel B) and schematic diagram of western blotting. Adapted from Bio-Rad.

Antibody	Source	Dilution
Anti-phospho-MLC-ser^{19}	Mouse	1:1000 in BSA
Anti-phospho-MYPT1-thr^{696}	Rabbit	1:500 in milk
Anti-phospho-MYPT1-thr^{853}	Rabbit	1:250 in milk
Anti-RhoA	Mouse	1:1000 in BSA
Anti- β-tubulin	Mouse	1:1000 in BSA
Anti-Syk	Mouse	1:1000 in BSA
Anti-GCR	Rabbit	1:250 in milk
Anti-GCR	Mouse	1:250 in milk
Anti-phospho-GCR-ser^{203}	Rabbit	1:1000 in milk
Anti-phospho-GCR-ser^{211}	Rabbit	1:1000 in milk
Anti-phospho-GCR-ser^{226}	Rabbit	1:1000 in milk

Table 2.5 Primary antibodies used in this study and the dilutions at which they were used.

2.8 Immunoprecipitation

Immunoprecipitation as the name suggests, is a biochemical technique, which is applied to 'precipitate' or pull a particular protein out from a complex heterogeneous mixture, such as that in any cell lysate, using an antibody raised against a specific epitope of the protein of interest. It is particularly useful to identify proteins, which are low in abundance in the cell by enriching its fraction and also to identify binding partners of protein(s) of interest. The antibody is allowed to bind to protein A or G immobilised to a solid matrix such as agarose or sepharose beads. Protein A and protein G are isolated from the cell wall of *Staphylococcus aureus* and capable of binding to the constant region (Fc) of most IgGs derived from a wide variety of species. Thus, when applied to a whole cell lysate, the antibody coupled to beads should bind to the protein of interest by recognising the antigen epitope to which it has been raised. Thus the protein of interest can be purified from the lysate by centrifugation and precipitation of the antigen-antibody-protein A/G complex bound to the beads, which can later be eluted from the beads by use of specific denaturation reagents and resolved through SDS-PAGE.

2.8.1 Sample preparation

Platelet count was adjusted to 8×10^8/ml and platelets were lysed with an equal volume of immunoprecipitation lysis buffer (150mM NaCl, 10mM Tris base, 1mM EGTA, 1mM EDTA, 1% Igepal (v/v), pH 7.4 containing 1mM PMSF, 2.5mM Na_3VO_4, 1:200 protease inhibitor cocktail (v/v), 1:200 phosphatase inhibitor cocktail (v/v)). Lysates were left on ice for at least 30 minutes and centrifuged briefly at 13000 rpm to remove insoluble cell debris. The protein concentration in the lysates was determined as indicated in 2.7.3.

2.8.2 Preparation of protein A sepharose beads

300 µl of protein A/G sepharose beads were briefly centrifuged at 13000 rpm and the supernatant ethanol was removed. Beads were washed twice in Tris-buffered

saline (TBS) buffer (75 µl) and resuspended in TBS containing 0.1% (v/v) tween 20 as 50% (w/v) slurry on ice.

2.8.3 Incubation of platelet lysates with antibody and beads

25 µl of the bead slurry was aliquoted in 0.5 ml eppendorf tubes on ice. An antibody raised against the protein of interest or a species-specific control IgG was added to the beads. The antibody-bead mix was then mixed gently on a rotator at 4°C for 3 hours to allow binding of antibody to protein A/G. The control IgG reaction serves as a control to ensure that any signal obtained is specific to the test antibody during data analysis. Meanwhile 300-400 µg of lysates was incubated with 25 µl of beads in a separate eppendorf tube for at least an hour at 4°C with rotation. This step is known as 'pre-clearing of lysates' and was performed to minimise non-specific binding of irrelevant antigens to protein A/G. The lysates were centrifuged briefly and the beads used for pre-clearing were discarded. The pre-cleared lysates were then added to the antibody-bead mix and incubated overnight at 4°C with gentle rotation. Lysates were then centrifuged briefly and antigen-antibody-protein A/G ternary complexes were precipitated with the sepharose beads. Supernatant containing unbound antigens were discarded. Beads were then washed once with standard lysis buffer (25 µl) and twice with TBS containing 0.1% (v/v) tween 20 to dissociate non-specific complexes. Supernatant were discarded after every wash step and 2X sample Laemmli buffer (65 µl) was added to the beads after the last wash to dissociate the ternary complexes and elute the antigen through the denaturing action of SDS. Beads were boiled at 100°C for 5 minutes to assist with dissociation and then precipitated by brief centrifugation. Supernatant containing antigens on target protein and antibody was loaded on 10% polyacrylamide gels and resolved and analysed by SDS-PAGE and western blotting as described before.

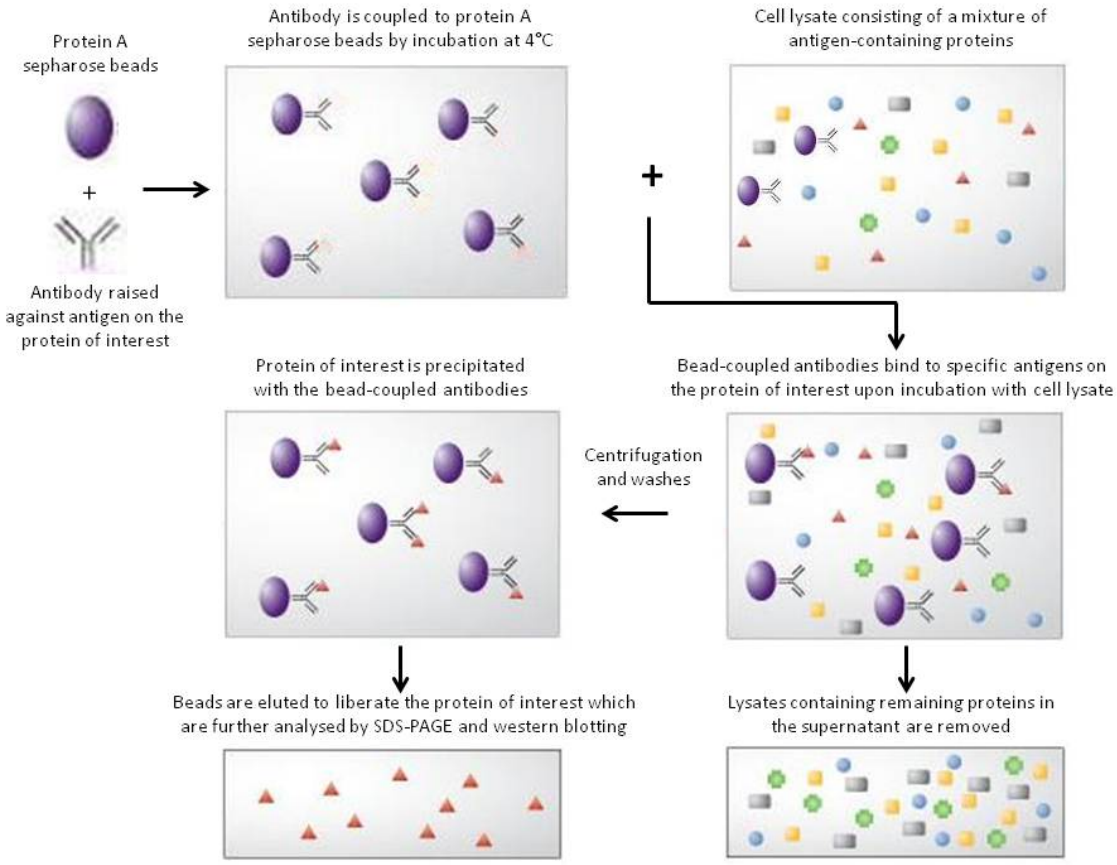


Figure 2.4: Schematic representation of protein immunoprecipitation. Adapted from Thermo Scientific.

2.9 Analysis of RhoA activation in platelets

2.9.1 Principle of RhoA pull down assay

RhoA is a small G-protein that belongs to the Rho family of GTPases which act as molecular switches and regulate a number of biochemical processes such as cytoskeletal reorganisations, transcriptional regulation, cell proliferation, and membrane trafficking. Like other members (Rac1 and Cdc42) of its family, RhoA participates in cellular signal transduction where it transmits signals through several effector proteins, which result in the above processes. In the process of transmitting signals, RhoA switches between an active form when it binds to GTP and an inactive form when it binds to GDP. The switch between RhoA-GTP active form and RhoA-GDP inactive form is regulated by two classes of proteins. GTPase activating proteins (GAPs) initiate the RhoA switch from its active to inactive state through GTP hydrolysis by its intrinsic GTPase activity, while guanine nucleotide exchange factors (GEFs) assist in GDP removal from RhoA thus leaving it free to bind to a new GTP molecule and hence switches RhoA from its inactive to active state. The various RhoA effector proteins specifically recognise and interact with only the active GTP-bound form of RhoA and this feature is used to develop an assay to examine RhoA activation. The assay kit contains RhoA binding domain (RBD) (amino acid residues 7-89) of the RhoA effector protein Rhotekin, which is expressed as a GST-fusion protein in E.coli and bound to coloured glutathione-sepharose beads. This domain has high affinity for RhoA-GTP and hence such beads can be used to pull down the active RhoA protein fraction from cell lysates. Levels and activity of RhoA are determined by eluting the beads and analysing the pulled RhoA fraction by SDS-PAGE and western blotting.

2.9.2 Preparation of platelet samples for activation of RhoA

Washed platelets were prepared and adjusted to a count of 5×10^8/ml with modified Tyrode's buffer. After warming at 37°C for a minute, platelets were then stimulated with 0.005 U/ml thrombin for 1 minute under stirring conditions in an aggregometer and lysed with equal volume of ice-cold lysis buffer provided with the kit. The lysis buffer was supplemented with protease (1:100) and phosphatase

inhibitor (1:200) cocktails. In selected samples, pre-warmed platelets were incubated with prednisolone (10 µM) for 1 minute followed by thrombin stimulation. Lysates were left on ice for at least 30 minutes and centrifuged briefly at 13000 rpm to precipitate insoluble cell debris. Supernatant was transferred in eppendorf tube and quantified for protein concentration. If the pull-down protocol was performed on the same day, lysates were processed immediately after determination of protein concentration, otherwise lysates were snap frozen in liquid nitrogen and immediately transferred to -80°C freezer.

2.9.3 Preparation of positive and negative controls for the assay

An aliquot of untreated washed platelets were lysed with an equal volume of ice-cold lysis buffer supplemented with protease and phosphatase inhibitor cocktails. 25 µl of loading buffer (150 mM EDTA; provided with the kit) was added to 225 µl of platelet lysates on ice. 2.5 µl of a non-hydrolysable GTP analogue, GTPγS (200 µM final concentration) or GDP (1 mM final concentration) provided in the kit was immediately added to the lysates to perform positive or negative controls, respectively. Lysates were incubated at 4°C for 15 minutes with gentle rotation. The reaction was terminated by adding 27 µl of stop buffer (600 mM $MgCl_2$; provided with the kit). These positive and negative controls were then subjected to the pull-down assay as detailed below.

2.9.4 Rhotekin-RBD bead pull down methodology

300-400 µg of lysates were added to 30 µg (9 µl) of Rhotekin-RBD sepharose beads (provided with the kit) in a 0.5 ml eppendorf tube on ice and incubated at 4°C for 90 minutes with gentle rotation. Lysates were centrifuged at 13000 rpm for 1 minute and the supernatant removed carefully. Beads were washed once gently with 0.5 ml of ice-cold wash buffer (25 mM Tris, 30 mM MgCl2, 40 mM NaCl, pH 7.5) provided with the kit and precipitated by centrifugation at 13000 rpm for 1 minute. Supernatant was removed and beads were eluted with 40 µl of 2X sample Laemmli buffer and boiled at 100°C for 5 minutes. Beads were precipitated again by centrifugation and supernatant was then loaded on 10 % polyacrylamide gels for analysis by SDS-PAGE and Western blotting using the mouse monoclonal anti-RhoA

primary antibody (1:500) provided with the kit. Total RhoA protein levels were also determined by SDS-PAGE and western blotting of platelet lysates from each sample using the same RhoA antibody.

2.10 Cell culture

2.10.1 Culture of primary mouse bone marrow derived megakaryocytes

2.10.1.1 Use of mice and isolation of bones

All the mice used in experiments were suitably caged, bred and maintained as per the UK Home Office Regulations. Wild type (WT) mice from C57BL/6 background were chosen for bone marrow isolation. Mice were sacrificed by a standard Schedule I method followed by cervical dislocation to confirm death. The skin of the mice abdomen was cut open with the help of a sharp scissors and forceps to expose the hind legs. The hip joints were then dislocated and the legs removed from the body. The knee and ankle joints were carefully dislocated, the tibia and femurs were separated and the muscles were stripped off. The bones were placed in an eppendorf tube containing sterile 1X phosphate buffered saline (PBS).

2.10.1.2 Preparation of bone marrow and isolation of megakaryocytic progenitors

Eppendorf tubes containing the isolated bones were transferred to a class II tissue culture cabinet (designated for aseptic mouse experiments) appropriately sterilised with 70% ethanol. The following procedures were carried out aseptically. Bones were flushed out using complete DMEM medium supplemented with 10% fetal bovine serum (FBS), L-Glutamine (2mM), penicillin (1X) and streptomycin (1X) with the help of a 25G needle. The bone marrow cells were centrifuged at 1200 rpm, 22°C for 5 minutes, resuspended in 6 ml of ammonium chloride potassium buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3) to lyse the red blood cells for 5 minutes at room temperature and filtered through a 70 µm nylon mesh in a falcon tube. The filtered cells were centrifuged again at 1200 rpm, 22°C for 5 minutes. The cell pellet was then resuspended in 1 ml of complete DMEM containing 3 µl each of anti-GR1, anti-B220, anti-CD16/32 and anti-CD11b

antibodies and incubated on ice or 30 minutes. These antibodies are raised in rat against epitopes of various mouse white blood cells to which they should bind. After antibody incubation, cells were centrifuged at 1200 rpm, 22°C for 5 minutes and resuspended in 1 ml of complete DMEM medium. 50 µl of immunomagnetic sheep anti-rat IgG was added to the DMEM and mixed thoroughly by pipetting. The cells were then centrifuged again at 1200rpm for 5 minutes. The cells were then resuspended via pipetting. This process was repeated. At this point the DMEM was placed in a magnetic field, and the supernatant removed. The pelleted beads were then resuspended with 0.5 ml of complete DMEM. The beads were then placed in the magnetic stand to allow the magnetic beads to be concentrated in a pellet. The supernatant was then removed. The cells were centrifuged at 1200 rpm, 22°C for 5 minutes, resuspended in 1 ml complete DMEM supplemented with 20 ng/ml stem cell factor (SCF) and incubated in a 24-well tissue culture plate at 37°C with 5% CO_2 supply for 2 days.

2.10.1.3 Differentiation of megakaryocytic progenitors and isolation of megakaryocytes

The progenitors were transferred to a falcon tube and centrifuged at 1200 rpm, 22°C for 5 minutes. Progenitors were resuspended in 1 ml complete DMEM supplemented with 20 ng/ml SCF and 50 ng/ml murine thrombopoietin (TPO) and incubated in a 24-well tissue culture plate at 37°C with 5% CO_2 supply for 3-4 days. When the megakaryocytes appear to have populated the culture, they were isolated from the cell mixture using a BSA gradient. 4 ml of a sterile 3% (w/v) BSA/PBS solution was added in a falcon tube on top of which 4 ml of a sterile 1.5% (w/v) BSA/PBS solution was carefully layered. The cell mixture was layered slowly on top of this BSA gradient and this set up was allowed to stand for 45 minutes in the cabinet. Owing to their large size, the megakaryocytes should fall down the gradient and settle at the bottom, while the undifferentiated progenitors as well as some residual white blood cells, being smaller in size, should reside in the upper layer. Thus the top 7 ml of the gradient was removed, centrifuged and resuspended in 1 ml complete DMEM with SCF and TPO for a second round of differentiation if

required. The megakaryocyte-enriched bottom 2 ml of the gradient was centrifuged and resuspended in reagents according to the appropriate experiment.

2.10.1.4 Determination of megakaryocyte ploidy

2.10.1.4.1 Staining of megakaryocytes with specific antibodies

The megakaryocytes purified through the BSA gradient were resuspended in 5 ml of a 0.5% (w/v) BSA/PBS solution and centrifuged at 1200 rpm, 22°C for 10 minutes. The cell pellet thus obtained was resuspended in 95 µl of 0.5% (w/v) BSA/PBS, transferred to an eppendorf tube and incubated for 1 hour in the dark at 4°C with 5 µl of an anti-mouse CD41 antibody (raised against GP IIb) conjugated to FITC. The cells were centrifuged at 300 g for 5 minutes and resuspended in 0.5 ml of PBS. 0.5 ml of 1% paraformaldehyde was added slowly to the cells to fix them and incubated on ice for 45 minutes. Cells were centrifuged and washed once in 0.5 ml PBS. Cells were resuspended in 200 µl of PBS containing saponin (0.05%, w/v), the DNA staining dye propidium iodide (PI) (10 µg/ml) and RNase A (10 µg/ml) (to destroy RNA fragments that might also stain with PI and contribute to a noise in the PI signal) and incubated overnight in the dark at 4°C. Cells were centrifuged, resuspended in PBS, transferred to FACS tubes and analysed in a BD LSRFortessa cell analyser.

2.10.1.4.2 Ploidy analysis by flow cytometry

2.10.1.4.2.1 Principle of flow cytometry

Flow cytometry is a very sensitive and powerful technique to assess multiple parameters of single cells within a cell suspension. It utilises the principles of light scattering and excitation and emission of fluorochrome molecules attached to cells to generate the multi-parameter data. A very useful application of flow cytometry is the detection of proteins in a cell population as well as their quantification at the single cell level. For this, fluorophore-conjugated antibodies are incubated with the cells to allow antibody-protein interaction following which the cells are fixed to preserve the fluorophores and allowed to run through the flow cytometer.

2.10.1.4.2.2 Flow cytometry methodology during ploidy analysis

Unlabelled, CD41-labelled, PI-labelled and CD41/PI doubly labelled megakaryocytes were run in the flow cytometer. Using an unlabelled sample a forward and side scatter dot plot was chosen where the forward scatter (FSC) was set in the linear scale and the side scatter (SSC) in the logarithm scale. Once the megakaryocyte population was identified by adjusting the FSC and SSC voltages, a gate was drawn on the plot around it. Another dot plot was created where PE-Texas red and FITC channels were selected to detect PI and CD41 binding, respectively. Using samples that were labelled with either CD41 or PI, regions on the dot plot, which are negative and positive for each of these, were identified. A new gate was drawn around the cell population that bound both CD41 and PI. A histogram was then drawn with the PE-Texas red on the x-axis and the cell count on the y-axis to analyse the cells identified as CD41/PI double positive cells. The different peaks obtained on this plot corresponded to different megakaryocyte populations with ploidy ranging from 2N, 4N, 8N, 16N and so on.

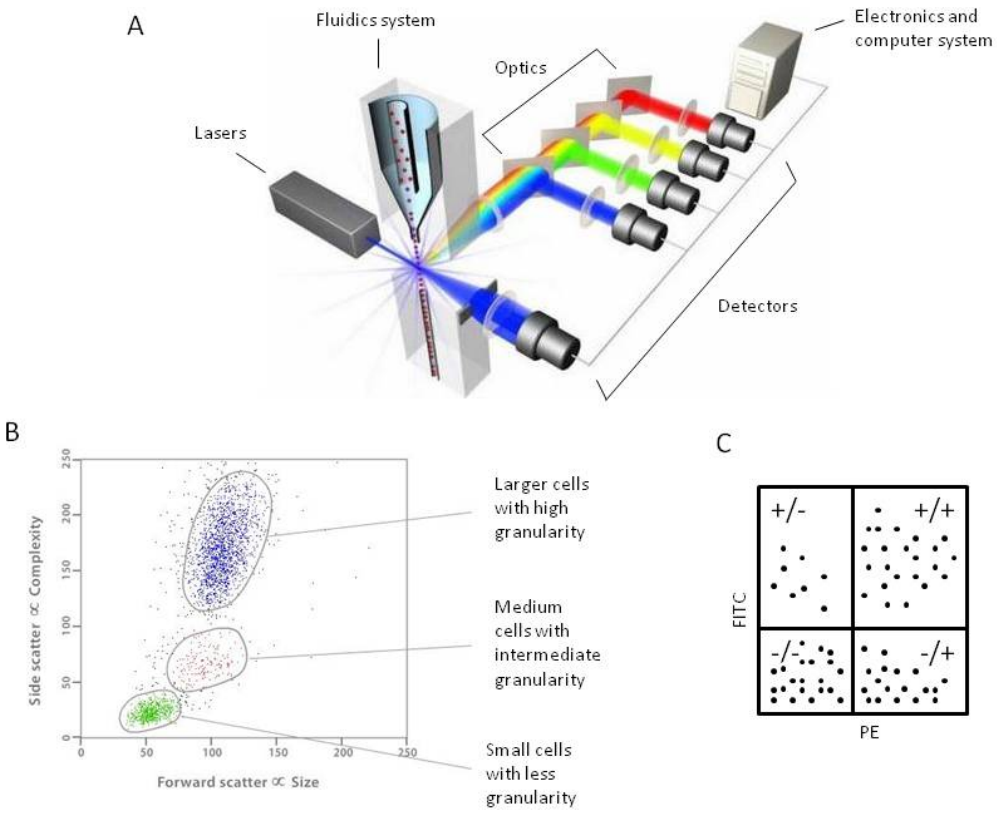


Figure 2.5: Diagram showing the design of a flow cytometer and its various components (panel A). The forward and side scatter dot plot (panel B) shows the distribution of individual cell population within a cell mixture. Panel C shows distribution of cell populations based on the signal emitted by the fluorescent antibodies, which bind them. FITC and PE are the fluorophores tagged to antibodies whose fluorescence is recorded and shown in the plot.

2.10.2 Culture of MEG-01 cell line

MEG-01 is a megakaryoblastic cell line derived from the bone marrow of a patient with blast crisis of Philadelphia (Ph1) chromosome-positive chronic myelogenous leukemia (Ogura et al., 1985). It has been shown to express the megakaryocytic and platelet glycoprotein GP IIb/IIIa complex (integrin $\alpha_{IIb}\beta_3$) uniformly on the cell surfaces using antibodies against the two respective hematopoietic markers CD41 and CD61. The platelet GP Ib has also been shown to be expressed in larger MEG-01 cells. It is reported not to express any myeloid or lymphoid markers. As it possesses similar phenotypic characteristics to a megakaryocyte and has been derived from a leukemic condition where the regulation on normal cell cycle has been lost, MEG-01 cell line has been used extensively in in-vitro research to study the process of megakaryocytic differentiation and platelet production as well as various signalling mechanisms that have known to be involved in megakaryocytic and platelet lineages. The advantage of using this cell-line is its ease of maintenance in culture throughout an indefinite timescale and improved efficiency of genetic manipulation.

2.10.2.1 Thawing of frozen MEG-01 cells

MEG-01 cells (HPA Culture Collections) were obtained in the form of a frozen vial. Upon receipt, the vial was thawed in a 37°C water bath and swiftly transferred to a class II tissue culture cabinet (designated for aseptic cell-line experiments) appropriately sterilised with 70% ethanol. The following procedures were carried out aseptically. The cells in the vial were added drop wise in 5 ml of pre-warmed RPMI medium (37°C) supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (1X) and streptomycin (1X). Cells were then centrifuged at 1500 rpm, 22°C for 5 minutes. Supernatant medium was discarded in 1% (w/v) virkon and the precipitated cell pellet was resuspended in 1 ml of the above complete RPMI medium. A viability count (section 2.10.2.4) was performed at this stage to determine the efficiency of the thawing process and estimate the percentage of live cells in the culture. The cells were seeded in a well of a 6-well tissue culture plate in a total volume of 3 ml of reconstituted RPMI medium.

2.10.2.2 Maintenance of MEG-01 cells

After 24 hours of establishing the culture as indicated in the previous section, the cells were transferred to a falcon tube and centrifuged at 1500 rpm, 22°C for 5 minutes. The supernatant was discarded in Virkon and the cell pellet resuspended in 1 ml of complete RPMI medium. Cells were then counted and seeded at a density of 2-4 $\times 10^5$/ml in a 6-well tissue culture plate. After the initial few subcultures, when the cells recovered from the thawing process and started dividing faster, MEG-01 cells were subcultured or passaged every 4-5 days by centrifugation of cells, removal of spent medium, resuspension of cells in fresh medium and maintenance in ventilated tissue culture flasks at 37°C in a humified incubator with 5% CO_2 supply. Cells were maintained in culture for 20-30 weeks after which they were discarded in Virkon and another frozen vial of cells were thawed and maintained in culture.

2.10.2.3 Freezing of MEG-01 cells

MEG-01 cells were harvested by centrifugation at 1500 rpm, 22°C for 5 minutes. The supernatant was discarded in virkon and the cells were resuspended in 1 ml of complete RPMI to perform a viability count. Cells were again harvested and resuspended at a density of 2-4 $\times$ 10^6/ml in freezing medium (90% (v/v) FBS and 10% (v/v) DMSO). Cells were aliquoted in cryovials with 1 ml cells per vial and immediately transferred in a -80°C freezer. The frozen vials were then transferred on dry ice in a liquid nitrogen tank in the gaseous phase for long-term storage.

2.10.2.4 Cell viability count

An aliquot of MEG-01 cell suspension was mixed well with an equal volume of Trypan blue and the cells were counted under an inverted light microscope using the 10X objective. Trypan blue is a viability stain that is excluded from live cells. Hence, live cells appear colourless and bright under phase contrast, while dead cells appear blue. The cells in the central larger square and each of the four larger corner squares were counted. The following calculations were applied to find the number of cells and their viability.

Cells/ml= (average count of cells in the 5 larger squares) × (dilution factor) × 10^4

% of viable cells= [(live or colourless cells) ÷ (live cells + dead or blue cells)] × 100

2.11 Statistical analyses

Data were expressed as mean ± SEM. Statistical comparisons of data were carried out using Student's t-test and the one-way analysis of variance (ANOVA) test available in Microsoft Excel 2010. Comparisons were considered to be statistically significant for values of $p<0.05$.

Chapter 3

Effect of prednisolone on platelet function

3.1 Introduction

Upon encountering a vascular injury, the otherwise quiescent platelets become exposed to subendothelial matrix proteins to which they adhere, become activated and participate in clot formation to prevent excessive blood loss. However, in certain pathological scenarios, such as a well-characterised endothelial inflammation in atherosclerotic lesions, platelet activation can occur in a dysregulated fashion leading to arterial thrombosis.

Glucocorticoids (GCs) are the most widely administered group of anti-inflammatory therapeutics that have also been shown to be effective in reducing atherosclerosis in animal models (Hagihara et al., 1991). GCs have been shown to act via genomic and non-genomic modes. Since GCs have been shown to modulate leukocyte-endothelial cell interactions, their effects on platelets should also be elucidated in order to dissect previously unknown pathways, which could be regulated by GCs. Owing to the lack of nuclei platelets serve as an apt model to study the non-genomic effects of GCs, a subject that gained increasing attention over the past two decades. Human platelets have been shown to express GC receptor (GCR) (Moraes et al., 2005), the classical receptor that is believed to mediate the genomic effects of GCs in the body. Interestingly, a synthetic GC prednisolone has earlier been shown to modulate human platelet function by suppressing platelet aggregation in platelet rich plasma (PRP) and whole blood, platelet-monocyte interactions and interaction of washed platelets with surfaces immobilised with collagen under static and flow conditions. These studies employed collagen, adenosine 5'-diphosphate (ADP) and the thromboxane A_2 (TxA_2) analogue U46619 to stimulate platelet activation. Thrombin, a serine protease generated at the site of vascular injury upon exposure of tissue factor to coagulation factors, is the most potent physiological platelet agonist that activates platelets by cleavage of their G-protein coupled protease-activated receptors (PARs). Since there were no previous reports on the

effect of glucocorticoids on thrombin-mediated platelet activation, we sought to characterise the outcome of prednisolone treatment on platelet function downstream of thrombin stimulation.

3.2 Aims

This principle aim of this chapter was to evaluate the effect of prednisolone on platelet function with a particular emphasis on platelet aggregation induced by physiological agonists such as thrombin and ADP and platelet adhesion on immobilised adhesive surfaces under static conditions.

3.3 Characterisation of platelet aggregation in washed platelets

3.3.1 Platelet aggregation in washed platelets induced by physiological agonists thrombin, collagen and ADP

Before studying the effects of prednisolone on platelet aggregation it was imperative to test that the washed platelet preparation was functional for subsequent experiments. For this, platelets were stimulated with physiological agonists such as thrombin, collagen and ADP. Multiple agonists were used to verify the sensitivity of platelet activation through both G-protein coupled receptors (GPCR) as well as tyrosine kinases.

The addition of thrombin (0.01-0.1 U/ml) caused a concentration-dependent increase in aggregation response in washed platelets. While 0.01 U/ml evoked only minor aggregation, 0.05 U/ml gave nearly 82±3.8% aggregation ($p=0.006$ compared to 0.01 U/ml) and 0.1 U/ml induced a maximal response of 84±0.9% ($p=0.005$ compared to 0.01 U/ml) (Figure 3.1; Ai and ii). Similar concentration-dependent increase in platelet aggregation was observed with the addition of collagen where the maximal aggregation response of 86±2% was induced by 10 µg/ml collagen ($p=0.000001$ compared to 1 µg/ml) (Figure 3.1; Bi and ii). ADP is a weak agonist, which is not capable of inducing a substantial aggregation response in washed platelets on its own. Hence, washed platelets were supplemented with human fibrinogen (500 µg/ml) before stimulation with ADP. In such conditions, ADP could also induce a concentration-dependent aggregation response in washed platelets with the maximal aggregation of 83±0.6% observed with 10 µM ADP ($p=0.0002$ compared to 1 µM) (Figure 3.1; Ci and ii).

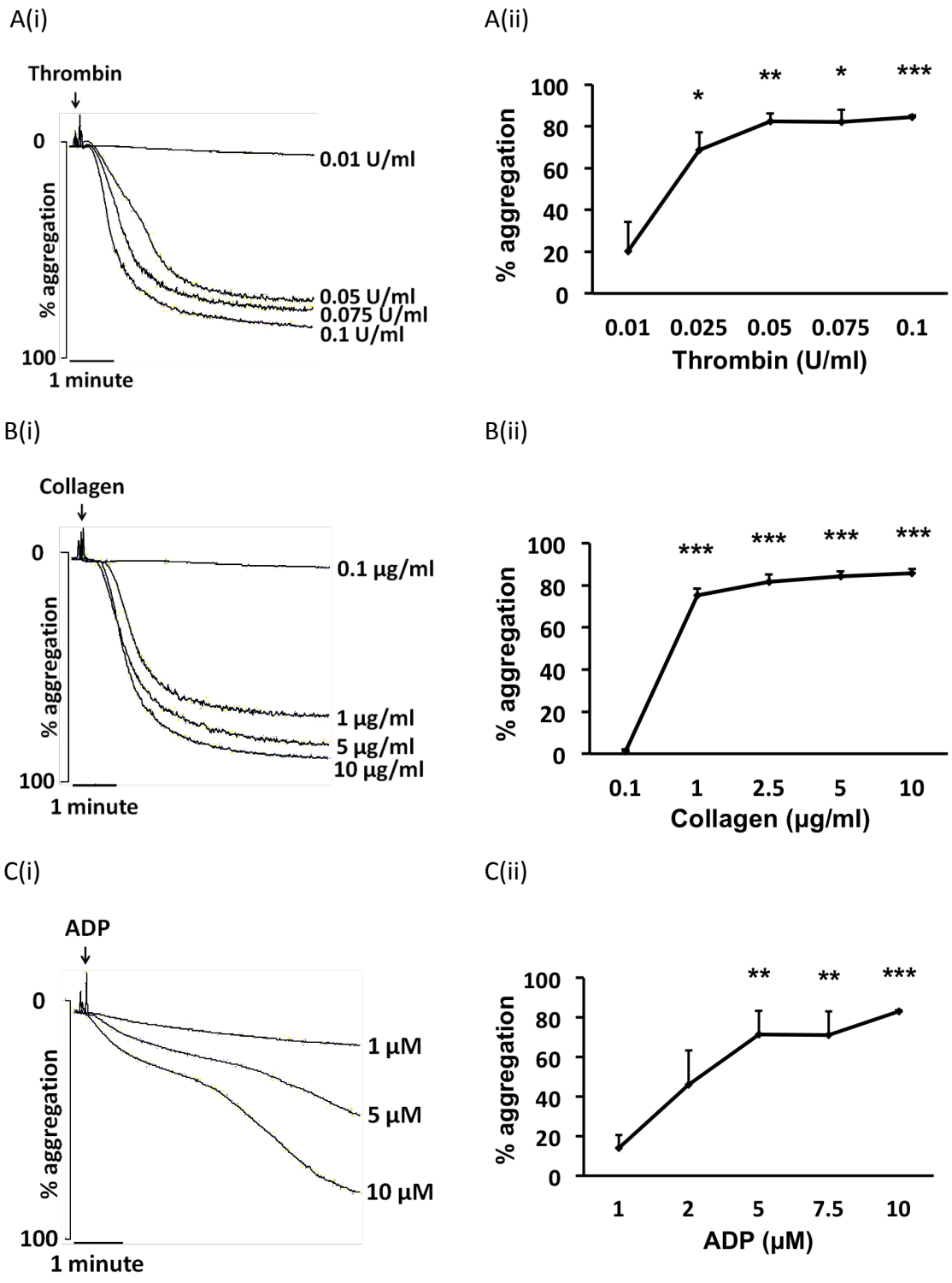


Figure 3.1 Platelet aggregation responses to thrombin, collagen and ADP. Washed platelets (2.5×10^8/ml) were stimulated with various concentrations of thrombin (A), collagen (B) and ADP (C) and aggregation was recorded for 4 minutes. (Ai), (Bi) and (Ci) show representative aggregation traces, while (Aii), (Bii) and (Cii) show the quantitative analysis. Data is presented as mean ± SEM, n=3. *** denotes p<0.005, ** p<0.01-0.005 and * p<0.01-0.05 when compared to the lowest concentration of the agonist.

3.3.2 GSNO-mediated inhibition of platelet aggregation

Although the above aggregation responses indicated that the washed platelet preparation was functionally competent, it was important to test if the aggregation responses by activated platelets could be dampened with the use of the physiological platelet inhibitor nitric oxide (NO). For this, platelets were incubated with an NO-donor S-nitrosoglutathione (GSNO) for 2 minutes at 37°C under stirring conditions prior to stimulation with agonists. GSNO (10 µM) reduced platelet aggregation response to each of the agonists at all the concentrations tested as evident from the rightward shifts in the concentration response curves in presence of GSNO (Figure 3.2). GSNO reduced platelet aggregation induced by 0.025 U/ml thrombin from 69±8.5% to 1±0% (99% inhibition; p=0.0007), while aggregation induced by 0.1 U/ml thrombin was reduced from 84±0.9% to 59±16.8% (30% inhibition; p=0.1) (Figure 3.2; Aii). Similarly, while GSNO inhibited platelet aggregation induced by 1 µg/ml collagen from 75±3.2% to 18±2.2% (76% inhibition; p=0.00006), it only reduced aggregation induced by 10 µg/ml collagen from 86±2% to 60±11.6% (30% inhibition; p=0.05) (Figure 3.2; Bii). In case of ADP stimulation, GSNO was able to reduce platelet aggregation with comparable efficiency at all concentrations of ADP tested. Thus while GSNO reduced aggregation induced by 5 µM ADP from 52±12.8% to 17±3.2% (67% inhibition; p=0.03), it reduced aggregation induced by 10 µM ADP from 83±2.9% to 22±1.7% (73% inhibition; p=0.00003) (Figure 3.2; Cii). Thus, as expected, the degree of inhibition of aggregation by GSNO was higher at low concentrations of agonists, while at higher agonist concentrations it was less efficient in reducing platelet aggregation. This was particularly applicable in case of strong agonists such as thrombin and collagen.

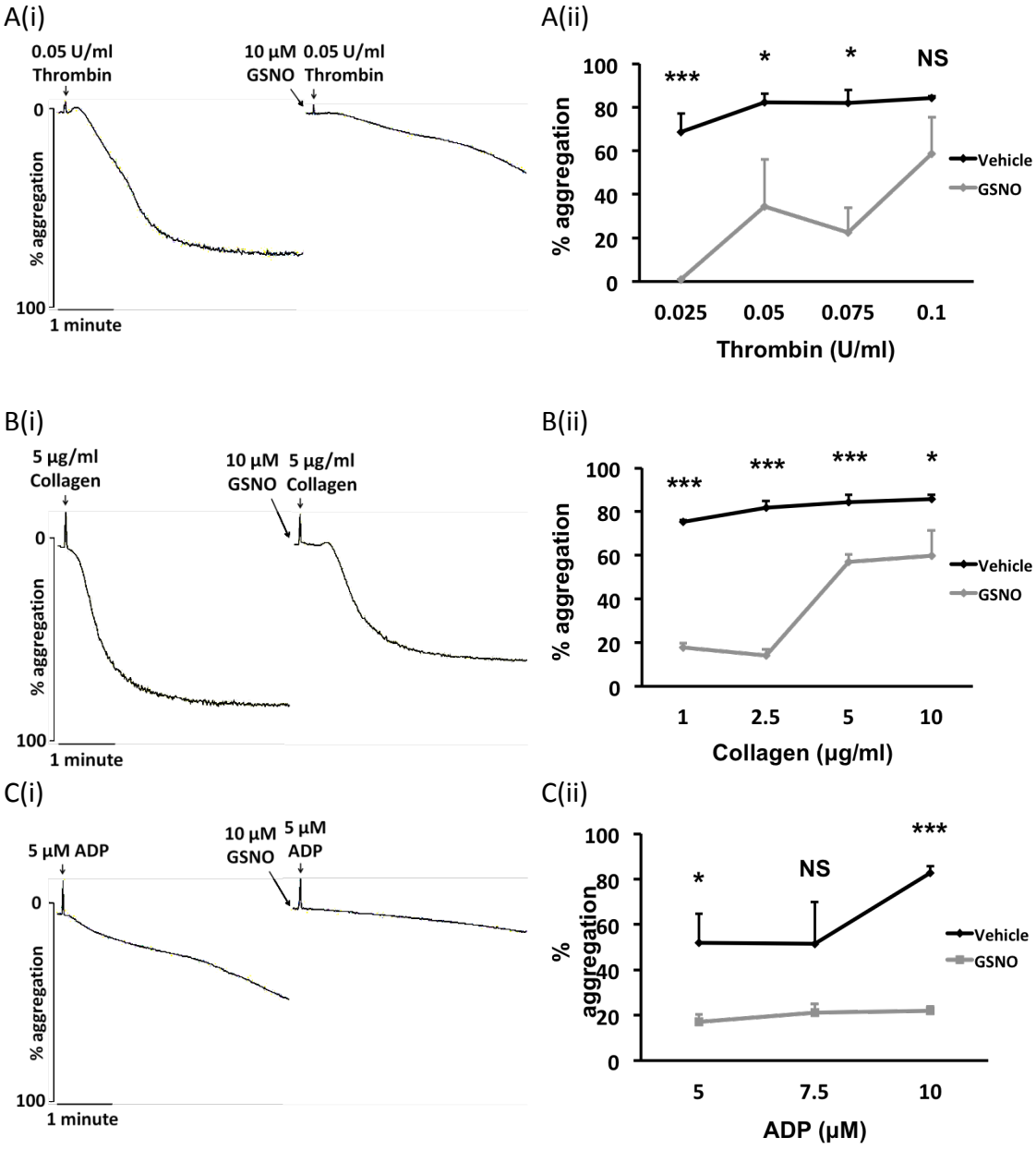


Figure 3.2: Platelet aggregation responses to various agonists were dampened by the physiological inhibitor nitric oxide (NO). Washed platelets (2.5 × 10^8/ml) were pre-treated with the NO-donating compound S-nitrosoglutathione (GSNO) (10 µM) for 2 minutes prior to stimulation with thrombin (A), collagen (B) and/or ADP (C). Aggregation was recorded for 4 minutes. Representative aggregation traces are shown in (Ai), (Bi) and (Ci), while quantitative analyses of the aggregation responses to thrombin, collagen and ADP in the presence or absence of GSNO are shown in (Aii), (Bii) and (Cii), respectively. Data is presented as mean ± SEM, n=3. *** denotes p<0.005, ** p<0.01-0.005 and * p<0.05-0.01. NS denotes non-significant.

3.3.3 Characterisation of the effects of prednisolone on thrombin-induced platelet aggregation

Having established the sensitivity of the platelet preparation to be stimulated and inhibited by physiological platelet agonists and antagonists respectively, we aimed to characterise the effect of the synthetic glucocorticoid prednisolone on thrombin-induced platelet aggregation.

3.3.3.1 Prednisolone suppressed thrombin-induced platelet aggregation in a concentration-dependent manner

Washed platelets were incubated with various concentrations of prednisolone at 37°C for 5 minutes with brief stirring followed by platelet stimulation with thrombin. The level of aggregation induced by thrombin was variable between donors at any particular thrombin concentration, hence platelets were stimulated with a thrombin concentration that gave approximately 50-60% aggregation. Prednisolone inhibited thrombin-induced platelet aggregation in a concentration-dependent manner (Figure 3.3; Ai and ii). Threshold inhibitory responses were observed at 0.1 μM prednisolone where thrombin-induced aggregation was reduced from 54±7.4% to 23±5.2% (57% inhibition; p=0.006). Maximal inhibitory effects were observed with 10 μM of the GC where aggregation was reduced from 54±7.4% to 13±3.1% (76% inhibition; p=0.003).

3.3.4 Prednisolone suppressed thrombin-induced platelet aggregation in a time-dependent manner

Prednisolone-mediated inhibition of platelet aggregation induced by thrombin was reversible (Figure 3.3; Bi and ii). Thrombin (0.02-0.05 U/ml) caused 63±5.1% aggregation, which after one minute of pre-incubation with prednisolone (10 μM) was reduced to 43±5.5% (32% inhibition; p=0.01). Platelet aggregation was further reduced to 36±7.8% (43% inhibition; p=0.007) with 5 minutes of pre-incubation with prednisolone. The aggregation response however returned to 58±3.2% (p=0.44 compared to untreated platelets and 0.05 compared to platelets treated with prednisolone for 5 minutes), similar to untreated platelets, after pre-incubation of

15 minutes or any longer. This data also suggests that prednisolone possesses a reversible but non-toxic effect on platelets.

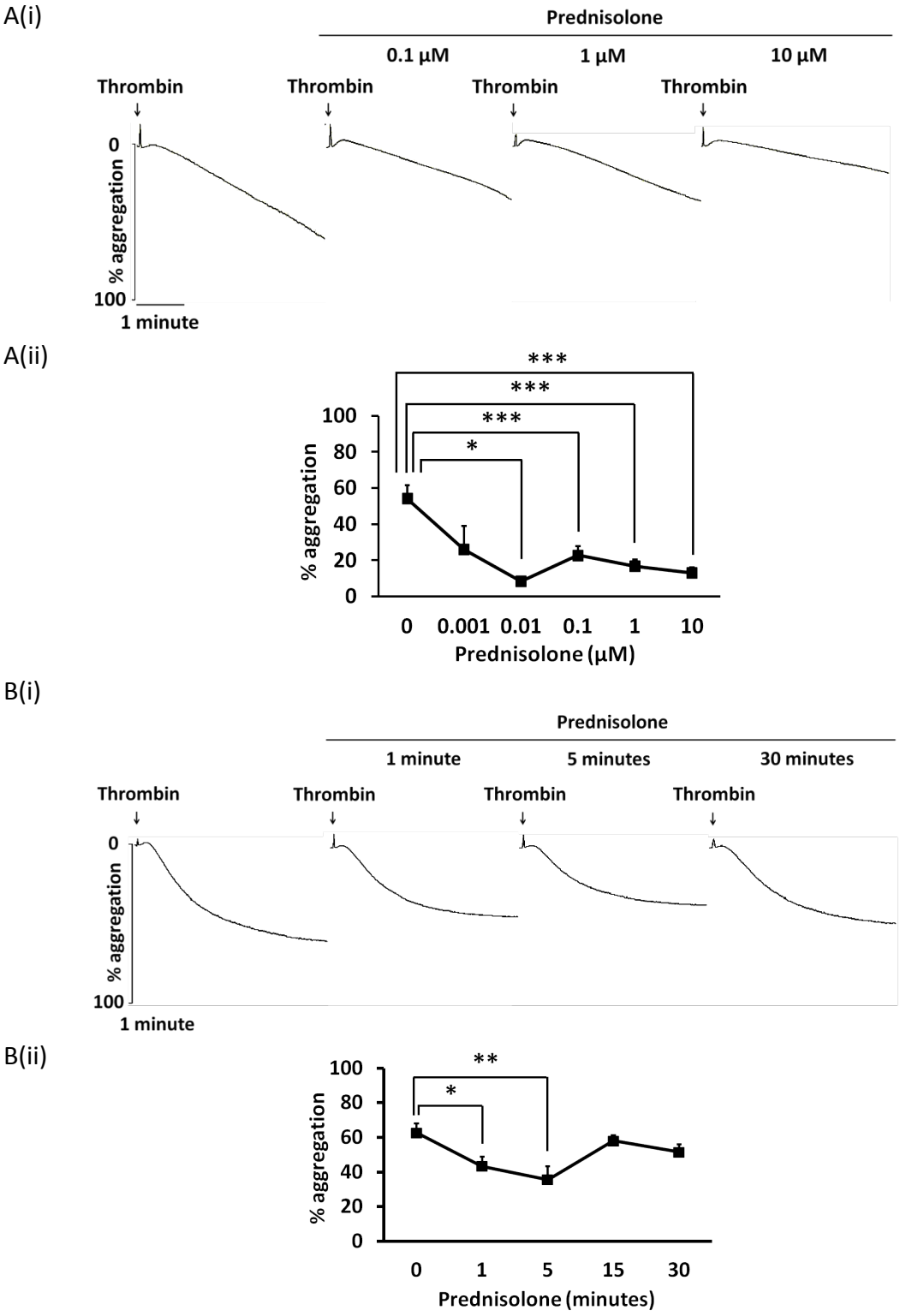


Figure 3.3. Concentration- and time-dependent inhibition of thrombin-induced platelet aggregation by prednisolone. Washed platelets (2.5 × 10^8/ml) were incubated with various concentrations of prednisolone for 5 minutes (Ai and ii) or 10 µM prednisolone for various time points (Bi and ii) prior to thrombin stimulation and aggregation was monitored for 4 minutes. (Ai) and (Bi) show representative aggregation traces, while (Aii) and (Bii) show the quantitative analyses. Data is presented as mean ± SEM, n=4. *** denotes p<0.005, ** p<0.01-0.005 and * p<0.05-0.01.

3.3.5 Involvement of the glucocorticoid receptor (GCR) in prednisolone-mediated suppression of thrombin-induced platelet aggregation

An earlier study (Moraes et al., 2005) demonstrated the presence of GCR in human platelets, which was confirmed in this study too by western blotting and immunoprecipitation (chapter 6). Having established the ability of prednisolone to inhibit thrombin-induced platelet aggregation, it was important to establish whether the effect was mediated by GCR. To achieve this, the GCR antagonist mifepristone (RU486) (Bertagna et al., 1984) was used. It is believed to attenuate GC-mediated cellular effects by blocking GC binding to the GCR through competitive inhibition. However, this compound has been reported to act as a partial agonist in other cells (Peeters et al., 2008; Zhang et al., 2007) and therefore the first series of experiments were designed to characterise its effects on platelet aggregation.

3.3.5.1 RU486 suppressed thrombin-mediated platelet aggregation in a concentration-dependent manner

Washed platelets were incubated with various concentrations of RU486 for 5 minutes followed by thrombin stimulation. RU486 inhibited platelet aggregation in a concentration-dependent manner similar to prednisolone. Maximal inhibitory effects were observed with 10 µM RU486, which reduced platelet aggregation from 56±3.5% to 24±7.4% (57% inhibition; p=0.009) (Figure 3.4; Ai and ii).

3.3.5.2 RU486-mediated inhibition of thrombin-induced platelet aggregation is reversible

Washed platelets were incubated with RU486 (10 µM) at 37°C for various time-points prior to thrombin stimulation. RU486-mediated inhibition of platelet aggregation was reversible. After a 1-minute incubation with RU486, thrombin-induced platelet aggregation was reduced from 56±3.5% to 41±4.2% (p=0.03). Maximal inhibition was seen when platelets were incubated with RU486 for 5 minutes when thrombin-induced aggregation was reduced from 56±3.5% to 24±7.4% (57% inhibition; p=0.009). Incubation with RU486 for longer time points could not significantly retain the inhibitory effect (Figure 3.4; Bi and ii).

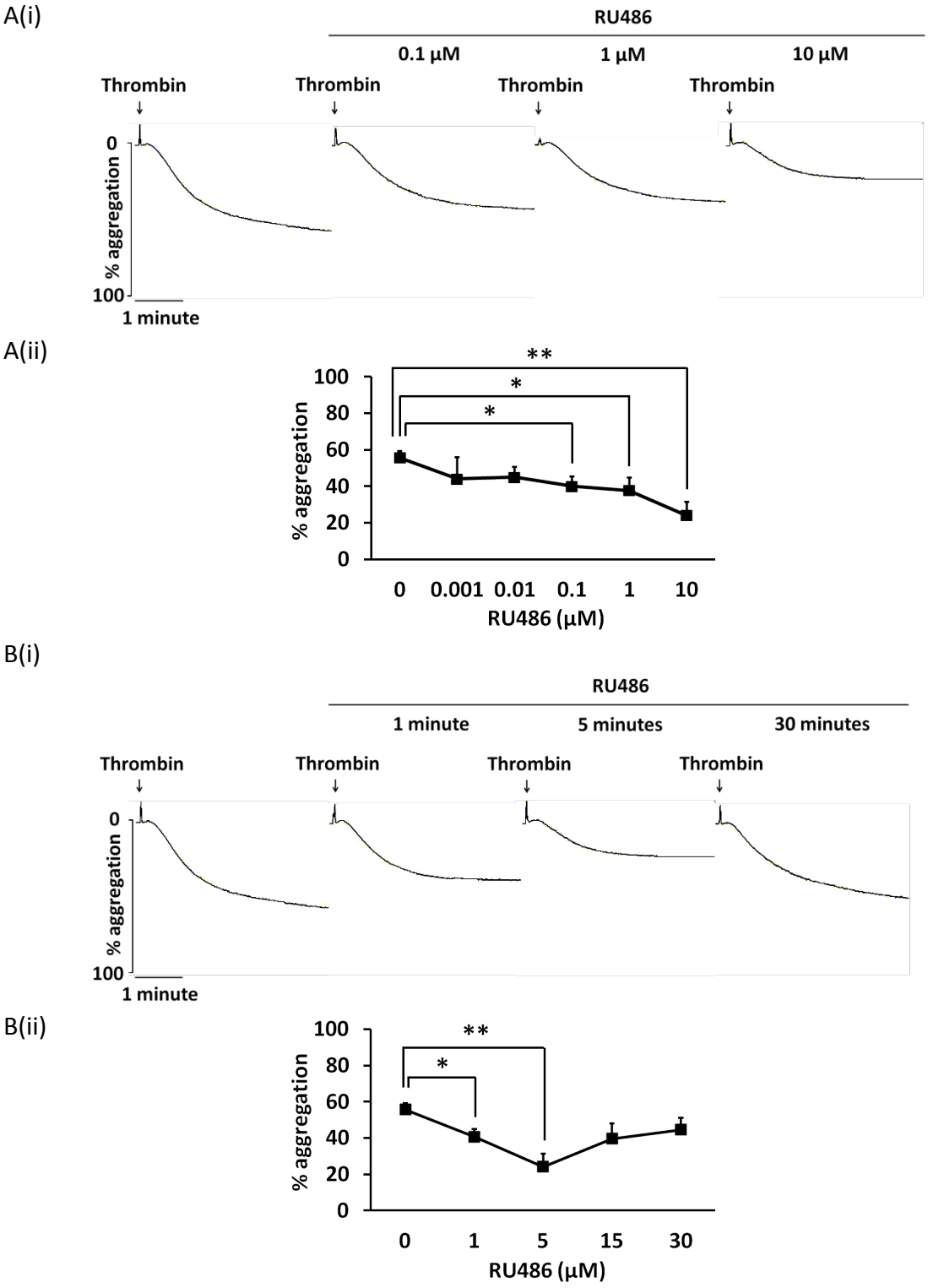


Figure 3.4. Concentration- and time-dependent inhibition of thrombin-induced platelet aggregation by RU486. Washed platelets (2.5×10^8/ml) were incubated with various concentrations of RU486 for 5 minutes (Ai and ii) or 10 µM RU486 for various time points (Bi and ii) prior to thrombin stimulation and aggregation was monitored for 4 minutes. (Ai) and (Bi) show representative aggregation traces, while (Aii) and (Bii) show the quantitative analyses. Data is presented as mean ± SEM, n=3. ** denotes p<0.01-0.005 and * p<0.05-0.01.

3.3.5.3 Prednisolone suppressed thrombin-induced platelet aggregation by acting through the GCR

After characterising the effects of RU486 on platelet aggregation, it was employed in platelet aggregometry in conjunction with prednisolone to determine whether the effects of prednisolone on platelet aggregation were GCR-mediated. Thus washed platelets were pre-incubated with RU486 (1 μM) at 37°C for 5 minutes with brief stirring followed by incubation with prednisolone (10 μM) at 37°C for 5 minutes and platelet stimulation with thrombin. Prednisolone reduced thrombin-induced aggregation from 53±6.9% to 20±2.8% (65% inhibition; p=0.006). RU486 reversed the inhibitory effects of prednisolone by restoring thrombin-induced platelet aggregation to 44±5.2% (p=0.008) (Figure 3.5; A and B). In these experiments, RU486 itself did not affect thrombin-induced platelet aggregation (data not shown). The data hence suggests that prednisolone exerts its inhibitory effects on platelets by acting, at least in part, through GCR.

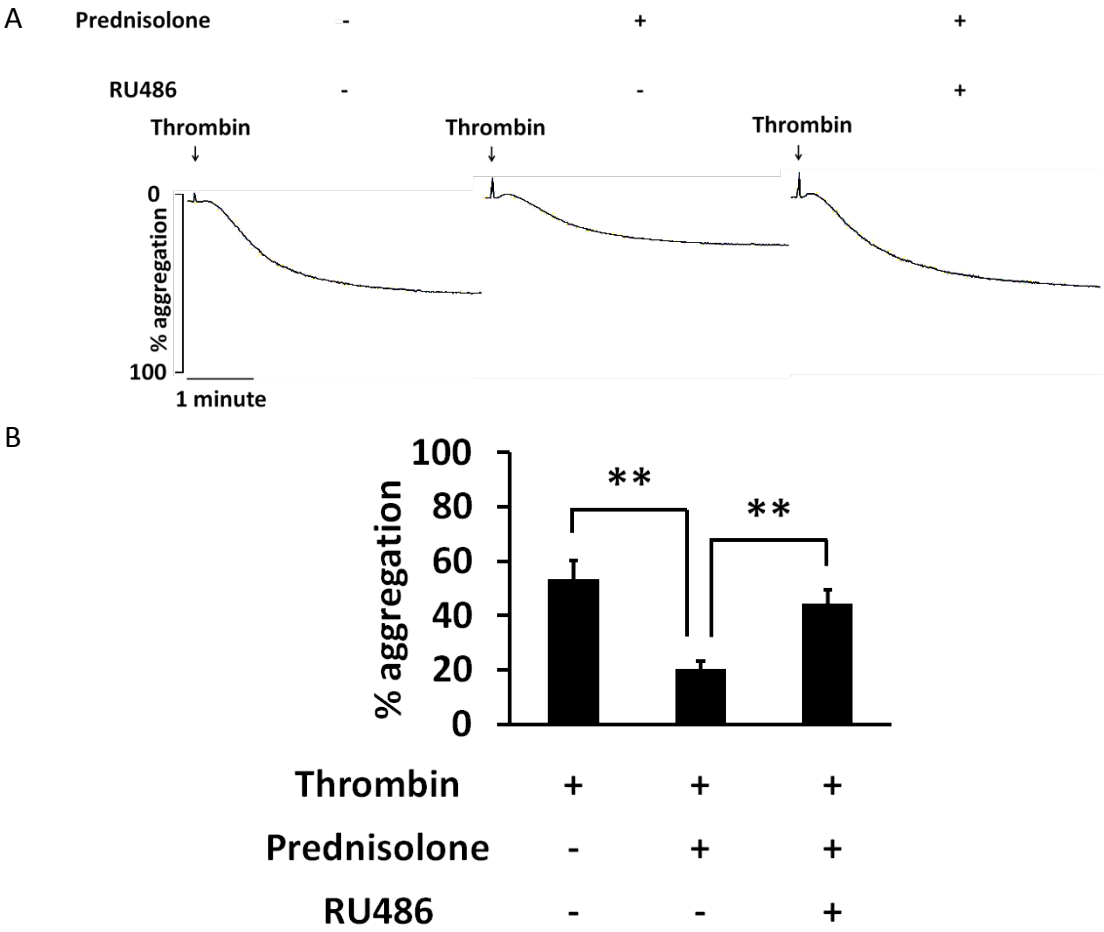


Figure 3.5. Reversal of prednisolone-mediated inhibition of thrombin-induced platelet aggregation by RU486. Washed platelets (2.5×10^8/ml) were pre-incubated with RU486 (1 µM) for 5 minutes followed by incubation with prednisolone (10 µM) for another 5 minutes and thrombin stimulation. Aggregation was monitored for 4 minutes. Representative aggregation traces (A) and quantitative analyses (B) are shown. Data is presented as mean ± SEM, n=3. ** denotes p<0.01-0.005.

3.4 Prednisolone targeted both the protease activated receptors (PARs) on human platelets

Thrombin activates platelets by cleavage of residues in the N-terminal exodomain of the two G-protein coupled protease activated receptors (PAR1 and PAR4) thereby exposing hidden residues in their N-terminal region. These new N-terminal regions then behave as tethered ligands bound to these receptors to generate molecular signals. Synthetic short peptides mimicking the first six amino acids of the unmasked N-terminal region of each PARs have been developed, which can activate these receptors independent of thrombin stimulation. Hence we decided to apply these PAR peptides in platelet aggregometry in order to determine the PAR that might be targeted by prednisolone during suppression of thrombin-stimulated platelet aggregation. This strategy was an alternative to using PAR antagonists.

Washed platelets from each donor were stimulated with PAR1 (SFLLRN) and PAR4 (AYPGKF) peptides at concentrations that induced approximately 50-70% aggregation response. Prednisolone was shown to suppress platelet aggregation induced by both these peptides. Maximal inhibition of aggregation was seen at 0.1 μM prednisolone when it reduced PAR1 (1.5-5 μM) peptide-induced aggregation from 49±0.3% to 25±4.9% (49% inhibition; p=0.004) (Figure 3.6; Ai and ii) and PAR4 (6-20 μM) peptide-induced aggregation from 68±4% to 32±11% (68% inhibition; p=0.05) (Figure 3.6; Bi and ii). Thus prednisolone suppressed platelet aggregation response downstream of both the PARs.

To provide further physiological relevance, the above experiments were also performed in PRP. As in washed platelets, prednisolone suppressed platelet aggregation in PRP induced by the PAR4 peptide (25-30 μM) at all concentrations tested. The maximal inhibitory response was seen with 1 μM prednisolone when aggregation was reduced from 92±14.6% to 38±17.5% (59% inhibition; p=0.04), although the other prednisolone concentrations brought about similar levels of inhibition (Figure 3.7; Bi and ii). In contrast to washed platelets, prednisolone at all concentrations failed to significantly suppress platelet aggregation in PRP induced by the PAR1 peptide (2-5 μM) (Figure 3.7; Ai and ii).

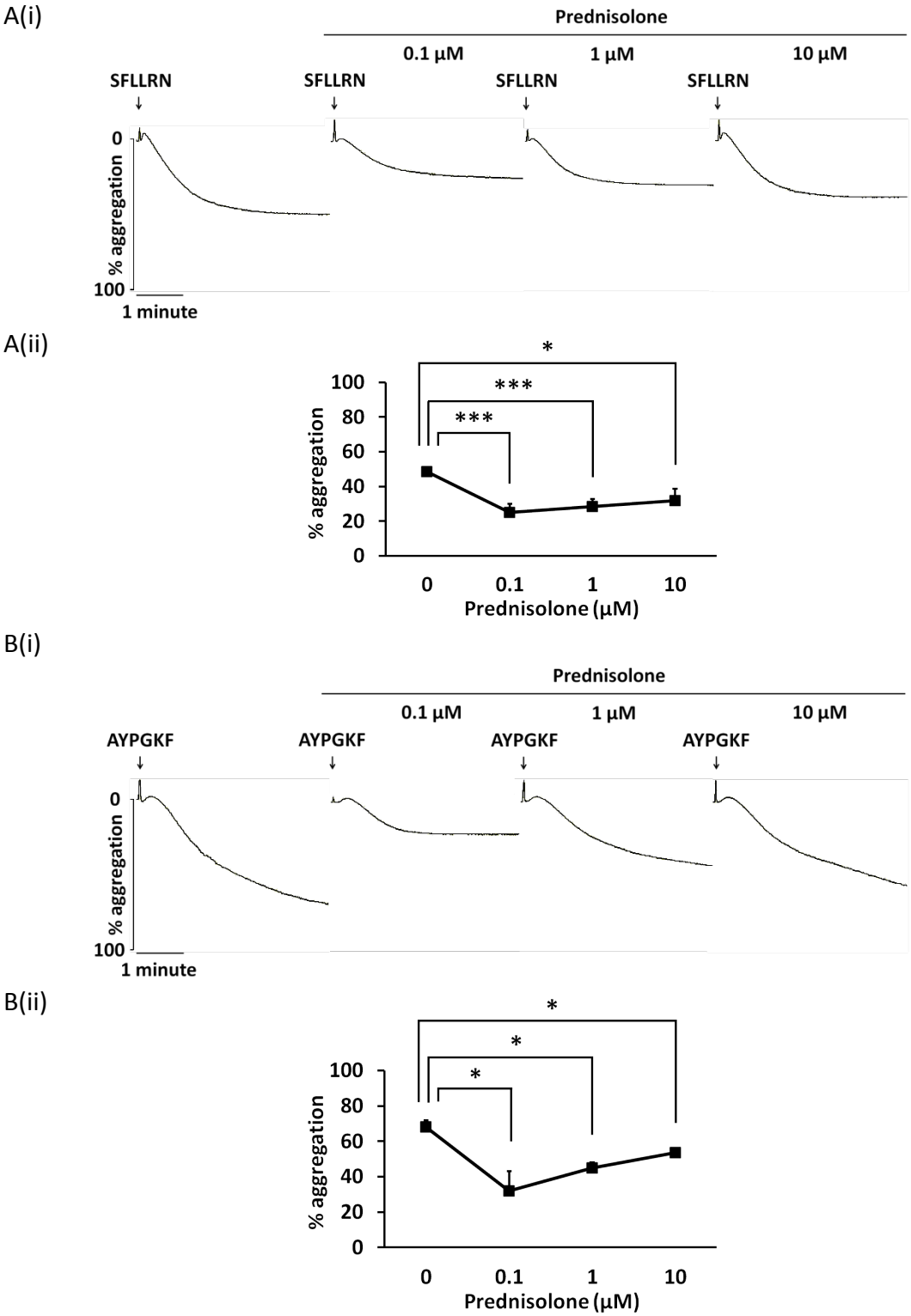


Figure 3.6. Effect of prednisolone on aggregation in washed platelets induced by PAR agonists. Washed platelets (2.5×10^8/ml) were incubated with various concentrations of prednisolone for 5 minutes prior to stimulation with PAR1 peptide SFLLRN (Ai and ii) or PAR4 peptide AYPGKF (Bi and ii) and aggregation monitored for 4 minutes. Representative aggregation traces (Ai and Bi) and quantitative analyses (Aii and Bii) are shown. Data is presented as mean ± SEM, n=3. *** denotes $p<0.005$ and * $p<0.05$-0.01.

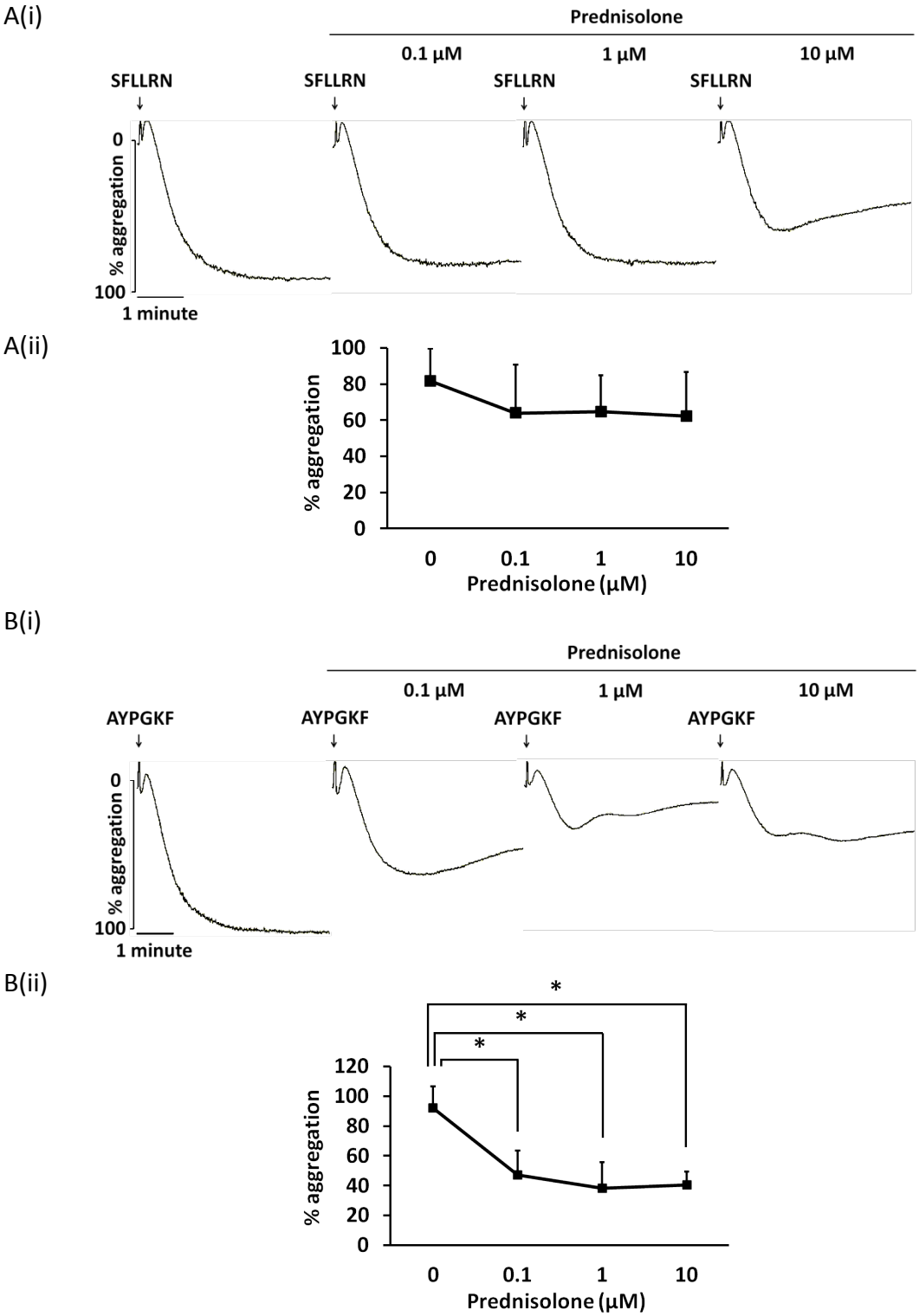


Figure 3.7. Effect of prednisolone on platelet aggregation in platelet rich plasma (PRP) induced by PAR agonists. Human PRP was incubated with various concentrations of prednisolone for 5 minutes prior to stimulation with PAR1 peptide SFLLRN (Ai and ii) or PAR4 peptide AYPGKF (Bi and ii) and aggregation monitored for 4 minutes. Representative aggregation traces (Ai and Bi) and quantitative analyses (Aii and Bii) are shown. Data is presented as mean ± SEM, n=3. * denotes p<0.05-0.01.

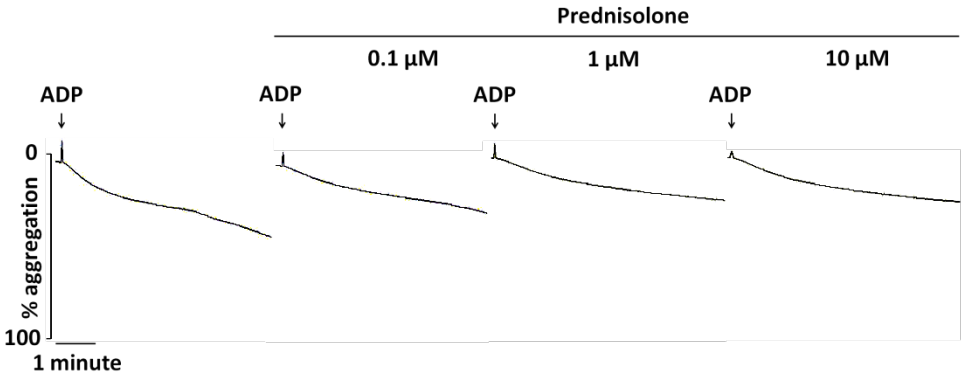
3.5 Prednisolone suppressed ADP-induced platelet aggregation

Having examined the effects of prednisolone on thrombin-induced aggregation, its effects on ADP were also tested. Prednisolone also inhibited aggregation induced by ADP in washed platelets and PRP. Maximal inhibition of platelet aggregation was caused by 10 μM prednisolone in both washed platelets and PRP, where aggregation was significantly reduced from 42±4.8% to 23±4.8% (45% inhibition; p=0.02) (Figure 3.8; Ai and ii) and from 75±8% to 51±11.4% (32% inhibition; p=0.05) (Figure 3.8; Bi and ii), respectively. Although lower prednisolone concentrations could significantly suppress ADP-induced platelet aggregation in washed platelets, they were ineffective in PRP.

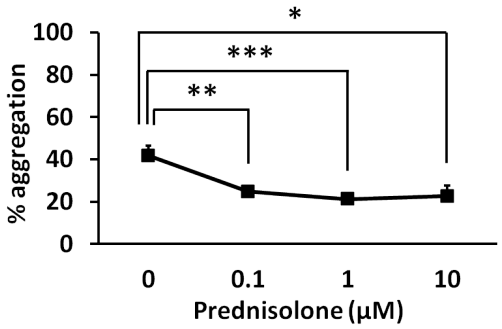
ADP, like thrombin, activates platelets by transmitting signals through two purinergic transmembrane G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$ expressed on the platelet surface. A possible reason behind the suppression of ADP-induced platelet aggregation by prednisolone is that it might block signalling pathways downstream of these receptors. To explore this possibility we employed the commercially available ADP receptor antagonists A3P5P and MRS2395 in platelet aggregometry. A3P5P and MRS2395 selectively block signalling events downstream of G_q-coupled $P2Y_1$ and G_i-coupled $P2Y_{12}$, respectively without affecting signalling events of their complementary receptors. Thus, the effect of prednisolone on $P2Y_{12}$ signalling could be studied in isolation in the presence of A3P5P, much as the same on $P2Y_1$ signalling in the presence of MRS2395. Washed platelets were treated with prednisolone (1 μM) for 5 minutes followed by a 1-minute incubation with either of these ADP receptor antagonists and ADP (5 μM) stimulation to determine if prednisolone targeted any of the ADP receptors. As shown in Figure 3.8 (Ci and ii), A3P5P (300 μM) and MRS2395 (10 μM) reduced ADP-induced platelet aggregation from 61±2.8% to 42±3.9% (30% inhibition) and 46±1.6% (23% inhibition), respectively (p=0.009 for A3P5P and 0.006 for MRS2395). Prednisolone (1 μM) alone reduced ADP-induced platelet aggregation from 61±2.8% to 46±3.7% (23% inhibition; p=0.02). Upon co-treatment with MRS2395, prednisolone reduced ADP-induced platelet aggregation even further to 28±4.9%, thus potentiating the degree of inhibition, when compared to treatment with either MRS2395 alone (p=0.01) or

prednisolone alone (p=0.02). However, no such potentiation of inhibition was observed with co-treatment of A3P5P and prednisolone. This indicated that prednisolone could potentially block the ADP receptor $P2Y_1$ and hence contributed to a reduced platelet aggregation response to ADP.

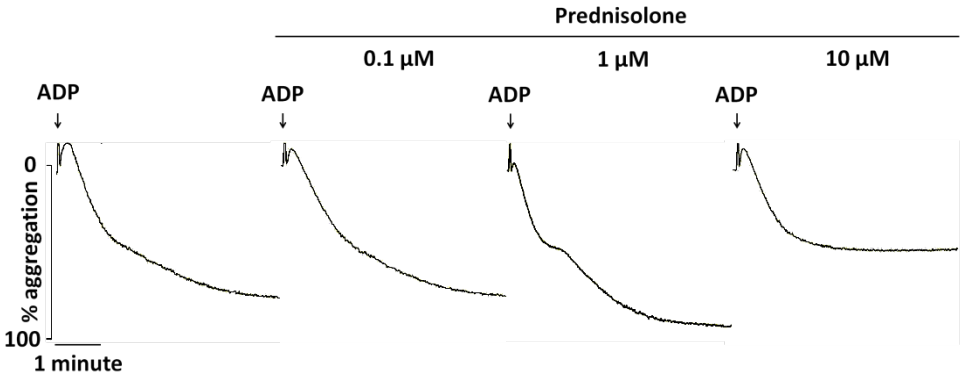
A(i)


Prednisolone
0.1 µM
1 µM
10 µM
ADP
ADP
ADP
ADP
0
% aggregation
100
1 minute

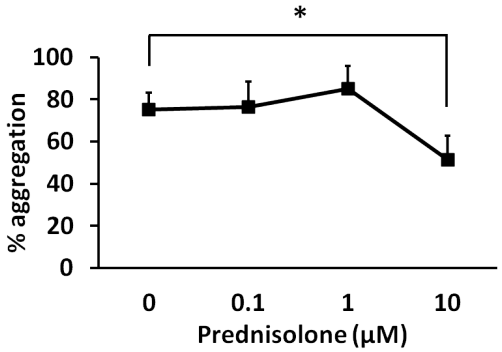
A(ii)


*
***
**
100
80
60
40
20
0
% aggregation
0
0.1
1
10
Prednisolone (µM)

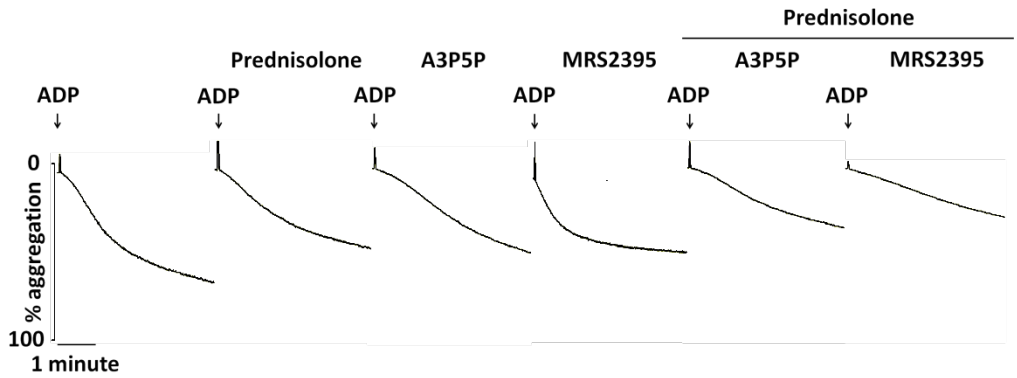
B(i)


Prednisolone
0.1 µM
1 µM
10 µM
ADP
ADP
ADP
ADP
0
% aggregation
100
1 minute

B(ii)


*
100
80
60
40
20
0
% aggregation
0
0.1
1
10
Prednisolone (µM)

C(i)



C(ii)

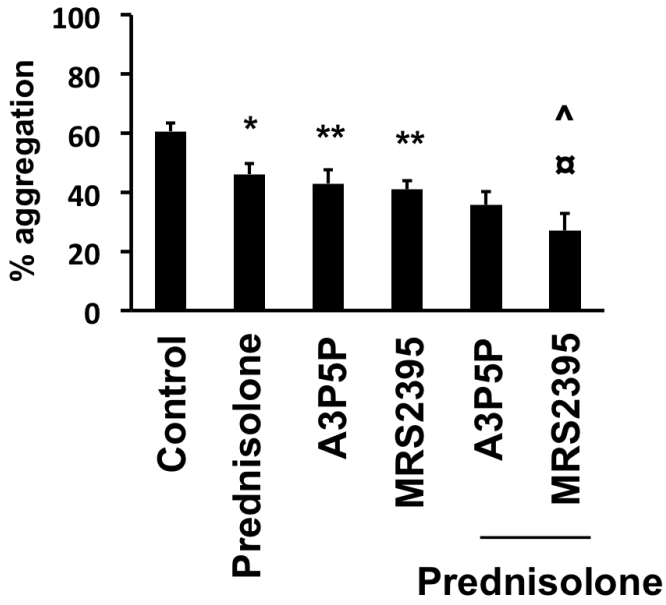


Figure 3.8 Effect of prednisolone on ADP-induced platelet aggregation. Washed platelets (2.5×10^8/ml) (Ai, Aii, Ci and Cii) or PRP (Bi and Bii) were incubated with various concentrations of prednisolone for 5 minutes prior to stimulation with ADP (2-10 µM) and aggregation monitored for 4 minutes. In conditions where ADP receptor antagonists were used (Ci and ii), washed platelets were incubated with the antagonists, A3P5P (300 µM) and MRS2395 (10 µM) for 1 minute prior to stimulation with ADP. When prednisolone was used in combination with the antagonists, prednisolone (1 µM) was added first to platelets and, during the last minute of its incubation, the ADP antagonists were added to the platelets followed by ADP (5 µM) stimulation. Representative aggregation traces (Ai, Bi and Ci) and quantitative analyses (Aii, Bii and Cii) are shown. Data is presented as mean ± SEM, n=3. *** denotes p<0.005, p<0.01-0.005 and * p<0.05-0.01. *, ¤ and ^ represent p-values when compared to control platelets, MRS2395-treated platelets and prednisolone-treated platelets.

3.6 Characterisation of the effect of prednisolone on platelet adhesion under static conditions

3.6.1 Washed platelets adhered and spread on immobilised fibrinogen in a time-dependent manner

Static adhesion provides a powerful tool to visualise the events in platelet shape change and actin assembly in response to contact with thrombogenic materials during the initial phase of thrombosis. An initial experiment was performed to determine the kinetics of platelet adhesion. Washed platelets were applied to human fibrinogen immobilised on glass slides for increasing time points from 5 minutes up to an hour. Platelets not only adhered to fibrinogen within 5 minutes of application (407 platelets per 0.1 mm^2), but most of them also attained their fully spread morphology (20.7 μm^2), which was indicative of their maximal activation on coming in contact with immobilised fibrinogen. When adhered for longer time points, increasing numbers of platelets adhered on fibrinogen albeit there was no substantial difference in their spread morphology (Figure 3.9).

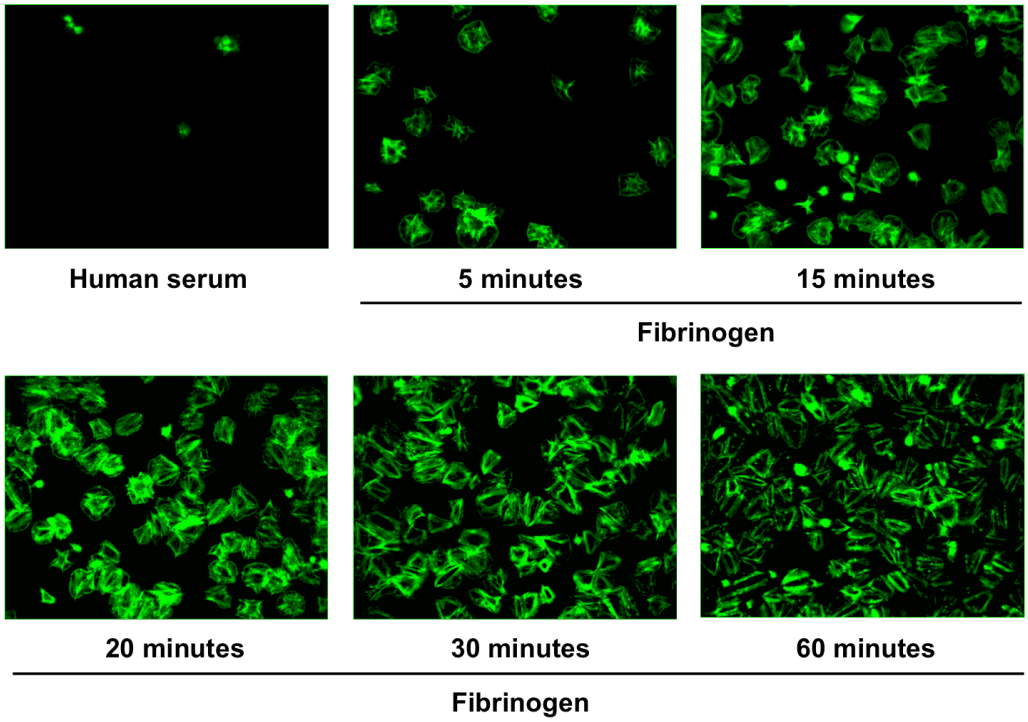


Figure 3.9. Washed platelets adhered and spread on immobilised fibrinogen under static conditions in a time-dependent manner. Wells on glass slides were coated overnight at 4°C with human fibrinogen (1 mg/ml) or human serum. After blocking uncoated surfaces with human serum, washed platelets (5×10^7/ml) were allowed to adhere on coated slides at 37°C for various time points from 5 minutes to an hour. Adherent platelets were fixed, permeabilised and stained for filamentous actin (F-actin) with FITC-phalloidin and viewed under a fluorescence microscope using the ×60 objective. Images of adherent platelets for each time point from a single experiment are shown. Scale bar=20 μm.

3.6.2 Prednisolone suppressed platelet adhesion on immobilised fibrinogen in a GCR-dependent manner

Next we wanted to investigate whether prednisolone could influence platelet adhesion to immobilised fibrinogen. Thus washed platelets (5×10^7/ml) were treated with prednisolone (0.1-10 μM) for 5 minutes and then allowed to adhere to immobilised fibrinogen for 5 minutes. When applied at a concentration of 1 μM, prednisolone reduced platelet adhesion to fibrinogen from 404±23 platelets per 0.1 mm^2 to 255±24 platelets per 0.1 mm^2 (p=0.0006) (Figure 3.10; Ai). Moreover, prednisolone also reduced platelet spreading on fibrinogen from 20.1±2.9 μm^2 to 14.9±0.9 μm^2 (p=0.03) (Figure 3.10; Aii). Other concentrations of prednisolone could not significantly affect platelet adhesion and spreading on fibrinogen (data not shown). The inhibitory effect of prednisolone (1 μM) on platelet adhesion and spreading was however lost when platelets were allowed to adhere on immobilised fibrinogen for an hour (Figure 3.11; A and B).

After establishing an inhibitory effect of prednisolone on platelet adhesion and spreading on fibrinogen, the involvement of GCR in this effect was examined by using the GCR antagonist RU486 in static adhesion assay. Washed platelets were pre-treated with RU486 (0.2, 2 and 5 μM) for 5 minutes prior to prednisolone treatment and application of platelets on fibrinogen-coated slides. In the presence of both RU486 (2 μM) and prednisolone, platelet adhesion on fibrinogen was enhanced to 319±14 platelets per 0.1 mm^2 when compared to 255±24 platelets per 0.1 mm^2 (p=0.02) in the presence of prednisolone alone (Figure 3.10; Ai). Platelet spreading was also increased to 17.8±1.4 μm^2 in the presence of both RU486 and prednisolone when compared to 14.1±0.8 μm^2 in the presence of prednisolone alone (p=0.02), thus restoring the spreading response similar to untreated platelets (p=0.3) (Figure 3.10; Aii). Thus prednisolone-mediated inhibition of platelet adhesion and spreading was reversed by RU486, which indicated that prednisolone mediated its effects through the GCR. However, RU486 itself had no effect on platelet adhesion on fibrinogen or spreading at any of the concentrations tested (Figure 3.12; A and B).

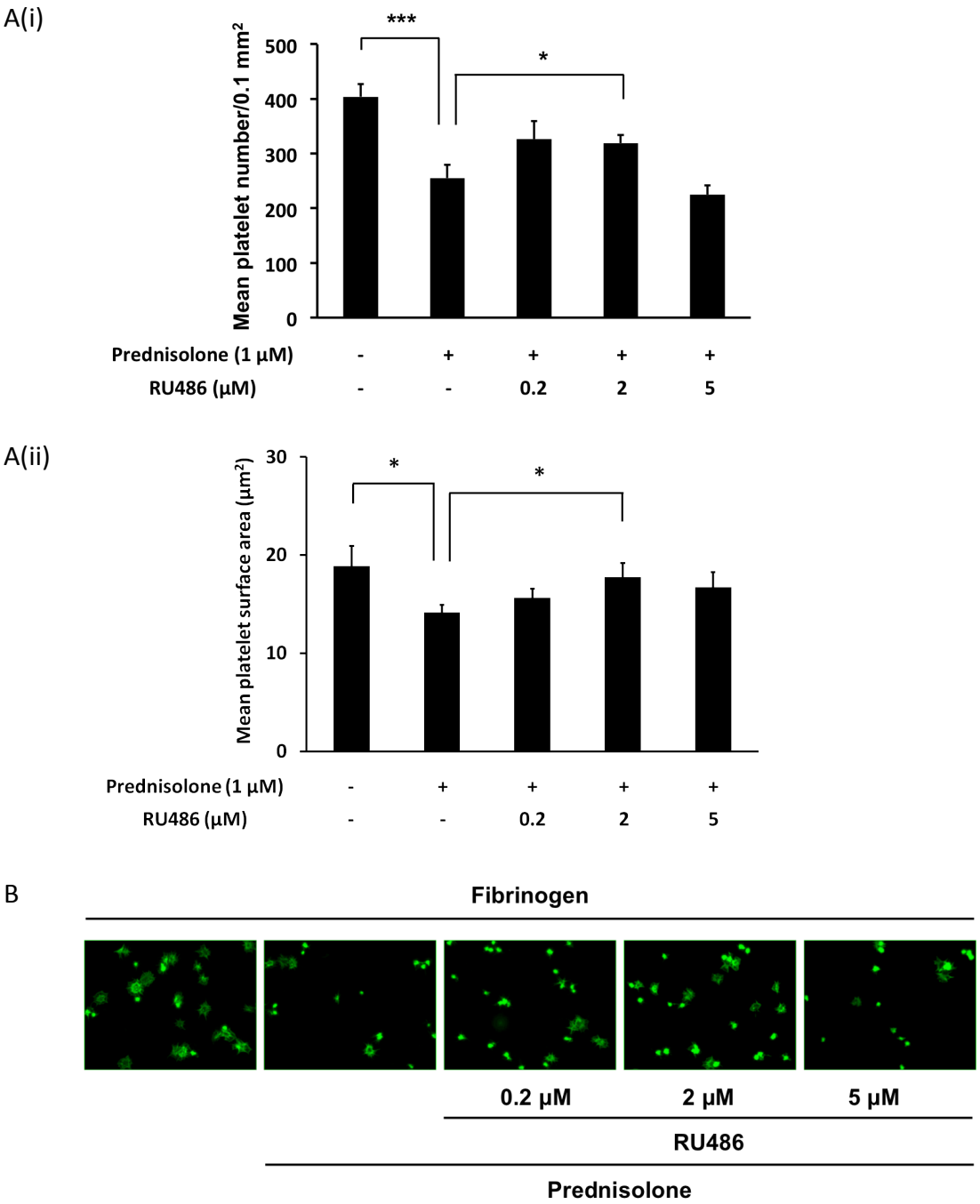


Figure 3.10. Reversal of prednisolone-mediated inhibition of platelet adhesion and spreading on immobilised fibrinogen by RU486. Washed platelets (5×10^7/ml) were treated with various doses of RU486 (0.2-5 μM) for 5 minutes followed by treatment with prednisolone (1 μM) for an additional 5 minutes. Platelets were then allowed to adhere on fibrinogen-coated glass slides at 37°C for 5 minutes. Adherent platelets were then stained for F-actin with FITC-phalloidin and viewed under a fluorescent microscope using a ×60 objective. (Ai) and (Aii) show quantitative analyses of the number of adherent platelets in a total area of 0.1 mm^2 and their mean surface area respectively, while (B) shows representative images (scale bar=20 μm). Data is presented as mean ± SEM, n=6. *** denotes $p<0.005$ and * $p<0.05$-0.01.

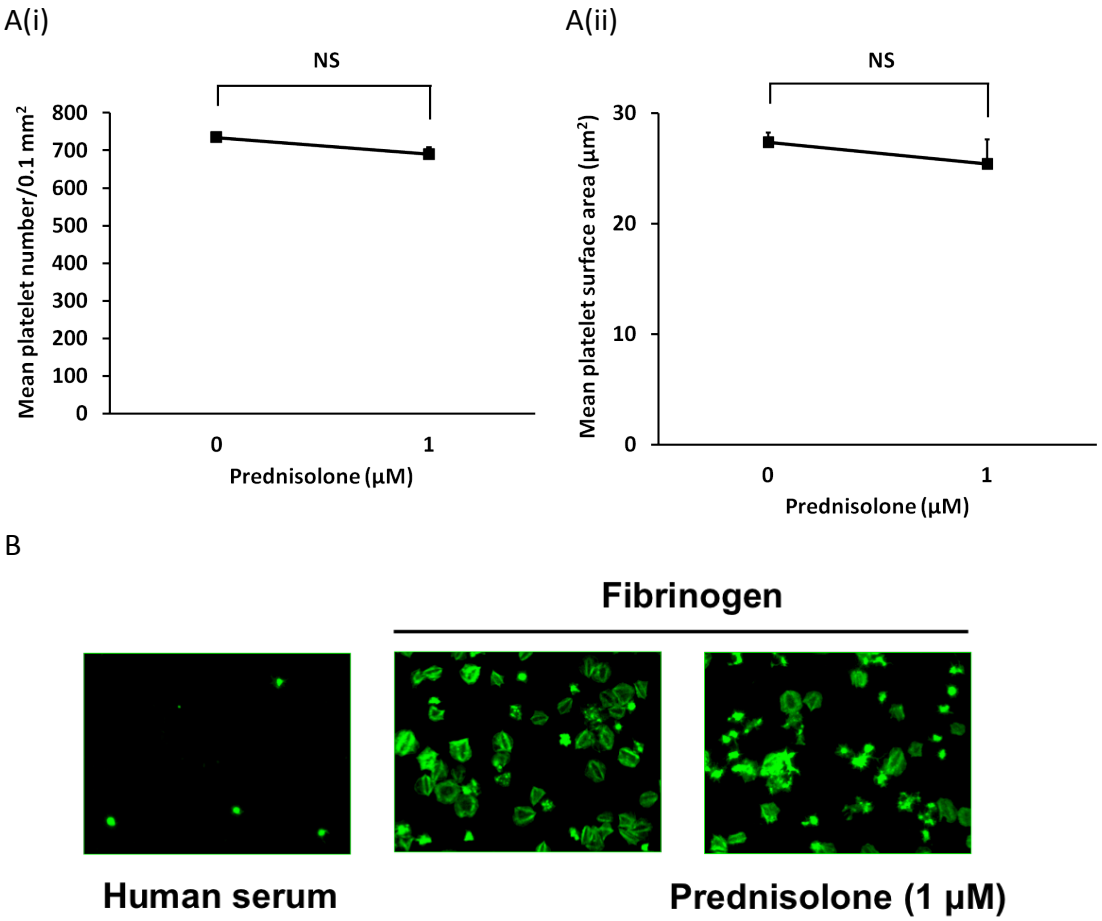


Figure 3.11. Prednisolone failed to affect platelet adhesion and spreading on immobilised fibrinogen when platelets were allowed to adhere for 1 hour instead of 5 minutes. Washed platelets (5×10^7/ml) were pre-treated with prednisolone for 5 minutes and allowed to adhere to fibrinogen-coated glass slides at 37°C for one hour (A and B). Adherent platelets were stained for F-actin with FITC-phalloidin and viewed under a fluorescent microscope using a ×60 objective. (Ai) and (Aii) show quantitative analyses of the number of adherent platelets in a total area of 0.1 mm^2 and their mean surface area respectively, while B shows representative images (scale bar=20 μm). Data is presented as mean ± SEM, n=2. NS denotes non-significant.

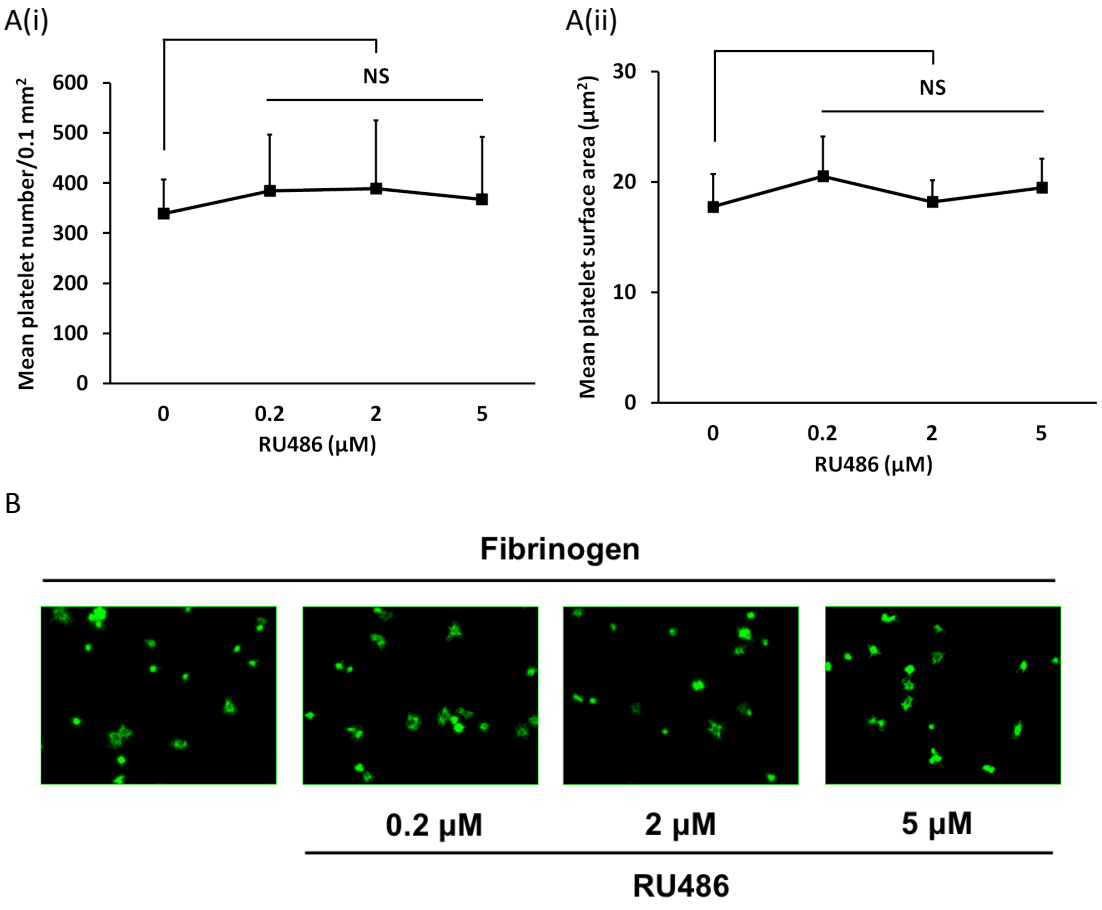


Figure 3.12 RU486 itself did not affect platelet adhesion and spreading on immobilised fibrinogen. Washed platelets (5×10^7/ml) were treated with various concentrations of RU486 (0.2-5 μM) for 5 minutes and allowed to adhere on fibrinogen-coated glass slides at 37°C for 5 minutes. Adherent platelets were stained for F-actin with FITC-phalloidin and viewed under a fluorescent microscope using a ×60 objective. (Ai) and (Aii) show quantitative analyses of the number of adherent platelets in a total area of 0.1 mm^2 and their mean surface area respectively, while (B) shows representative images (scale bar=20 μm). Data is presented as mean ± SEM, n=2. NS denotes non-significant.

3.7 Discussion

The initial objective of this study was to characterise the effects of prednisolone on platelet function in depth. This chapter confirmed the inhibitory properties of prednisolone and extended the initial findings of Moraes et al. (2005) to show that prednisolone targeted thrombin-induced platelet aggregation at the receptor level. From the data it is likely that the inhibitory actions were mediated by the classical GCR.

3.7.1 Prednisolone reduced thrombin-induced platelet aggregation by acting through the classical GCR

Prednisolone was shown to suppress thrombin-induced aggregation of washed platelets. The inhibition appeared to be rapid and transient, since the inhibitory effect was observed with 1 minute of incubation, reached its maximal with 5 minutes of incubation and then was lost with longer incubation. The rapid onset of prednisolone-mediated inhibition also indicated a possible non-genomic mode of action of this glucocorticoid, which is well established in literature (Buttgereit and Scheffold, 2002; Falkenstein et al., 2000; Groeneweg et al., 2011; Lee et al., 2012). The reversal of inhibition pointed out to a non-toxic effect of prednisolone, since platelets remained functional to respond normally to thrombin.

The observations, that prednisolone suppressed platelet aggregation in a concentration-dependent fashion and that this effect was saturated at the concentration of 10 µM, suggested that a receptor could be involved in mediating the effects of prednisolone. Some of the non-genomic effects of GCs are thought to be non-specific and receptor-independent. Such effects had been shown to be carried out by very high therapeutic concentrations of GCs through alteration of the physicochemical properties of the plasma membrane (Buttgereit et al., 1997, 1994). The possibility of such an effect of prednisolone in platelets seems weak, as the maximal amount of prednisolone used in any of the aggregation experiments is at least about 500 fold less in comparison to the amounts used in the studies cited in the above references. Hence the involvement of the classical glucocorticoid receptor (GCR) was examined, similar to reports from other cell types. The GCR

antagonist RU486 was appointed to address this question. Interestingly, prednisolone-mediated inhibition of platelet aggregation was partially reversed in the presence of RU486, which suggested a possibility that GCR in platelets might be involved in mediating the effects of prednisolone, at least in part. The identity of the GCR, which could be responsible in mediating the effects of prednisolone, yet remains elusive since the effects were non-genomic in nature contrary to the classical GCR. This raises a possibility that such effects could rather be mediated by a GPCR endowed with GC activity. However, in most cases RU486 caused only a partial reversal of prednisolone-mediated inhibition of platelet aggregation suggesting the possibility of the involvement of another receptor in mediating the effects of prednisolone. There is increasing evidence in the literature about interaction of prednisolone with another class of very closely related steroid receptors called mineralocorticoid receptor (MCR) as well as MCR activity induced by prednisolone. Thus it can be suggested that in platelets too prednisolone might exert a fraction of its effects through the MCR. The obvious way to validate this possibility is to employ MCR antagonists (such as spironolactone and/or eplerenone) in combination with RU486 and prednisolone in platelet aggregation assays and monitor reversal of prednisolone-mediated inhibition. Unfortunately we were unable to perform these experiments, but would be required for future investigations of the mechanism.

3.7.2 The conventional GCR antagonist RU486 as a partial agonist in platelets

RU486 has been earlier shown to possess GCR agonistic properties in a number of previous studies. An important feature of GCR activation upon agonistic GC binding is its dissociation from the basal machinery followed by nuclear translocation. This GCR nuclear translocation had been observed upon RU486 treatment in various cell-lines (Pariante et al., 2001; Peeters et al., 2008). Another study suggested that the agonistic property of RU486 is mediated by c-AMP signalling pathways (Beck et al., 1993). It had been suggested that RU486 might act as an active antagonist since not only can it compete with GC ligands for binding to the GCR in the cytoplasm, but the RU486-GCR complex can compete with the GC-GCR complex for binding to specific sequences (glucocorticoid-responsive elements; GREs) in the promoters of

the GCR-target genes (Wagner et al., 1999). Thus it was important to characterise the effects of RU486 on platelet aggregation before assuming that it would necessitate the reversal of prednisolone-mediated effects. RU486 was shown to suppress thrombin-induced platelet aggregation in a concentration-dependent manner, an observation similar to that of prednisolone. This concentration-dependent effect implied that RU486 indeed acted through the GCR and hence its use as a GCR antagonist in further experiments to evaluate the role of GCR in mediating the effects of prednisolone was well justified.

It was further observed that the reversal of prednisolone-mediated inhibition of platelet aggregation was dependent on the concentration of RU486 used. At concentrations (10 µM) where RU486 itself affected thrombin-induced platelet aggregation, it not only failed to reverse the inhibitory effects of prednisolone but, in some cases, was shown to potentiate prednisolone-mediated inhibition of platelet aggregation (data not shown). This observation was probably not unexpected, since as discussed earlier, prednisolone had been known to act not only via GCR, but also via MCR in other cell-types (Juruena et al., 2010). Thus although RU486 might specifically act through the GCR, thereby blocking GCR interaction with prednisolone, and inhibit platelet aggregation when used at higher concentrations, prednisolone could still exert a proportion of its effects through the MR, which cannot be blocked by the use of RU486. The synergistic activities through the GCR and MCR could thus result in an aggravated inhibition of platelet aggregation in response to thrombin in experiments where high concentrations of RU486 were used.

3.7.3 Prednisolone targeted PARs on platelets

Having established that thrombin induced aggregation was inhibited by prednisolone, we wished to explore the mechanisms underlying these observations. In order to achieve this we first attempted to define whether a specific thrombin receptor was targeted. Interestingly prednisolone suppressed platelet aggregation induced by the PAR1 and PAR4 peptides, which was in coherence with the observation of suppression of thrombin-induced aggregation, as these PARs are the

main signal transmitters in human platelets downstream of thrombin stimulation. In washed platelets, the data suggested that PAR4 stimulated platelet aggregation was more sensitive to the effects of prednisolone than PAR1. This is important, as it is believed that PAR1 initiates the activation response in platelets upon thrombin stimulation while PAR4 sustains the response. Thus, prednisolone at lower concentrations may not have a strong influence on initial activation upon thrombin stimulation mediated by PAR1, but might still be able to suppress the sustained thrombin response mediated by PAR4. At higher concentrations, prednisolone might possibly suppress the very initial activation response to thrombin mediated by PAR1 and hence be more effective in dampening the overall aggregation response of platelets to thrombin compared to lower concentrations. This supposition is supported by experimental data obtained using PRP. Here prednisolone failed to significantly reduce platelet aggregation induced by PAR1 peptide, while it maintained its inhibitory effect on aggregation induced by PAR4 peptide. Since prednisolone seems to consistently target PAR4 in both washed platelets and PRP, it might thus assist in restricting the overall response to thrombin instead of exerting a dramatic dampening effect comparable to that of the physiological platelet inhibitors such as prostanoids and nitric oxide.

3.7.4 Prednisolone suppressed adhesion and spreading of platelets on fibrinogen possibly via the GCR

Under physiological conditions platelets undergo adhesion and spreading rather that aggregation in suspension. Prednisolone could significantly reduce platelet adhesion and spreading on immobilised fibrinogen under static conditions, which indicated that the effects of prednisolone were not only restricted to aggregation but also to much earlier events of platelet activation. However, the effect was subtle with the inhibitory effect of prednisolone on platelet adhesion and spreading on immobilised fibrinogen was completely lost when platelets were allowed to adhere on fibrinogen for an hour. This is possibly because the inhibitory effects of prednisolone are so short-lived and the platelets could adhere and spread normally once they recovered from the effect of prednisolone. This observation correlates with the time-dependent inhibition of prednisolone on thrombin-induced platelet

aggregation, showing a rapid and transient effect of prednisolone. This weak effect on adhesion and spreading was also observed with collagen where prednisolone had no effect on platelet adhesion or spreading (data not shown) and is probably explained by collagen being a much more potent thrombogenic stimuli than fibrinogen. However, consistent with the aggregation data, the inhibitory effect of prednisolone on platelet adhesion and spreading was possibly mediated by GCR, at least in part, as the effects were reversed partially in presence of RU486.

3.8 Conclusion

Data presented in this chapter demonstrated that the synthetic glucocorticoid prednisolone, which is popular in the clinic as an anti-inflammatory and immunosuppressive drug possessed anti-platelet attributes as it suppressed key stages of platelet activation like platelet aggregation induced by thrombin as well as platelet adhesion and spreading on immobilised fibrinogen under static conditions. These inhibitory effects were rapid in onset, transient in nature and much weaker compared to physiological platelet inhibitors. A part of these anti-platelet effects were likely to be mediated by the classical GCR.

Chapter 4

Effect of prednisolone on thrombin-induced signalling pathways in platelets

4.1 Introduction

From chapter 3 it was established that prednisolone could suppress thrombin-induced platelet aggregation. Hence it was important to study the underlying biochemical processes following thrombin stimulation to understand the potential molecular targets of prednisolone in platelets. Thrombin stimulates platelets by cleaving the first few N-terminal residues of two platelet transmembrane protease activated receptors PAR1 and PAR4. This event activates these G-protein coupled receptors, which in turn transmit activation signals to platelets via various signalling cascades, which ultimately lead to platelet shape change and dense granule release.

Platelet activation involves a number of morphological changes driven by cytoskeletal rearrangements and are transformed from their quiescent discoid shape to a spheroid by formation of finger-like projections called filopodia and web-like structures in between filopodia called lamellipodia (Hartwig, 1992). This shape change assists the activated platelets to attach and spread on adhesive matrices exposed at the site of vascular injury, which provides an increased surface area on which additional circulating platelets can be recruited for the formation of a blood clot. The contractile protein myosin IIA, which is believed to interact with actin polymers is critical to initiating cytoskeletal rearrangements (Daniel et al., 1984). The regulation of myosin activity is believed to be driven by its post-translational modification, that is, phosphorylation of its light chains on serine 19 and threonine 18 residues, of which the former residue plays a more important role (Getz et al., 2010). Myosin light chain (MLC) phosphorylation is performed by Ca^{+2}/calmodulin-dependent myosin light chain kinase (MLCK) through a Gq-mediated pathway. Another key regulator of myosin phosphorylation is the myosin light chain

phosphatase (MLCP), which dephosphorylates the light chains. Hence for morphological changes to occur, it is important to prevent MLCP from dephosphorylating MLC (Benjamin Z. S. Paul et al., 1999). The activity of MLCP is regulated by a separate Ca^{+2}-independent pathway, which proceeds by activation of the $G\alpha_{12/13}$-coupled receptors. Engagement of these receptors leads RhoA activation whereby the small GTPase RhoA switches from an inactive (GDP-bound) state to an active (GTP-bound) state by the involvement of RhoGEFs. RhoA then activates Rho-kinase (ROCK), which inhibits MLCP activity by phosphorylation of two key residues within its MYPT1 domain (threonines 696 and 853) (Feng et al., 1999; Lincoln, 2007). These phosphorylation events switches MLCP to an inactive state whereby it fails to dephosphorylate MLC. Hence a net increase in MLC phosphorylation can take place, which promotes platelet cytoskeletal rearrangement. As the platelet thrombin receptors (PAR1 and PAR4) are coupled to both $G\alpha_q$ and $G\alpha_{12/13}$, thrombin stimulation of platelets provides a wide platform to study the entire regulation of myosin phosphorylation through the two separate pathways.

Signalling events downstream of $G_{\alpha q}$ activation form the underlying basis of secretion of platelet granular contents and thromboxane A_2 (TxA_2) generation from membrane phospholipids, which in turn results in an irreversible secondary wave of platelet aggregation and stabilisation of thrombus formation. Stimulation of platelets with thrombin and other physiological agonists such as ADP and thromboxane A_2 (TxA_2), which act through $G_{\alpha q}$-coupled receptors, leads to activation of $G_{\alpha q}$, which in turn stimulates the enzyme phospholipase Cβ (PLCβ). The activated enzyme catalyses the formation of second messengers such as inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) following the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), a phospholipid component of the platelet membrane. IP_3 participates in raising platelet cytosolic calcium concentrations by promoting calcium mobilisation from intracellular platelet stores as well as store-operated calcium entry from outside. DAG activates protein kinase C (PKC), which in turn phosphorylates a number of substrates and promotes platelet granule secretion. Thus $G_{\alpha q}$-mediated signalling cascades downstream of

thrombin not only contribute to platelet shape change, but also are crucial primarily for secretion and secretion-dependent platelet aggregation. Thus we decided to investigate the impact of prednisolone on both $G_{\alpha q}$- and $G_{\alpha 13}$-mediated signalling events in platelets downstream of thrombin stimulation.

Hence the biochemical pathways leading to platelet shape change in response to thrombin stimulation were studied and the impact of prednisolone on each of the key regulators was examined.

4.2 Aims

The objective of this chapter were

- to focus on signalling mechanisms, which lead to platelet activation downstream of thrombin stimulation, with a particular emphasis on the $G\alpha_{12/13}$ pathway.
- to investigate whether prednisolone could modulate these signalling pathways.
- to establish whether prednisolone could affect platelet shape change
- to focus on signalling mechanisms in the $G_{\alpha q}$ pathway in platelets following thrombin stimulation.

4.3 Characterisation of myosin light chain phosphorylation in washed platelets in response to thrombin stimulation

In the first instance, the ability of the most potent physiological agonist to induce phosphorylation of the light chains of the platelet contractile protein myosin IIA at the key residue serine 19 (MLC-ser^{19}) was examined. Washed platelets were treated with the calcium chelator EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 μM) in order to eliminate the contribution of platelet aggregation and secretion to the observed effects. In line with earlier reports, thrombin stimulation led to an increase in platelet myosin light chain phosphorylation at serine 19 (MLC-ser^{19}) when compared to unstimulated or basal sample (Figure 4.1). The increase in phospho-MLC-ser^{19} was dependent on the concentration of thrombin used with threshold effect observed at 0.01 U/ml (lowest concentration tested) and maximal effect observed at 0.1 U/ml (Figure 4.1 i).

MLC-ser^{19} phosphorylation in response to thrombin was detectable strongly as early as 15 seconds after thrombin stimulation and reached its peak after 1 minute of stimulation. Phosphorylation started to decline at later time-points tested and was barely detectable after 10 minutes or later (Figure 4.1 ii). Thus, thrombin-induced MLC-ser^{19} phosphorylation was very rapid in occurrence, which is perhaps expected owing to its role in platelet shape change.

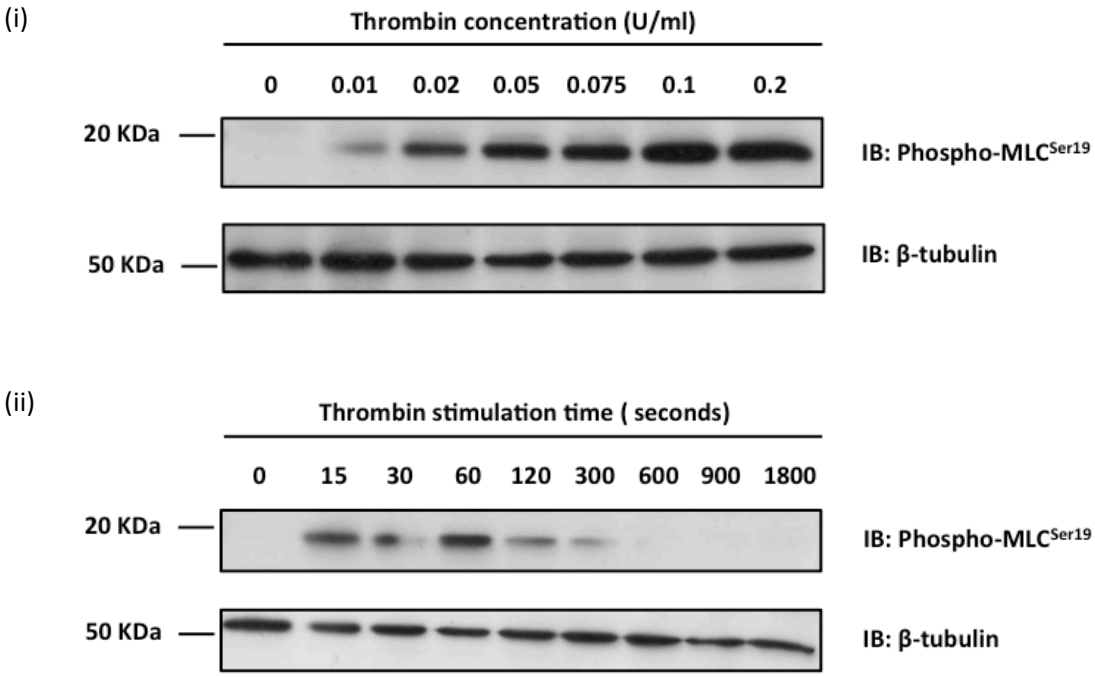


Figure 4.1. Thrombin stimulation resulted an increase of myosin light chain phosphorylation at serine 19 residue (MLC-ser^{19}) in washed platelets in a concentration and time-dependent manner. Washed platelets (3×10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then stimulated with (i) various concentrations of thrombin (0.01-0.2 U/ml) for 1 minute or (ii) 0.05 U/ml thrombin for various time points (15 seconds to 30 minutes) under stirring conditions. Samples were then lysed, resolved by SDS-PAGE and immunoblotted for phospho-MLC-ser^{19}. Membranes were later re-probed for β-tubulin. Immunoblots (i and ii) from a single experiment are shown.

4.4 Effect of prednisolone on thrombin-induced myosin light chain phosphorylation in washed platelets

4.4.1 Prednisolone reduced thrombin-induced myosin light chain phosphorylation in washed platelets

The next objective was to determine the effect of prednisolone on thrombin-induced MLC-ser^{19} phosphorylation. From chapter 3 (Figure 3.1 A), we observed that 0.01 U/ml thrombin could not induce any detectable platelet aggregation. The same thrombin concentration was however capable of inducing modest MLC-ser^{19} phosphorylation (Figure 4.1). The differential efficacy of thrombin to induce platelet aggregation and MLC-ser^{19} phosphorylation at a particular concentration led us to consider the possibility that lower concentrations of thrombin could also induce MLC-ser^{19} phosphorylation. Thus we stimulated platelets with lower thrombin concentrations (0.001-0.01 U/ml). Thrombin was indeed shown to significantly increase MLC-ser^{19} phosphorylation in washed platelets over basal levels at concentrations of 0.002 U/ml and over (Figure 4.2). We then incubated washed platelets with prednisolone (10 μM) for 5 minutes before stimulation with various concentrations of thrombin. Prednisolone reduced MLC-ser^{19} phosphorylation induced by thrombin (0.01 U/ml) ($p=0.01$) (Figure 4.2). In case of MLC-ser^{19} phosphorylation induced by 0.005 U/ml thrombin there was a slight trend in reduction by prednisolone, but the data was non-significant ($p=0.07$). Thus, it was observed that prednisolone could induce a very modest reduction of MLC-ser^{19} phosphorylation induced by thrombin at certain conditions as discussed above.

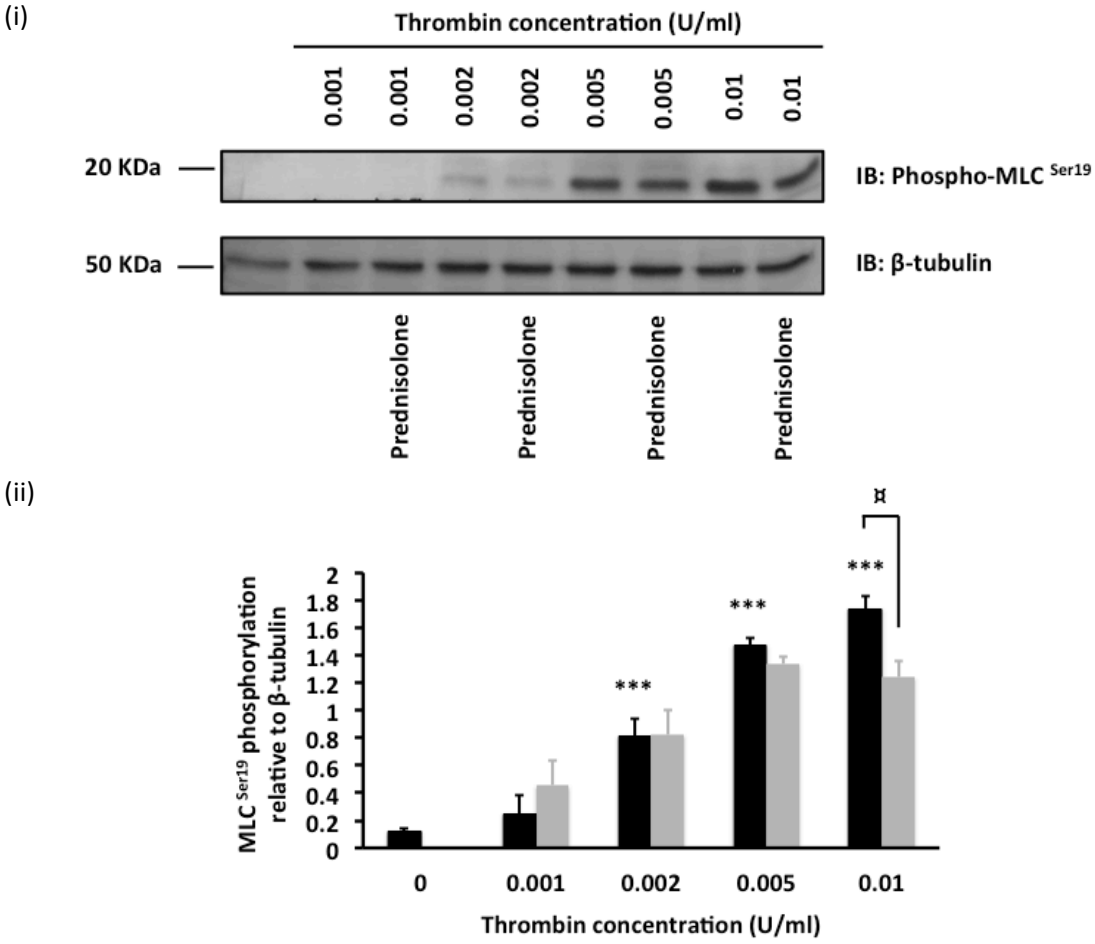


Figure 4.2. Prednisolone reduced thrombin-induced myosin light phosphorylation (MLC-ser^{19}) in washed platelets. Washed platelets (3 × 10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with prednisolone (10 µM) for 5 minutes and stimulated with various concentrations of thrombin (0.001-0.01 U/ml) for 1 minute. Samples were then lysed, resolved by SDS-PAGE and immunoblotted for phospho-MLC-ser^{19}. Membranes were later re-probed for β-tubulin. A representative immunoblot (i) and quantitative analysis (ii) are shown. Data is presented as mean ± SEM, n=3. Black bars represent untreated samples, while grey bars prednisolone-treated samples. *** denotes $p<0.005$ when compared to unstimulated sample while ¤ denotes $p<0.05$-0.01 when compared with thrombin (0.01 U/ml) sample.

4.4.2 Prednisolone reduced thrombin-induced myosin light chain phosphorylation in a time-dependent manner

We next sought to characterise the kinetics of the effects of prednisolone on thrombin-induced MLC-ser^{19} phosphorylation. It was thought to be a logical approach to select a lower concentration of thrombin for future experiments for data consistency throughout the series of experiments to be performed. Keeping that in mind, we chose the concentration of 0.005 U/ml thrombin for subsequent experiments. Washed platelets were treated with prednisolone for various time points (1-30 minutes) and then stimulated with thrombin. We observed a rapid and transient reduction of thrombin-induced MLC-ser^{19} phosphorylation by prednisolone, which was maximal with an incubation time of 1 minute ($p=0.04$), but was not maintained for longer incubation time periods (Figure 4.3, Ai and Aii). This data partially correlated with the rapid and transient reduction of thrombin-induced platelet aggregation by prednisolone (chapter 3, Figure 3.3 A).

4.4.3 Prednisolone reduced thrombin-induced myosin light chain phosphorylation in a concentration-dependent manner

We then incubated washed platelets with various concentrations of prednisolone for one minute as this time point gave maximal inhibition of MLC-ser^{19} phosphorylation (Figure 4.3 A). Prednisolone was shown to reduce thrombin-induced MLC-ser^{19} phosphorylation in a concentration-dependent manner, with threshold effects seen with the lowest concentration (1 nM), although it was non-significant. Significant reduction was observed at concentrations of 1-100 µM ($p=0.02$, 0.002 and 0.0008 for 1, 10 and 100 µM, respectively) (Figure 4.3, Bi and Bii). Although maximal reduction was obtained with 100 µM prednisolone, it was felt that this supraphysiological concentration was unlikely to provide physiologically meaningful results and therefore 10 µM prednisolone, which reduced MLC-ser^{19} phosphorylation substantially, was selected for subsequent experiments. At this concentration, prednisolone did not induce platelet MLC-ser^{19} phosphorylation on its own without thrombin stimulation (Figure 4.3, Bi and Bii).

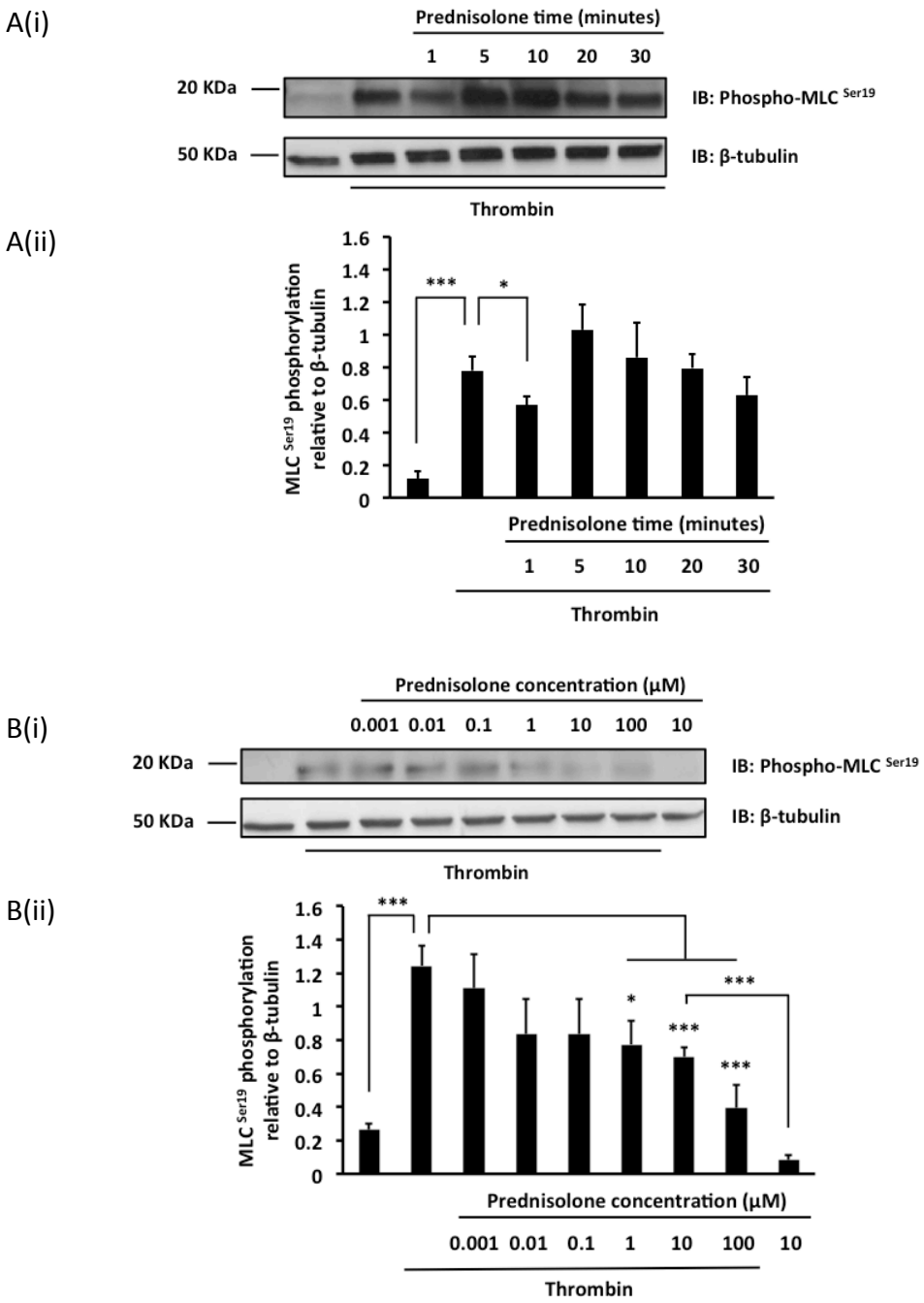
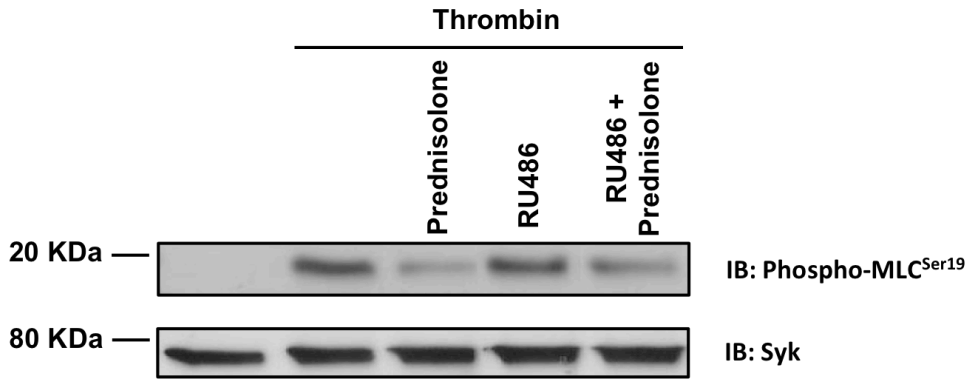


Figure 4.3. Prednisolone reduced thrombin-induced myosin light chain phosphorylation (MLC-ser^{19}) in washed platelets in a time and concentration-dependent manner. Washed platelets (3×10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 μM) for at least 20 minutes before performing further experiments. Platelets were then treated with (A) prednisolone (10 μM) for various time points or (B) various concentrations of prednisolone for 1 minute and stimulated with thrombin (0.005 U/ml) for 1 minute. Samples were then lysed, resolved by SDS-PAGE and immunoblotted for phospho-MLC-ser^{19}. Membranes were later re-probed for β-tubulin. Representative immunoblots (Ai and Bi) and quantitative analyses (Aii and Bii) are shown. Data is presented as mean ± SEM, n=4 (A) and n=5 (B). *** denotes $p<0.005$ and * $p<0.05$-0.01.

4.4.4 Reduction of thrombin-induced MLC-ser^{19} phosphorylation by prednisolone was mediated by glucocorticoid receptor (GCR)

As prednisolone-mediated suppression of thrombin-induced platelet aggregation was found to be mediated by the glucocorticoid receptor (GCR) as documented in chapter 3 (section 3.3.5.3), hence the next question was whether GCR had a similar involvement in mediating the effects of prednisolone on signalling events in platelets following thrombin stimulation. Thus, the GCR antagonist mifepristone (RU486) was employed to address the above possibility. Washed platelets were incubated with RU486 (10 μM) for 5 minutes followed by incubation with prednisolone (10 μM) for 1 minute and thrombin stimulation and MLC-ser^{19} phosphorylation was studied. The conditions applied for RU486 incubation were optimised from platelet aggregometry (chapter 3, section 3.3.5). Thrombin stimulation caused a significant increase in MLC-ser^{19} phosphorylation when compared to basal ($p=0.006$), which was substantially reduced by prednisolone ($p=0.02$) (Figure 4.4, i and ii). RU486 was able to partially reverse the reduction mediated by prednisolone ($p=0.04$) compared to treatment with prednisolone alone (Figure 4.4, i and ii), thus indicating that prednisolone reduced thrombin-induced MLC-ser^{19} phosphorylation in platelets by acting through the GCR. Although RU486 at a concentration of 1 μM could reverse prednisolone-mediated inhibition of thrombin-induced platelet aggregation, it failed to reverse prednisolone-mediated inhibition of thrombin-induced phospho-MLC-ser^{19}, suggesting that different levels of GCR occupancy were implicated in different platelet physiological events.

(i)



(ii)

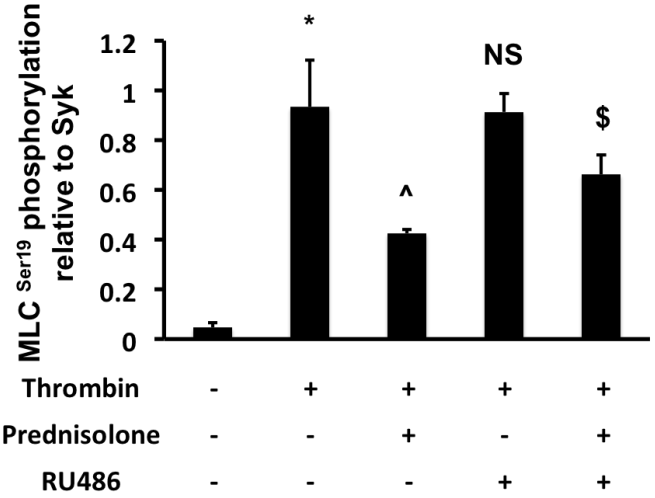


Figure 4.4. Prednisolone-mediated reduction of thrombin-induced MLC-ser^{19} phosphorylation was partially reversed by the GR antagonist RU486. Washed platelets (3 × 10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with RU486 (10 µM) for 5 minutes followed by treatment with prednisolone (10 µM) for 1 minute and thrombin (0.005 U/ml) stimulation for 1 minute. Samples were then lysed, resolved by SDS-PAGE and immunoblotted for phospho-MLC-ser^{19}. Membranes were later re-probed for Syk. Representative immunoblot (i) and quantitative analysis (ii) are shown. Data is presented as mean ± SEM, n=3. ** denotes p<0.01-0.005 and * p<0.05-0.01.

4.5 Prednisolone targeted signalling events mediated by Rho kinase (ROCK) following thrombin stimulation in washed platelets

The observation that prednisolone caused a modest but consistent inhibition of MLC-ser^{19} phosphorylation was followed by investigation of the underlying mechanisms. As described earlier in section 4.1 of this chapter, thrombin stimulation culminates in myosin phosphorylation through a Ca^{+2}-dependent activation of MLCK and a Ca^{+2}-independent activation of RhoA/ROCK-dependent phosphorylation and inactivation of MLCP (Bauer et al., 1999). Thus, the effects of prednisolone on each of these pathways were tested. To achieve this we appointed inhibitors to block each pathway specifically and study the isolated effects from the other pathway. The intracellular Ca^{+2} chelator BAPTA-AM (20 µM) was used to block the Ca^{+2}-dependent pathway allowing the ROCK-mediated pathway only to be active. Likewise, the widely used ROCK inhibitor Y27632 (10 µM) (Ishizaki et al., 2000) was used to block the ROCK-dependent signalling, thereby allowing MLC-ser^{19} phosphorylation to proceed specifically through the Ca^{+2} pathway. Thus the effect of prednisolone on the ROCK-mediated pathway could be studied in isolation in the presence of BAPTA-AM, while that on the Ca^{+2} pathway could be determined specifically in the presence of Y27632. The concentration of these chemicals was determined from earlier studies carried out in the laboratory (Aburima et al., 2013; Wraith et al., 2013).

Thrombin-induced MLC-ser^{19} phosphorylation was significantly reduced to near basal levels by incubation of platelets with Y27632 ($p=0.002$). Incubation with BAPTA-AM also slightly reduced MLC-ser^{19} phosphorylation, albeit the data was not significant ($p=0.09$) (Figure 4.5). Co-incubation with Y27632 and BAPTA-AM completely abolished MLC-ser^{19} phosphorylation ($p=0.001$). Together these data suggest that both pathways are involved in generation of phospho-MLC-ser^{19}, but that RhoA/ROCK play a more substantive role. Prednisolone caused a substantial reduction of thrombin-induced MLC-ser^{19} phosphorylation ($p=0.05$). When prednisolone was combined with Y27632, we could not detect any further reduction of MLC-ser^{19} phosphorylation compared to reduction by Y27632 alone ($p=0.45$). Interestingly however, we observed an additional reduction in MLC-ser^{19}

phosphorylation when prednisolone was co-incubated with BAPTA-AM compared to that seen with BAPTA-AM alone (p=0.05) (Figure 4.5). This implied that in the above conditions, prednisolone seemed to preferentially target ROCK-mediated signalling downstream of thrombin stimulation.

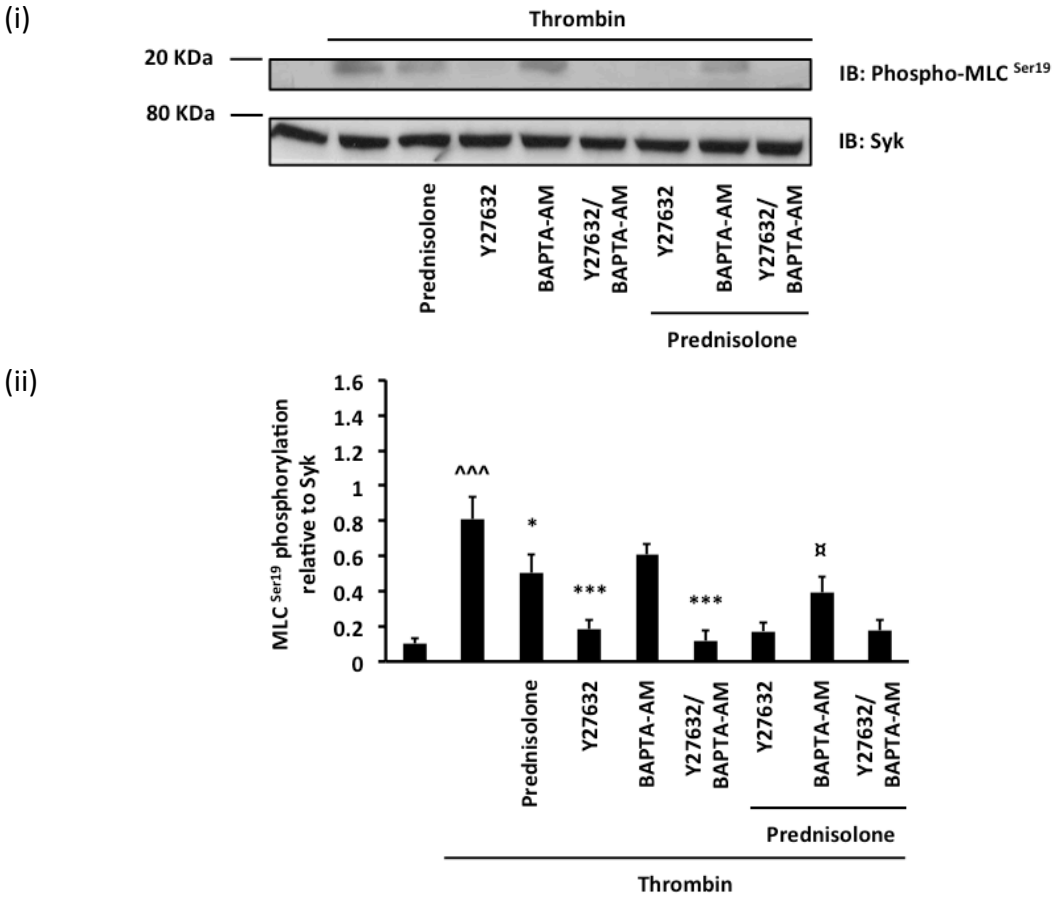
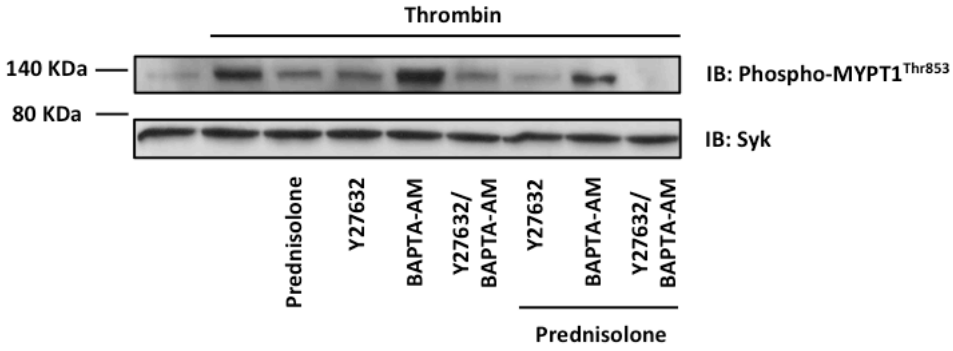


Figure 4.5. Prednisolone reduced thrombin-induced myosin light chain phosphorylation (MLC-ser^{19}) by targeting ROCK-mediated signalling. Washed platelets (3 × 10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with either prednisolone (10 µM) for 1 minute, Y27632 (10 µM) for 20 minutes, or BAPTA-AM (20 µM) for 20 minutes alone or a selected combination of these reagents as indicated above and stimulated with thrombin (0.005 U/ml) for 1 minute. In conditions where prednisolone was applied in the presence of the other inhibitors, platelets were treated first with Y27632 and/or BAPTA-AM followed by addition of prednisolone during the last minute of their incubation and thrombin stimulation. Samples were then lysed, resolved by SDS-PAGE and immunoblotted for phospho-MLC-ser^{19}. Membranes were later re-probed for Syk. Representative immunoblots (i) and quantitative analyses (ii) are shown. Data is presented as mean ± SEM, n=3. ^^^ denotes p<0.005 when compared with unstimulated sample. *** denotes p<0.005 and * p<0.01-0.005 when compared with thrombin-stimulated sample. ¤ denotes p<0.05-0.01 when compared to the sample treated with BAPTA-AM and stimulated with thrombin.

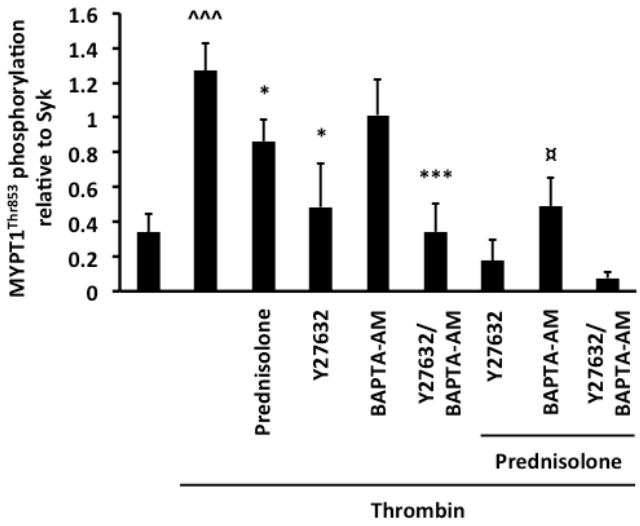
4.6 Effect of prednisolone on thrombin-induced phosphorylation of myosin light chain phosphatase (MLCP) in washed platelets

Phosphorylation of MLCP at two key threonine residues within its targeting subunit MYPT1, thr^{696} and thr^{853} occurs downstream of ROCK activation and inactivates the MLCP, which results in the net increase of MLC-ser^{19} phosphorylation. We next used the same approach to examine the effects prednisolone on the RhoA/ROCK pathway. In the first instance the phosphorylation of MLCP was used as a downstream marker of RhoA activity. Thrombin stimulation was shown to encourage phosphorylation of MLCP at both thr^{696} and thr^{853} ($p=0.003$ for MYPT1-thr^{696} and 0.001 for MYPT1-thr^{853}) (Figure 4.6 A and B). This data correlated to the increase in thrombin-induced phospho-MLC-ser^{19}. The ROCK inhibitor Y27632 reduced thrombin-induced MLCP phosphorylation at both the threonine residues to near basal levels ($p=0.002$ for MYPT1-thr^{696} and 0.02 for MYPT1-thr^{853}), while the calcium chelator BAPTA-AM had no detectable effect ($p=0.35$ for MYPT1-thr^{696} and 0.18 for MYPT1-thr^{853}) showing that MLCP phosphorylation was entirely a RhoA/ROCK-mediated event. Prednisolone was able to reduce thrombin-induced MLCP phosphorylation at both thr^{696} and thr^{853} ($p=0.05$ for MYPT1-thr^{696} and 0.04 for MYPT1-thr^{853}). Thus it was confirmed that prednisolone targeted ROCK-mediated signalling, as its immediate downstream events were also suppressed. Thus we decided to investigate and characterise further the regulation of MLCP phosphorylation at the two above residues.

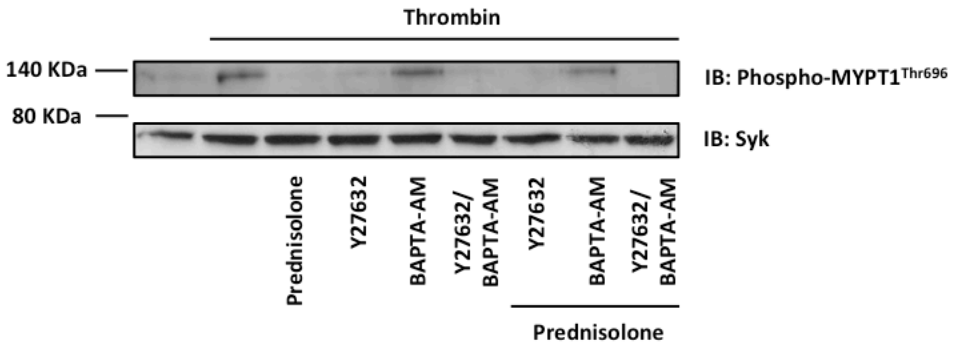
A(i)


Thrombin
140 KDa
80 KDa
IB: Phospho-MYPT1Thr853
IB: Syk
Prednisolone
Y27632
BAPTA-AM
Y27632/ BAPTA-AM
Y27632
BAPTA-AM
Y27632/ BAPTA-AM
Prednisolone

A(ii)


MYPT1Thr853 phosphorylation relative to Syk
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
^^^
*
*
***
¤
Prednisolone
Y27632
BAPTA-AM
Y27632/ BAPTA-AM
Y27632
BAPTA-AM
Y27632/ BAPTA-AM
Prednisolone
Thrombin

B(i)


Thrombin
140 KDa
80 KDa
IB: Phospho-MYPT1Thr696
IB: Syk
Prednisolone
Y27632
BAPTA-AM
Y27632/ BAPTA-AM
Y27632
BAPTA-AM
Y27632/ BAPTA-AM
Prednisolone

B(ii)

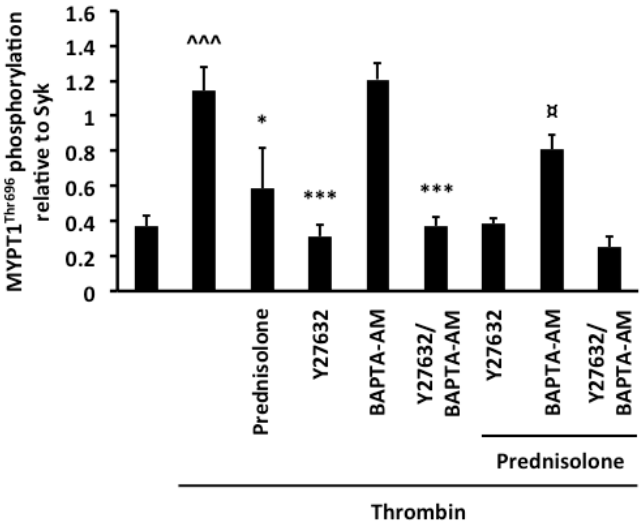

MYPT1Thr696 phosphorylation relative to Syk
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
^^^
*
***
***
¤
Prednisolone
Y27632
BAPTA-AM
Y27632/ BAPTA-AM
Y27632
BAPTA-AM
Y27632/ BAPTA-AM
Prednisolone
Thrombin

Figure 4.6 Prednisolone reduced thrombin-induced phosphorylation of myosin light chain phosphatase (MLC-ser^{19}) by targeting ROCK-mediated signalling. Washed platelets (3 × 10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with either prednisolone (10 µM) for 1 minute, Y27632 (10 µM) for 20 minutes, or BAPTA-AM (20 µM) for 20 minutes alone or a selected combination of these reagents as indicated above and stimulated with thrombin (0.005 U/ml) for 1 minute. In conditions where prednisolone was applied in the presence of the other inhibitors, platelets were treated first with Y27632 and/or BAPTA-AM followed by addition of prednisolone during the last minute of their incubation and thrombin stimulation. Samples were then lysed, resolved by SDS-PAGE and immunoblotted for phospho- MYPT1-thr^{696} and phospho-MYPT1-thr^{853}. Membranes were later re-probed for Syk. Representative immunoblots (Ai and Bi) and quantitative analyses (Aii and Bii) are shown. Data is presented as mean ± SEM, n=3. ^^^ denotes p<0.005 when compared with unstimulated sample. *** denotes p<0.005 and * p<0.01-0.005 when compared with thrombin-stimulated sample. ¤ denotes p<0.05-0.01 when compared to the sample treated with BAPTA-AM and stimulated with thrombin.

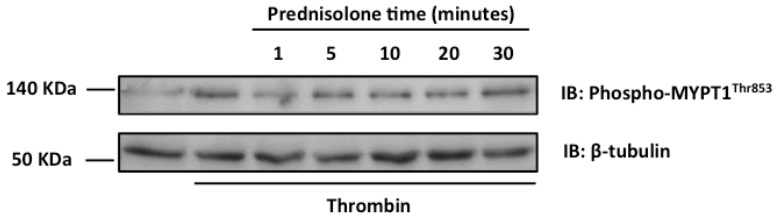
4.6.1 Prednisolone reduced thrombin-induced phosphorylation of myosin light chain phosphatase in a time-dependent manner

We chose platelet lysates that were prepared during the study of MLC-ser^{19} phosphorylation to look for changes in the phosphorylation status at these two residues following prednisolone incubation for various time points. Thrombin stimulation of platelets caused a significant increase of phosphorylation of MLCP at both the residues (p=0.03 and 0.002 for MYPT1-thr^{696} and MYPT1-thr^{853}, respectively). A reduction in phosphorylation was observed at both the residues by prednisolone treatment, the kinetics of which were similar to that of MLC-ser^{19} phosphorylation. Thus, maximal reduction of phosphorylation of MLCP at both the residues was observed with one minute of incubation, although the effects were significant only for MYPT1-thr^{853} phosphorylation (p=0.008). Prednisolone-mediated reduction of MLCP phosphorylation was lost completely with longer prednisolone incubation time points (Figure 4.7, A and C). These data again indicate the rapid and transient nature of prednisolone-mediated effects of platelets.

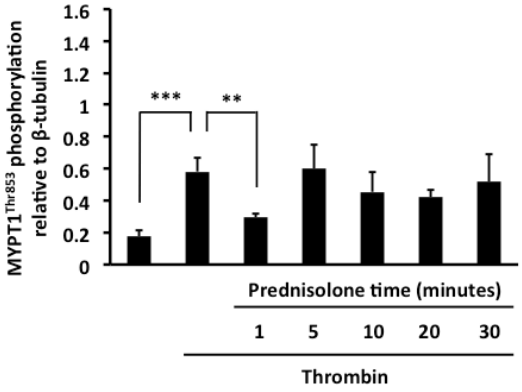
4.6.2 Prednisolone reduced thrombin-induced phosphorylation of myosin light chain phosphatase in a concentration-dependent manner

We then chose platelet lysates that were prepared by incubation with various concentrations of prednisolone for 1 minute prior to thrombin stimulation and looked into the phosphorylation status of MLCP at the threonine residues (696 and 853). Threshold reduction of thrombin-induced phosphorylation at both the residues were observed with prednisolone concentration of 1 nM (p=0.14 and 0.04 for MYPT1-thr^{696} and MYPT1-thr^{853}, respectively), while maximal reduction was observed with prednisolone concentrations of 10 µM for MYPT1-thr^{696} (p=0.009) and 100 µM for MYPT1-thr^{853} (p=0.002). Nevertheless, prednisolone at 10 µM could substantially reduce MLCP phosphorylation at MYPT1-thr^{853} also (p=0.005) (Figure 4.7, B and D). Thus prednisolone incubation conditions, which were optimised to give maximal reduction of thrombin-induced MLC-ser^{19} phosphorylation, also reduced MLCP phosphorylation at the two key threonine residues within its MYPT1 domain with comparable efficiency.

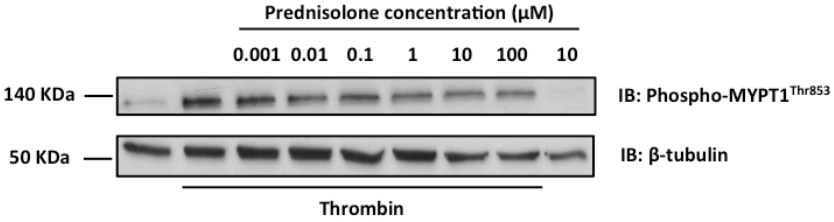
A(i)


Prednisolone time (minutes)
1 5 10 20 30
140 KDa
IB: Phospho-MYPT1Thr853
50 KDa
IB: β-tubulin
Thrombin

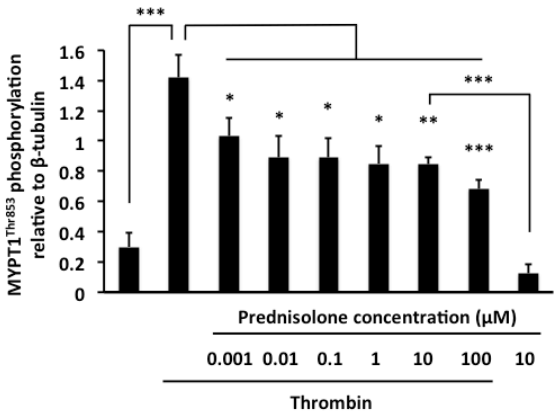
A(ii)


MYPT1Thr853 phosphorylation relative to β-tubulin
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
**
Prednisolone time (minutes)
1 5 10 20 30
Thrombin

B(i)


Prednisolone concentration (μM)
0.001 0.01 0.1 1 10 100 10
140 KDa
IB: Phospho-MYPT1Thr853
50 KDa
IB: β-tubulin
Thrombin

B(ii)


MYPT1Thr853 phosphorylation relative to β-tubulin
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
*
*
*
*
**
***
***
Prednisolone concentration (μM)
0.001 0.01 0.1 1 10 100 10
Thrombin

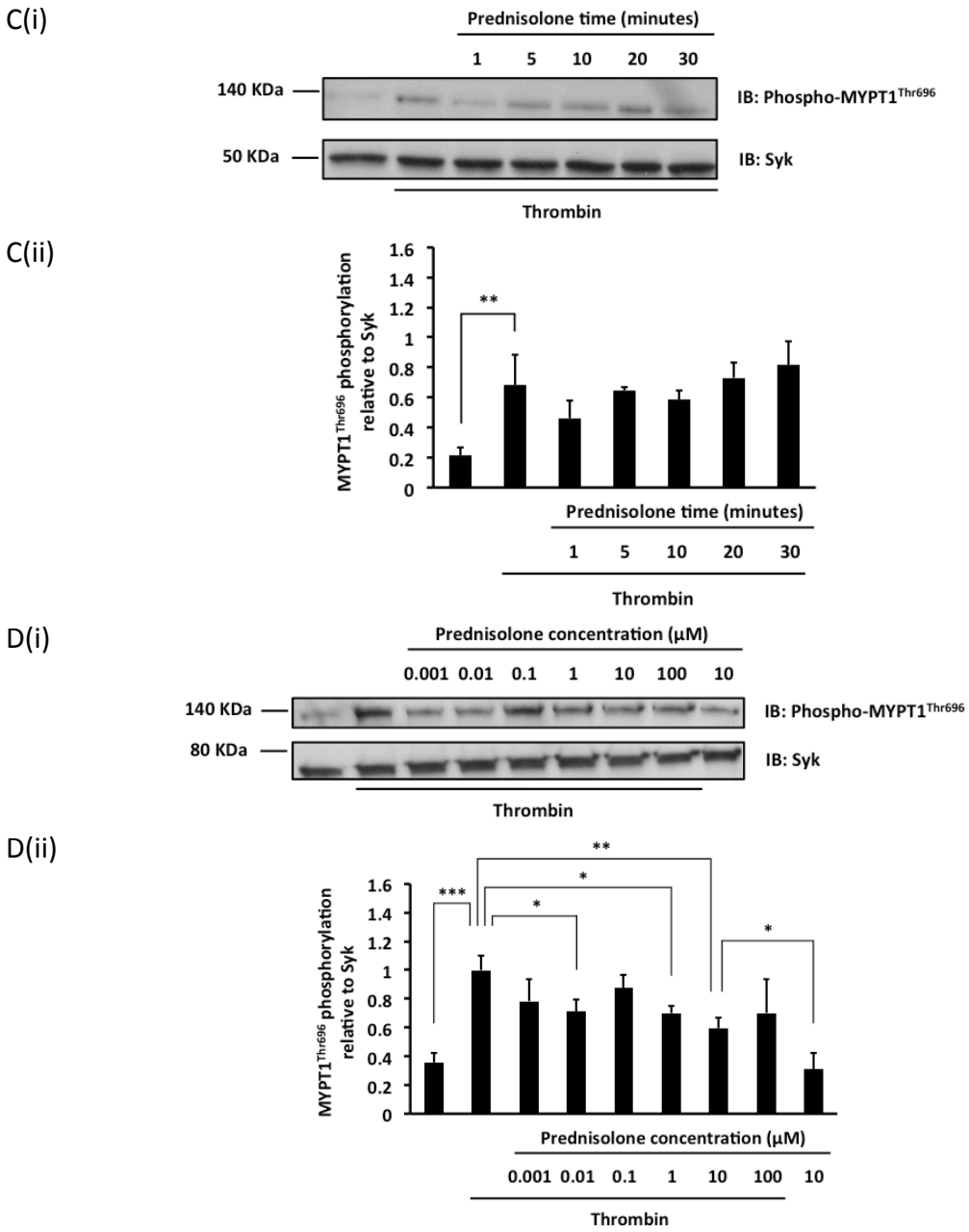


Figure 4.7. Prednisolone reduced thrombin-induced phosphorylation of myosin light chain phosphatase (MYPT1-thr^{696} and MYPT1-thr^{853}) in washed platelets in a time and concentration-dependent manner. Washed platelets (3 × 10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 μM) for at least 20 minutes before performing further experiments. Platelets were then treated with (A) prednisolone (10 μM) for various time points or (B) various concentrations of prednisolone for 1 minute and stimulated with thrombin (0.005 U/ml) for 1 minute. Samples were then lysed, resolved by SDS-PAGE and immunoblotted for phospho-MYPT1-thr^{696} and phospho-MYPT1-thr^{853}. Membranes were later re-probed for β-tubulin or Syk. Representative immunoblots (Ai, Bi, Ci and Di) and quantitative analyses (Aii, Bii, Cii and Dii) are shown. Data is presented as mean ± SEM, n=3. *** denotes p<0.005, ** p<0.01-0.005 and * p<0.05-0.01.

4.6.3 Reversal of prednisolone-mediated reduction of thrombin-induced MLCP phosphorylation by RU486

It was then questioned whether the observed reduction of thrombin-induced MLCP phosphorylation by prednisolone was mediated by GCR, as was in the case of MLC-ser^{19} phosphorylation. Thus platelet lysates, prepared by incubation with RU486 followed by prednisolone and thrombin stimulation (section 4.4.4), were prepared and MLCP phosphorylation at the two key residues (threonine 696 and 853) examined. Thrombin stimulation caused a significant increase of MLCP phosphorylation at both these residues ($p=0.001$ and 0.01 for MYPT1-thr^{696} and MYPT1-thr^{853}, respectively) relative to basal levels. Prednisolone caused a significant reduction of phosphorylation at these residues ($p=0.003$ and 0.01 for MYPT1-thr^{696} and MYPT1-thr^{853}, respectively), which was reversed partially by RU486 ($p=0.04$ and 0.004 for MYPT1-thr^{696} and MYPT1-thr^{853}, respectively). This data suggested that prednisolone exerted its inhibitory effects of MLCP phosphorylation by acting through the GCR (Figure 4.8, A and B).

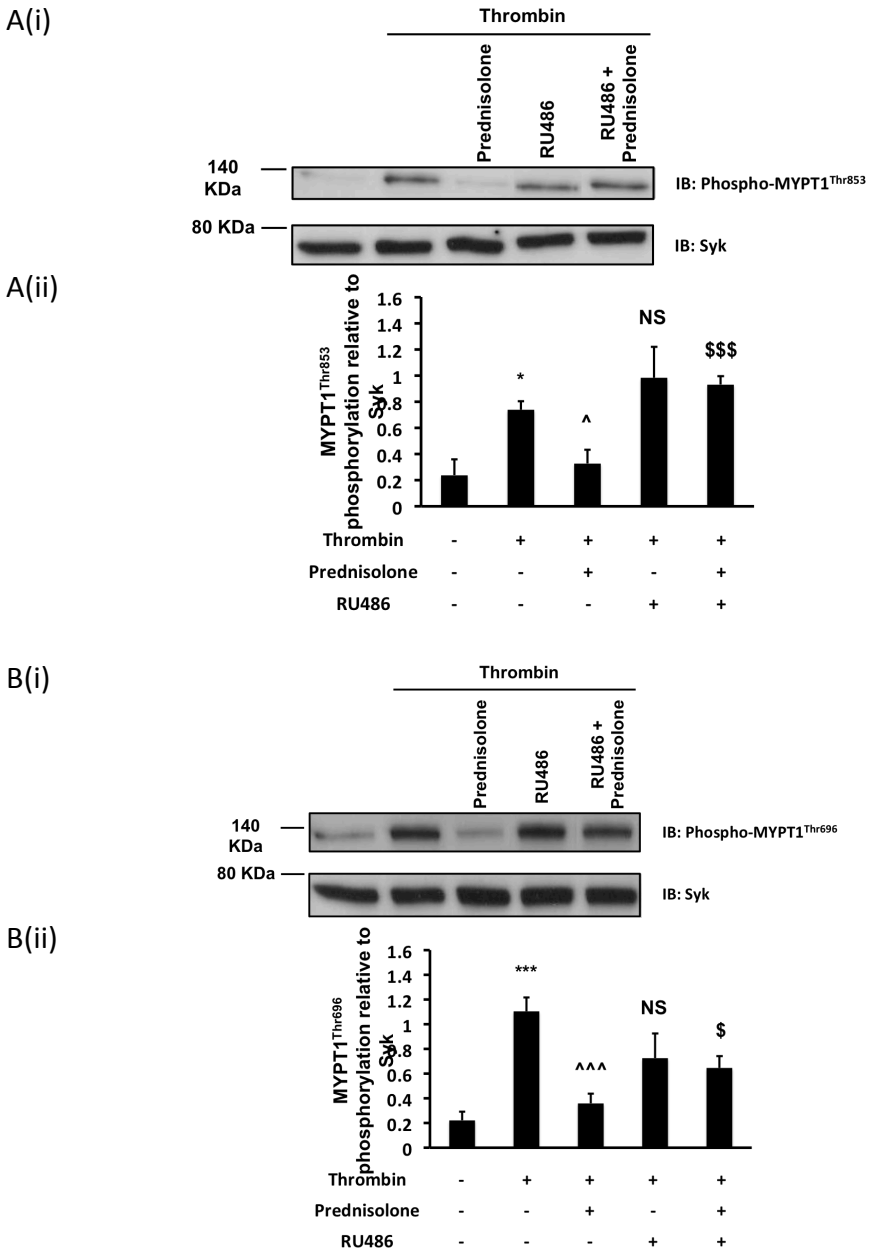


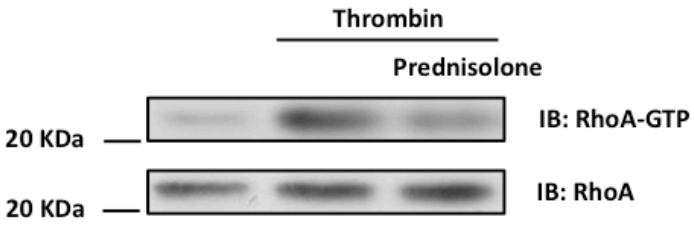
Figure 4.8. Prednisolone-mediated reduction of thrombin-induced phosphorylation of MYPT1-thr^{696} and MYPT1-thr^{853} were partially reversed by the GR antagonist RU486. Washed platelets (3×10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with RU486 (10 µM) for 5 minutes followed by treatment with prednisolone (10 µM) for 1 minute and thrombin (0.005 U/ml) stimulation for 1 minute. Samples were then lysed, resolved by SDS-PAGE and immunoblotted for phospho-MYPT1-thr^{853} (A) and phospho-MYPT1-thr^{696} (B). Membranes were later re-probed for Syk. Representative immunoblots (Ai and Bi) and quantitative analyses (Aii, and Bii) are shown. Data is presented as mean ± SEM, n=3. *** denotes p<0.005, ** p<0.01-0.005 and * p<0.05-0.01.

4.7 Prednisolone reduced thrombin-induced activation of RhoA in washed platelets

The next objective was to determine if prednisolone could target signalling events upstream of ROCK activation. Hence, we decided to investigate the activation of the small GTPase RhoA in washed platelets following thrombin stimulation and activation of the $G_{12/13}$ protein-coupled PARs. This was achieved by performing a pull-down assay in which platelet lysates, prepared by incubation with prednisolone (10 μM) for 1 minute followed by thrombin stimulation for another minute, were incubated with Rhotekin-RBD conjugated glutathione sepharose beads. RBD domain of the RhoA effector protein Rhotekin is believed to possess high affinity for active RhoA and thus these beads should precipitate and isolate the active RhoA-GTP fraction (Ren, 1999). Following thrombin (0.005 U/ml) stimulation, a significant increase of RhoA-GTP in platelets was observed ($p=0.03$), which was reduced significantly by prednisolone ($p=0.04$) (Figure 4.9, i and ii).

Thus, prednisolone was shown to inhibit RhoA activation, the very upstream signalling event of the RhoA/ROCK pathway, which probably accounted for the suppression of the downstream events of the pathway such as ROCK-mediated MLCP phosphorylation and inactivation and myosin phosphorylation.

(i)



(ii)

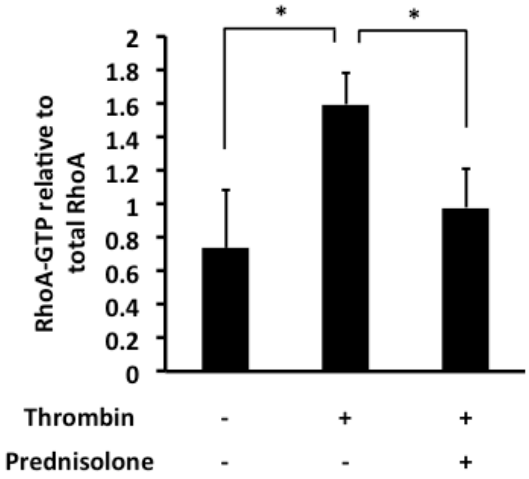
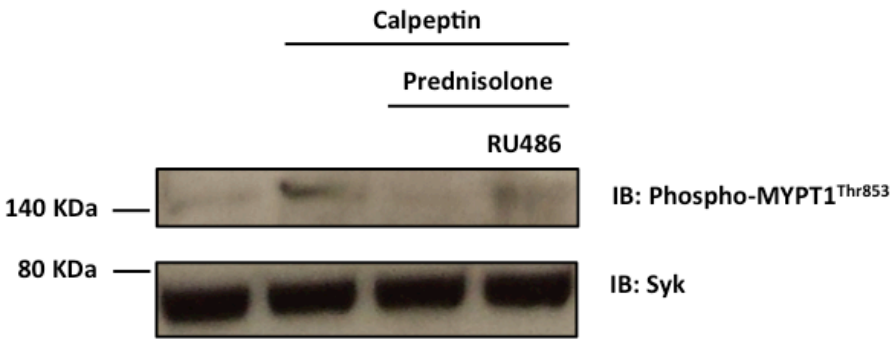


Figure 4.9. Prednisolone reduced thrombin-induced RhoA activation in washed platelets. Washed platelets (5 × 10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with prednisolone (10 µM) for 1 minute and stimulated with thrombin (0.005 U/ml) for 1 minute. Samples were then lysed and the RhoA-GTP (active RhoA) fraction was precipitated using Rhotekin-RBD bound glutathione-sepharose beads. Active RhoA was then resolved by SDS-PAGE and immunoblotted for RhoA. A small amount of the samples were resolved and immunoblotted for RhoA to determine total RhoA levels. Representative immunoblots (i) and quantitative analyses (ii) are shown. Data is presented as mean ± SEM, n=3. * denotes $p<0.05$-0.01.

4.8 Inhibitory effects of prednisolone on the RhoA/ROCK-mediated signalling were maintained independent of $G\alpha_{12/13}$ activation

It was possible that the inhibitory effects of prednisolone on the RhoA/ROCK signalling pathway downstream of thrombin stimulation might arise from direct inhibition of the $G\alpha_{12/13}$ protein. To investigate whether prednisolone targeted the RhoA/ROCK pathway directly independent of targeting $G\alpha_{12/13}$-receptor activation, we used a specific and direct RhoA activator, Calpeptin (Schoenwaelder and Burridge, 1999) to stimulate signalling events downstream of RhoA activation. Stimulation of washed platelets with calpeptin (10 μM) for 15 minutes caused a significant increase of MYPT1-thr^{853} phosphorylation (p=0.04). Calpeptin-induced phosphorylation was reduced significantly by prednisolone (10 μM) (p=0.05). The ability of prednisolone to inhibit phospho-MYPT1-thr^{853} was reversed partially by the GCR antagonist RU486 (10 μM) (p=0.03) (Figure 4.10, i and ii). Hence the data revealed that prednisolone could indeed target RhoA activation independent of its upstream regulator and hence contribute to the inhibition of the RhoA/ROCK-mediated signalling events in platelets. It also confirmed that these effects of prednisolone were mediated by signalling events requiring the GCR.

(i)



(ii)

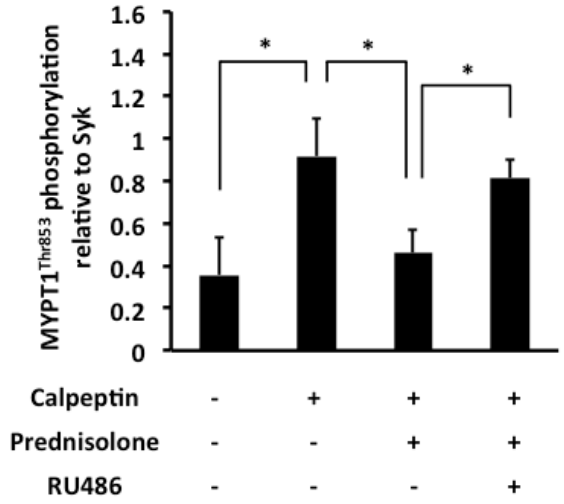


Figure 4.10. Prednisolone reduced phosphorylation of myosin light chain phosphatase (MYPT1^{Thr853}) in washed platelets induced by the RhoA activator calpeptin in a GR-mediated manner. Washed platelets (3×10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with prednisolone (10 µM) for 1 minute and stimulated with calpeptin (10 µM) for 15 minutes. In conditions where RU486 was used, platelets were treated first with RU486 (10 µM) for 5 minutes and then with prednisolone followed by calpeptin stimulation as indicated above. Samples were then lysed, resolved by SDS-PAGE and immunoblotted for phospho-MYPT1^{Thr853}. Membranes were later re-probed for Syk. Representative immunoblots (i) and quantitative analyses (ii) are shown. Data is presented as mean ± SEM, n=3. * denotes $p<0.05$-0.01.

4.9 Effect of prednisolone on platelet shape change

Since prednisolone was shown to substantially reduce MLC-ser^{19} phosphorylation in washed platelets and as phosphorylation of myosin is believed to assist in platelet shape change, hence it was important to study the effect of prednisolone on platelet shape change. For this, we appointed the direct RhoA activator calpeptin in order to investigate RhoA/ROCK-mediated platelet shape change in isolation. We purposely excluded thrombin in this experiment as it promotes shape change through the activation of the Ca^{+2}-dependent MLCK also besides the stimulation of RhoA/ROCK-mediated signalling pathways.

Calpeptin (50 µM and 100 µM) induced shape change in washed platelets as characterised by an initial bulge in the trace that appeared more distinct upon stimulation with higher calpeptin concentration (Figure 4.11 i and ii, indicated by a black arrowhead). Prednisolone (10 µM, 1 minute incubation) could indeed abolish shape change induced by both calpeptin concentrations (Figure 4.11, i and ii). Thus, prednisolone could block platelet shape change downstream of RhoA activation.

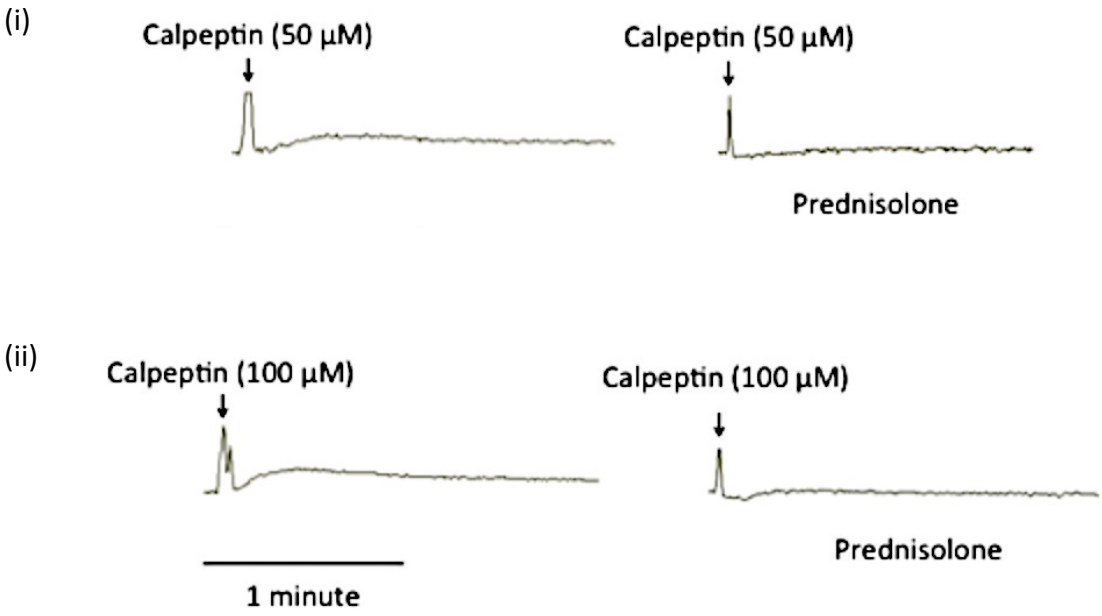


Figure 4.11. Effect of prednisolone on platelet shape change. Washed platelets (2.5×10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with prednisolone (10 µM) for 1 minute and stimulated with increasing concentrations of calpeptin (50-100 µM). Shape change responses were recorded for 2 minutes. Representative shape change traces from three independent experiments are shown.

4.10 Characterisation of PKC activation in washed platelets in response to thrombin stimulation and effect of prednisolone

Platelets are known to express a number of protein kinase C (PKC) isoforms. Thrombin stimulation of platelets has been shown to activate PKC isoforms, which in turn phosphorylate several protein substrates. Hence, the ability of PKC to phosphorylate its substrate proteins in platelets in response to thrombin stimulation was studied. Platelet samples prepared during the study of RhoA-ROCK-mediated signalling events were subjected to western blotting using a polyclonal antibody which can specifically recognise substrate proteins that are phosphorylated at serine residues by PKC, referred to as phospho-PKC substrate antibody. Samples were pre-incubated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) in order to eliminate the contribution of secretory mediators and platelet aggregation to the observed effects and stimulated with various concentrations of thrombin. As shown in Figure 4.12, thrombin stimulation caused a concentration-dependent increase in phosphorylation of a number of PKC-phosphorylated platelet proteins (indicated by small arrowheads) when compared to unstimulated or basal sample. Threshold increase in phosphorylation was observed at 0.005 U/ml (lane 6) and maximal effect observed at 0.01 U/ml (lane 8). Lower thrombin concentrations (0.001 and 0.002 U/ml) were ineffective to increase phosphorylation over basal levels for most of these proteins (Figure 4.12, lanes 2 and 4). Thus, thrombin led to a concentration-dependent activation of PKC in platelets.

The effect of prednisolone on PKC activation was then studied. When pre-incubated for 5 minutes before thrombin stimulation, prednisolone (10 µM) was able to reduce thrombin-induced phosphorylation of some of the PKC substrate proteins (approximately 100, 70, 47, 36 and 27 KDa) and completely abolish phosphorylation of the others (approximately 140, 120, 55, 40 and 25 KDa) indicating that it reduced PKC activity. Similar extent of prednisolone-mediated reduction of phosphorylation of the PKC phosphorylated proteins was observed when platelets were stimulated with either 0.005 U/ml (lane 7 compared with lane 6) or 0.01 U/ml thrombin (lane 9

compared with lane 8). The reduction of phosphorylation of some proteins (36 and 27 KDa) was however more dramatic when stimulated with 0.01 U/ml thrombin.

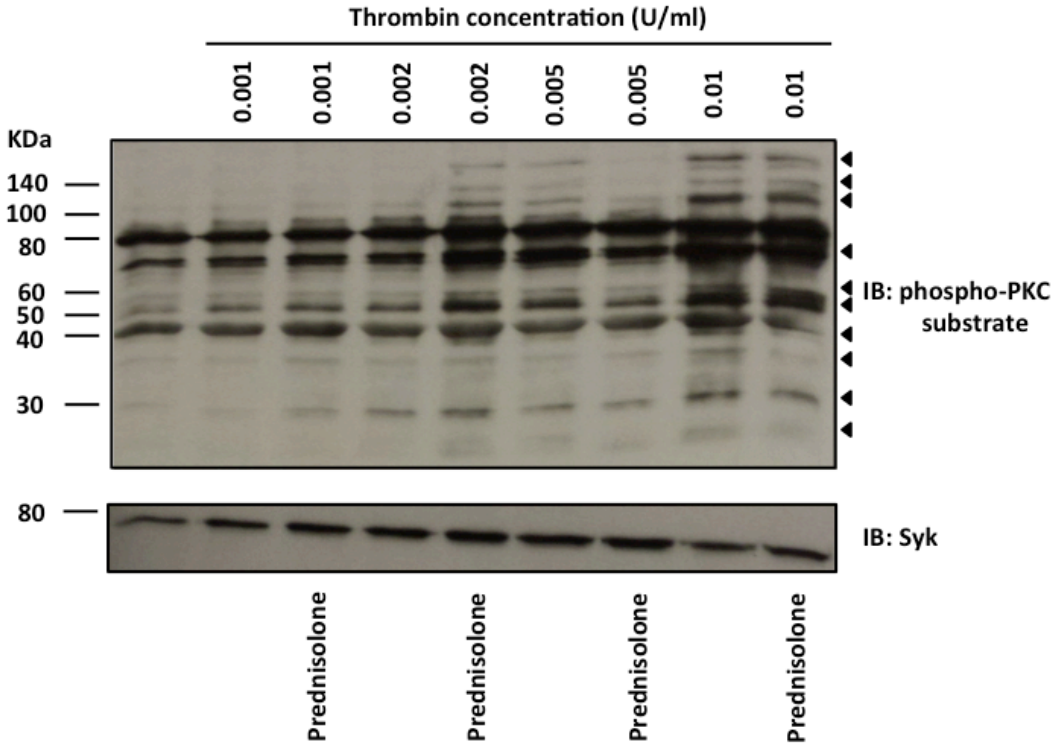


Figure 4.12. Prednisolone reduced thrombin-induced phosphorylation of PKC substrate proteins in washed platelets. Washed platelets (3 × 10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with prednisolone (10 µM) for 5 minutes and stimulated with various concentrations of thrombin (0.001-0.01 U/ml) for 1 minute. Samples were lysed, resolved by SDS-PAGE and immunoblotted with rabbit polyclonal phospho-(Ser) PKC substrate antibody. Membranes were later re-probed for Syk. Representative immunoblot from four independent experiments is shown. Small arrowheads indicate protein bands whose phosphorylation levels got enhanced with thrombin stimulation.

4.11 Characterisation of the effect of prednisolone on thrombin-induced PKC activation in washed platelets

4.11.1 Prednisolone reduced thrombin-induced PKC activation in a time-dependent manner

We next sought to characterise the kinetics of prednisolone-mediated effects on phosphorylation of PKC substrates proteins in response to thrombin stimulation. Platelet samples prepared during the study of RhoA/ROCK-mediated signalling events (Chapter 4, section 4.4.2) were analysed by western blotting using the phospho-PKC substrate antibody. A rapid and transient reduction of thrombin-induced phosphorylation of some PKC substrate proteins by prednisolone was observed, which was maximal with an incubation time of one minute, but was lost with longer incubation time periods (Figure 4.13 A). The reduction observed was however modest in comparison to that observed in the RhoA/ROCK-mediated signalling cascades (chapter 4). The data again highlighted the rapid and transient nature of prednisolone-mediated effects on platelet function and signalling events downstream of thrombin stimulation in line with the findings in chapter 3 and 4.

4.11.2 Prednisolone reduced thrombin-induced PKC activation in a concentration-dependent manner

Platelet samples incubated with various concentrations of prednisolone for one minute and stimulated with thrombin were next analysed to look for phosphorylation of PKC substrate proteins. Prednisolone was again shown to modestly reduce thrombin-induced phosphorylation of PKC substrates in a concentration-dependent manner with threshold effects seen with 1 μM prednisolone and maximal reduction obtained with either 10 or 100 μM prednisolone (Figure 4.13 B), depending on the donor. Prednisolone did not appear to induce phosphorylation of the PKC substrate proteins, as phosphorylation levels in samples treated with prednisolone (10 μM) on its own were similar to that in basal samples (Figure 4.13 B). Thus prednisolone reduced thrombin-induced PKC activity in a concentration-dependent manner, without affecting PKC activity on its own.

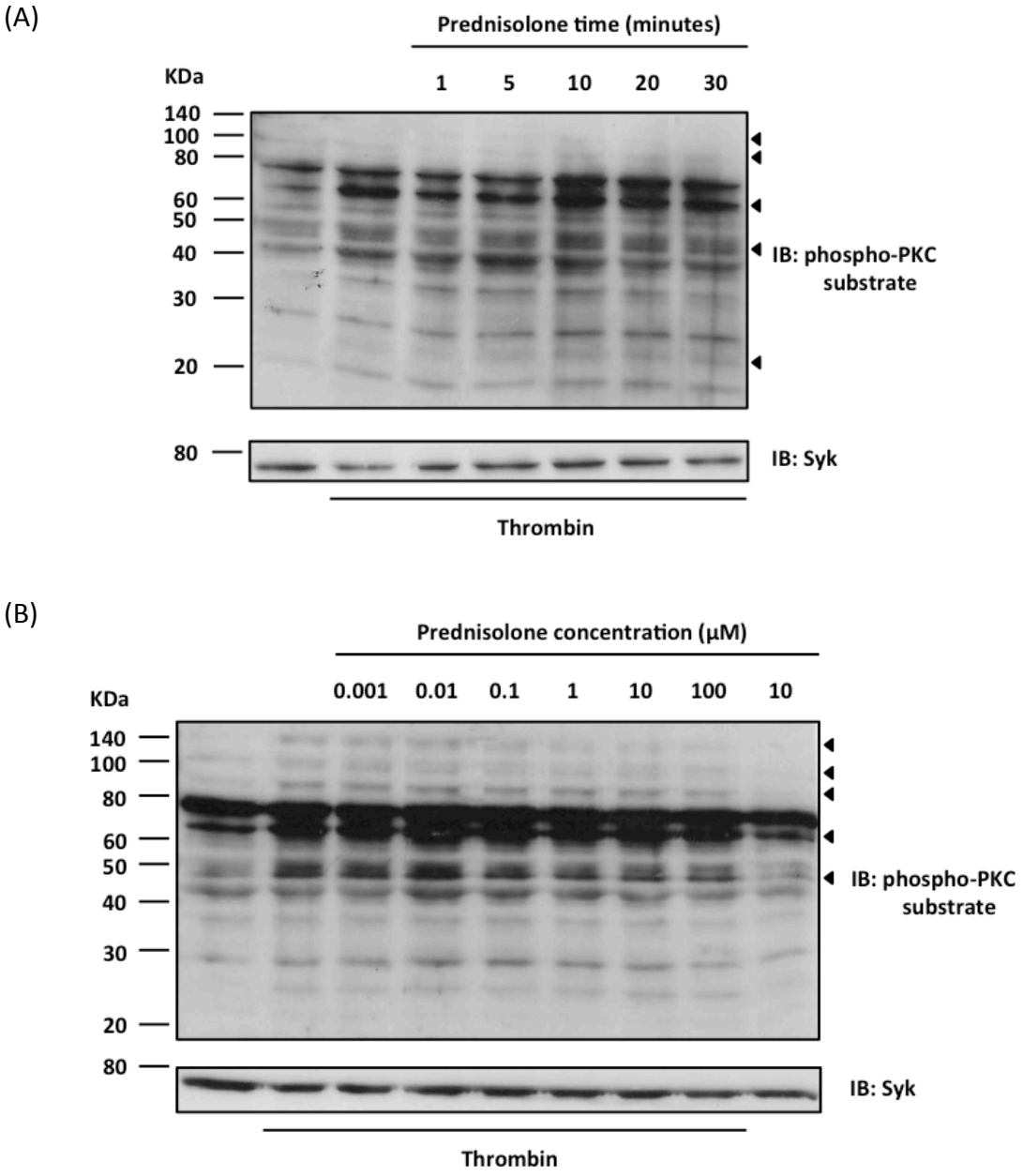


Figure 4.13. Prednisolone reduced thrombin-induced phosphorylation of PKC substrate proteins in washed platelets in a time and concentration-dependent manner. Washed platelets (3×10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with (A) prednisolone (10 µM) for various time points or (B) various concentrations of prednisolone for 1 minute and stimulated with thrombin (0.005 U/ml) for 1 minute. Samples were lysed, resolved by SDS-PAGE and immunoblotted with rabbit polyclonal phospho-(Ser) PKC substrate antibody. Membranes were later re-probed for Syk. Representative immunoblots (A and B) from two independent experiments are shown. Small arrowheads indicate protein bands whose phosphorylation levels were reduced by prednisolone.

4.12 Characterisation of ERK2-MAPK signalling in washed platelets in response to thrombin stimulation and effect of prednisolone

Different groups of mitogen-activated protein kinases (MAPK), such as the extracellular signal-regulated kinases (ERK1 or p44 and ERK2 or p42), p38 MAPK ($p38^{MAPK}$) and c-Jun N-terminal kinases (JNK-1) have known to be expressed in human platelets. Moreover, thrombin stimulation of platelets has been shown to activate ERK2 (Papkoff et al., 1994), $p38^{MAPK}$ (Kramer et al., 1995) and JNK-1 (Bugaud et al., 1999). Hence, the effect of prednisolone on thrombin-induced MAPK activation, particularly ERK2 in washed platelets was investigated. The ability of thrombin to activate ERK2 was first confirmed by looking into changes in ERK2 phosphorylation following thrombin stimulation of platelets. Platelet samples used for the study of thrombin-induced PKC activation were analysed by western blotting using an antibody that selectively recognised phosphorylated forms of ERK1/2 (phospho-ERK1/2 antibody). Thrombin stimulation was shown to cause an increase in phosphorylation of ERK2 or p42 (indicated by arrowhead) over basal condition (Figure 4.14) in agreement with previous findings, albeit the increase was modest. The increase in ERK2 phosphorylation was observed with the lowest thrombin concentration of 0.001 U/ml (lane 2) and appeared to remain constant throughout the higher thrombin concentrations tested (lanes 4, 6 and 8).

Prednisolone (10 μM) was shown to reduce ERK2 phosphorylation in platelets induced by 0.01 U/ml thrombin (lane 9 compared with lane 8) to basal levels (Figure 4.14). When platelets were stimulated with 0.005 U/ml thrombin, prednisolone caused a slight reduction in ERK2 phosphorylation (lane 7 compared with lane 6). Prednisolone however failed to affect ERK2 phosphorylation induced by lower thrombin concentrations.

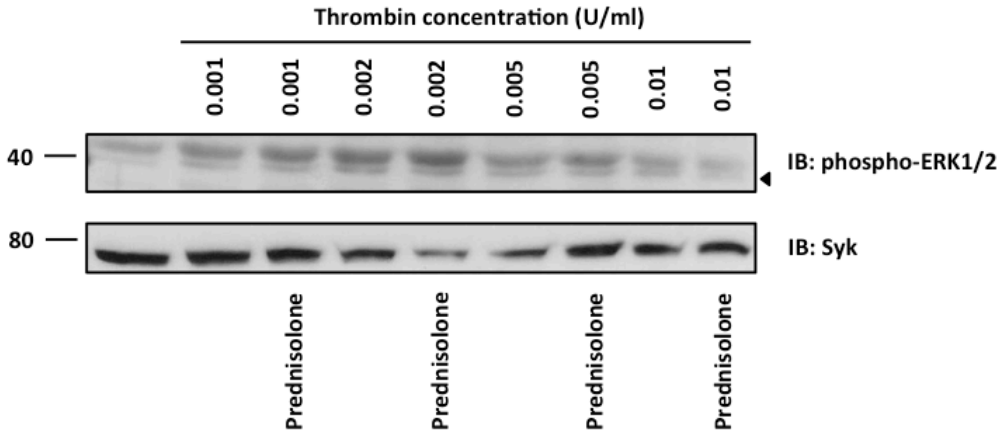


Figure 4.14. Prednisolone reduced thrombin-induced ERK2 phosphorylation in washed platelets. Washed platelets (3×10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 µM) for at least 20 minutes before performing further experiments. Platelets were then treated with prednisolone (10 µM) for 5 minutes and stimulated with various concentrations of thrombin (0.001-0.01 U/ml) for 1 minute. Samples were lysed, resolved by SDS-PAGE and immunoblotted for phospho-ERK1/2. Membranes were later re-probed for Syk. Representative immunoblot from four independent experiments is shown. Small arrowhead indicates phospho-ERK2 or p42.

4.13 Characterisation of the effect of prednisolone on thrombin-induced ERK2 phosphorylation in washed platelets

4.13.1 Prednisolone reduced thrombin-induced ERK2 phosphorylation in a time-dependent manner

The final step was to characterise the kinetics of prednisolone-mediated effects on ERK2 phosphorylation in response to thrombin stimulation using platelet samples used in the study of PKC activation. Prednisolone reduced thrombin-induced ERK2 phosphorylation following kinetics different from that of PKC activation and RhoA/ROCK-mediated signalling. Incubation with prednisolone (10 μM) for 1 minute did not affect thrombin-induced ERK2 phosphorylation. Detectable reduction of ERK2 phosphorylation was observed with prednisolone incubation of 5 minutes and with longer prednisolone incubation, ERK2 phosphorylation was found to reduce even further (Figure 4.15 A). No recovery of ERK2 phosphorylation in the presence of prednisolone at the concentration tested was found in our experimental conditions.

4.13.2 Prednisolone reduced thrombin-induced ERK2 phosphorylation in a concentration-dependent manner

Since prednisolone was found to reduce thrombin-induced ERK2 phosphorylation with 5 minutes of incubation, a concentration-response of prednisolone on ERK2 phosphorylation was performed by incubating platelets with various concentrations of prednisolone for 5 minutes prior to thrombin (0.005 U/ml) stimulation. Prednisolone was found to reduce thrombin-induced ERK2 phosphorylation in a concentration-dependent manner with threshold effects observed at a concentration of 1 nM prednisolone and maximal reduction observed with either 10 or 100 μM prednisolone depending on the donor. Prednisolone itself did not seem to induce ERK2 phosphorylation in platelets, as ERK2 phosphorylation in the presence of prednisolone on its own was similar to that in the basal sample (Figure 4.15 B). Prednisolone could not reduce thrombin-induced ERK2 phosphorylation at any concentration tested when it was pre-incubated for 1 minute prior to thrombin stimulation (Figure 4.15 C), consistent with the time-course data (Figure 4.15 A).

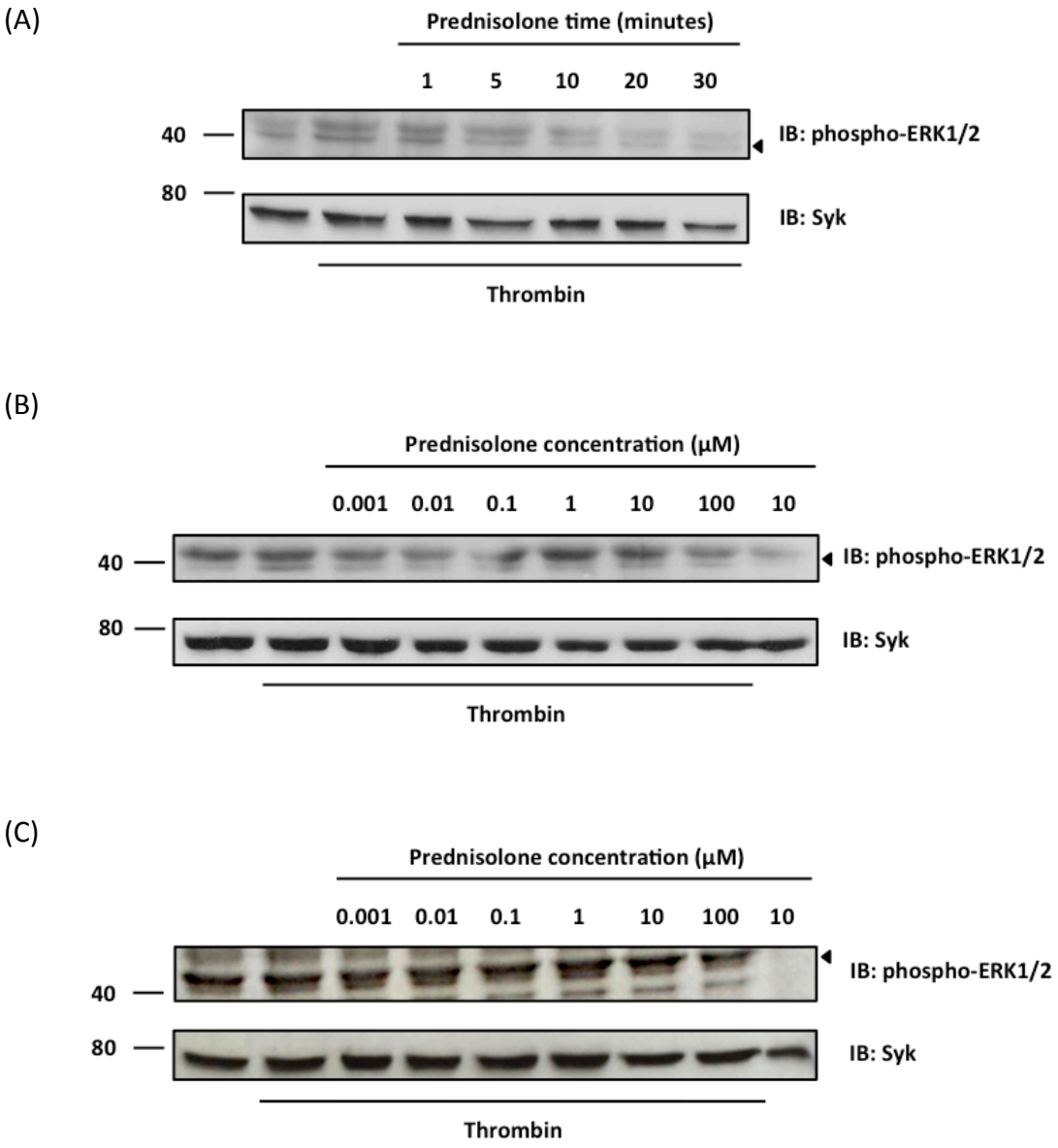


Figure 4.15. Prednisolone reduced thrombin-induced ERK2 phosphorylation of PKC substrate proteins in washed platelets in a time and concentration-dependent manner. Washed platelets (3×10^8/ml) were treated with EGTA (1 mM), apyrase (2 U/ml) and indomethacin (10 μM) for at least 20 minutes before performing further experiments. Platelets were then treated with (A) prednisolone (10 μM) for various time points or (B) various concentrations of prednisolone for 1 minute and stimulated with thrombin (0.005 U/ml) for 1 minute. Samples were lysed, resolved by SDS-PAGE and immunoblotted for phospho-ERK1/2. Membranes were later re-probed for Syk. Representative immunoblots (A, B and C) from two independent experiments are shown. Small arrowheads indicate phospho-ERK2 or p42.

4.14 Discussion

This chapter presented data, which revealed for the first time that the synthetic glucocorticoid prednisolone could target the RhoA/Rho kinase (ROCK)-mediated signalling cascades in platelets downstream of thrombin stimulation. Besides, we also showed that prednisolone could potentially target PKC activation in platelets downstream of thrombin stimulation. Thus, this chapter unfolds new molecular events that can be regulated by prednisolone, besides its well-characterised pro-inflammatory and immune-responsive molecular targets. To our knowledge, this is the first report on the effect of prednisolone on PKC-mediated signalling in human platelets.

4.14.1 Prednisolone reduced thrombin-induced myosin light chain (MLC-ser^{19}) phosphorylation in platelets

Chapter 3 established the inhibitory effects of prednisolone on thrombin-induced platelet aggregation and it was important to understand the molecular basis of this inhibition. Thrombin stimulation triggers distinct molecular pathways leading to the cytoskeletal rearrangement, which converge on and are driven by the phosphorylation of MLC. Hence we began our investigation by examining the effects of prednisolone on thrombin-induced MLC phosphorylation. Although platelet MLC has been shown to be diphosphorylated at serine 19 (MLC-ser^{19}) and threonine 18 (MLC-thr^{18}) (Itoh et al., 1992), platelet shape change is believed to be driven primarily by MLC-ser^{19} phosphorylation, with little role of MLC-thr^{18} phosphorylation in this physiological platelet response (Getz et al., 2010). Besides, phosphorylation at MLC-ser^{19} precedes that at MLC-thr^{18} and MLC-ser^{19} phosphorylation is much more robust compared to the weak phosphorylation at MLC-thr^{18} (Getz et al., 2010). Hence, we decided to focus on the phosphorylation of MLC-ser^{19} only as an apt marker of platelet shape change and study the effects of prednisolone on this marker.

In chapter 3 (section 3.7.5) we observed that the inhibitory effects of prednisolone on thrombin-induced platelet aggregation was modest compared to physiological platelet inhibitors. Hence, it was essential to use a moderate concentration of

thrombin to induce signalling so that prednisolone-mediated effects would not be masked in the presence of the potent agonist. Hence we chose to stimulate platelet signalling events with 0.005 U/ml thrombin for all further experiments, which was potent enough to induce phosphorylation events but not to such as extent that the potential effects of prednisolone would be masked. The time-course of prednisolone-mediated effects on thrombin-induced MLC-ser^{19} phosphorylation (Figure 4.3, A i and ii) correlated with its time-course on platelet aggregation (Chapter 3, Figure 3.3, B i and ii). However, in case of aggregation, prednisolone-mediated inhibition was sustained for slightly longer. Reduction of aggregation was observed after a 1-minute incubation and peaked with 5 minutes incubation before declining. Inhibition of MLC-ser^{19} phosphorylation also peaked after 1 minute of prednisolone incubation, but in contrast was completely lost with 5 minutes incubation or longer. Here also prednisolone exhibited rapid inhibitory effects on platelets, which is indicative of a non-genomic mode of action, as observed in platelet aggregation. A more stringent time-course experiment should have been designed where prednisolone-mediated inhibition of these responses could be monitored from 15 seconds of prednisolone incubation up to a maximum of 5 minutes with narrower time-points (15 or 30 seconds) between them. Such a design might have clarified and detailed the time of prednisolone activity as well as account for the discrepancy observed in the duration of its action on MLC-ser^{19} phosphorylation and aggregation.

A recent article from our group showed that the physiological prostanoid PGE_1 abolished thrombin-induced platelet shape change, which correlated well with the total abolishment of MLC-ser^{19} phosphorylation (Aburima et al., 2013). To some degree this was to be expected since PGE_1 activate a cAMP signalling cascade, which is know to been a potent regulators of platelet function. In our study, prednisolone failed to abolish platelet shape change in response to thrombin (Figure 4.11, A i and ii) at the same concentration, which was used to induce MLC-ser^{19} phosphorylation. The reason for this is unclear but could potentially be explained by the observation that prednisolone only partially reduced thrombin-induced MLC-ser^{19} phosphorylation in platelets. It is also possible that thrombin

activates additional pathways that are insensitive to prednisolone (Figure 4.3, A). This possibility is strengthened by the observation that prednisolone is able to inhibit shape change when it is induced by a direct activation of RhoA independently of thrombin. This again indicates the low potency of prednisolone to inhibit platelet signalling or functional responses compared to the physiological inhibitors.

4.14.2 Prednisolone reduced Rho kinase (ROCK)-mediated signalling in platelets

To further understand the effects of prednisolone on signalling events upstream of MLC phosphorylation, it was important to dissect whether prednisolone was able to target the Ca^{+2}-dependent and RhoA/Rho kinase (ROCK)-dependent pathways independently of each other. Thus we employed inhibitors to these pathways reasoning that blocking one pathway would reveal the effect of prednisolone on the other in isolation. Consistent with previous studies, both the ROCK inhibitor Y27632 and the intracellular Ca^{+2} chelator BAPTA-AM individually reduced MLC-ser^{19} phosphorylation (Figure 4.5 A) (Aburima et al., 2013; Bauer et al., 1999). We observed a stronger inhibition with Y27632 compared to other studies and this is probably related to the ten-fold lower thrombin concentration used in the present study. BAPTA-AM could slightly reduce MLC-ser^{19} phosphorylation and the data was not significant. This indicated that the ROCK-mediated was more important in our experimental settings. MLC as also been shown to be phosphorylated by ROCK directly in an earlier report (Amano et al., 1996). The study revealed that upon RhoA activation ROCK was activated and it stoichiometrically phosphorylated MLC at the same site (ser^{19}) as phosphorylated by the Ca^{+2}/calmodulin-dependent MLCK. This phosphorylation event promoted actin-dependent activation of myosin-ATPase. This possibility can be verified by an in-vitro kinase assay, where recombinant ROCK can be appointed to phosphorylate recombinant MLC at ser^{19} in the absence or presence of prednisolone.

Data presented in Figure 4.5 A identified that prednisolone targeted the ROCK-mediated pathway, as co-incubation of BAPTA-AM and prednisolone caused an additional reduction of MLC-ser^{19} phosphorylation compared to either of these

alone. In our experimental settings, we used a relatively low thrombin concentration (0.005 U/ml) to induce MLC-ser^{19} phosphorylation, which was almost completely blocked by Y27632. Hence any additional inhibitory effect of prednisolone on MLC-ser^{19} phosphorylation combined with that of Y27632 was undetectable. Thus the possibility that prednisolone could also target the Ca^{+2}-dependent pathway of thrombin-mediated platelet activation cannot be completely ruled out. To clarify whether prednisolone targeted the Ca^{+2}-mediated pathway, it would have been useful to test the specific MLCK inhibitor, ML-7 in combination with prednisolone, which could not be pursued due to time constraints. However, because of the prominent contribution of the RhoA/ROCK pathway we focussed on examining how this was influenced by prednisolone.

4.14.3 Prednisolone reduced thrombin-induced RhoA activation in platelets

The observation that prednisolone reduced thrombin-induced RhoA activation (Figure 4.9) is consistent with inhibition of its downstream effector, ROCK. An earlier study reported inhibitory effects of prednisolone on the upregulation of RhoA and RhoA promoter activity caused in human bronchial smooth muscles upon antigen-induced airway hyper-responsiveness in allergic bronchial asthma (Goto et al., 2010). Our results highlighted the inhibitory effects of prednisolone on RhoA activation in a non-genomic mode without the involvement of gene regulation. The total RhoA expression was not however affected by prednisolone treatment in our study (Figure 4.9).

Although our data established the inhibitory effects of prednisolone on thrombin-stimulated RhoA activation, the mode of inhibition remains to be determined. RhoA activation is regulated by phosphorylation of ser^{188} through enhanced recruitment of Rho guanine dissociation inhibitors (RhoGDIs) or prevention of its nuclear localisation (Ellerbroek et al., 2003; Forget et al., 2002). Another study revealed that RhoA phosphorylation (ser^{188}) precedes its membrane localisation and GTP-loading (Aburima et al., 2013). While a larger fraction of RhoA is localised in cell cytoplasm, its membrane localisation is important for its activation. Thus, prednisolone could target RhoA activation by either promoting RhoA ser^{188} phosphorylation or reducing

thrombin-stimulated membrane compartmentalisation of RhoA. Two different strategies should be appointed here to further elaborate how prednisolone could target RhoA activation. Firstly, phosphorylation of RhoA at ser^{188} in the platelet cytosol should be investigated in the presence of prednisolone by use of a phospho-specific RhoA antibody that can recognise the phosphorylated form of RhoA. Secondly, subcellular distribution of RhoA in control and prednisolone-treated platelets following thrombin stimulation should be analysed. Moreover, prednisolone could also affect GTP loading on RhoA by blocking Rho guanine nucleotide exchange factors (RhoGEFs) that catalyse the exchange of GDP for GTP (Ren, 1999). This possibility can be addressed by identifying the association of RhoA with RhoGEFs in control and prednisolone-treated platelets following thrombin-stimulation through co- immunoprecipitation studies.

We could not assess ROCK activity downstream of RhoA activation in response to thrombin stimulation owing to time constraints. Thus, we examined the phosphorylation of MLCP at threonines 853 and 696 within its MYPT1 domain (MYPT1-thr^{853} and MYPT1-thr^{696}) as markers of ROCK activity. RhoA/ROCK activation in response to thrombin stimulation leads to phosphorylation of the MLCP at these two residues (Feng et al., 1999; Lincoln, 2007). Since phosphorylation at these residues has been reported to inactivate the MLCP (Kimura et al., 1996; Murányi et al., 2005; Suzuki et al., 1999), these sites are referred to as inhibitory sites and phosphorylation at these sites as inhibitory phosphorylation for MLCP activity. Consistent with published studies MLCP was significantly phosphorylated at both MYPT1-thr^{696} and MYPT1-thr^{853} upon thrombin stimulation (Aburima et al., 2013; Wraith et al., 2013). These phosphorylation events were reduced by prednisolone following a similar kinetics as that of MLC-ser^{19} phosphorylation (Figure 4.7). The reduction of MLCP phosphorylation at the inhibitory sites by prednisolone meant that MLCP inactivation following thrombin stimulation could be prevented. Thus, in the presence of prednisolone MLCP could again dephosphorylate MLC-ser^{19} induced by thrombin. Thus the reduction of inhibitory phosphorylation of MLCP could account for reduction of thrombin-mediated MLC-ser^{19} phosphorylation by prednisolone. Since MLCP phosphorylation

at the inhibitory threonine residues is mediated by activated ROCK downstream of RhoA activation, reduction in these phosphorylation events indicated that ROCK activation might have been hindered by prednisolone. This possibility can be further explored by measuring ROCK activity in platelets following thrombin stimulation in the presence of prednisolone. Commercial assay kits have been designed to measure ROCK activity from cell lysates or purified kinase preparations through the chromogenic or chemiluminescent detection of phosphorylation of the recombinant ROCK substrate MYPT1 at thr^{696} residue. Another strategy to determine how prednisolone inhibits ROCK-mediated MLCP inactivation is to investigate the association of RhoA, ROCK and the MYPT1 subunit of MLCP in control and prednisolone-treated platelets following thrombin stimulation. The formation of this complex is believed to be crucial for ROCK-mediated MLCP inactivation *in vivo* (Kawano et al., 1999).

4.14.4 Prednisolone reduced MLCP phosphorylation in platelets independent of targeting $G\alpha_{12/13}$

The observation that prednisolone reduced MLCP phosphorylation ($MYPT1^{Thr853}$) induced by the specific RhoA activator Calpeptin (Figure 4.10) specified that prednisolone targeted ROCK-mediated signalling in platelets independent of its possible inhibitory impact on $G\alpha_{12/13}$-coupled receptor activation. Not only signalling, prednisolone also appeared to abolish platelet shape change induced by calpeptin, irrespective of the concentration used to induce this response (Figure 4.11, B i and ii). This observation was important, since it implied that prednisolone might hinder this entire signalling cascade in platelets irrespective of the agonist employed. A recent article from our group established that the pathological agent oxidised LDL (oxLDL) induced platelet shape change and activation via tyrosine kinase-mediated and RhoA/ROCK-mediated pathways (Wraith et al., 2013). Having established that prednisolone targets RhoA/ROCK-mediated signalling cascades in platelets independent of the stimulus used to induce these events, it will be interesting to determine if it can exert inhibitory properties on oxLDL-mediated platelet signalling too. This is important since oxLDL is present in abundance in atherosclerotic lesions and glucocorticoids are proven to be effective against

atherosclerosis. Thus, if the inhibitory effects of prednisolone are also prevalent in oxLDL-mediated platelet signalling, yet another mechanism of its anti-inflammatory properties can be explained.

4.14.5 Prednisolone reduced thrombin-induced PKC activation in platelets

The inhibitory effects of prednisolone on thrombin-induced platelet aggregation demonstrated in chapter 3 were paralleled by a reduction of RhoA/ROCK-mediated signalling events caused by prednisolone in response to thrombin stimulation of platelets. In an attempt to extend our findings on the effect of prednisolone on thrombin-mediated signalling in platelets, we decided to study $G_{\alpha q}$-mediated signalling events in response to thrombin stimulation. Due to time constraints, characterisation of the entire $G_{\alpha q}$-mediated cascade was not feasible. Hence we decided to study PKC activation following thrombin stimulation. Human platelets are known to express several isoforms of PKC such as conventional isoforms (α, β and γ), novel isoforms (δ, ε, η and θ) and atypical isoforms (ξ and ι/λ). These isoforms become activated in platelets upon agonist stimulation. Activation of the conventional isoforms is dependent on both Ca^{+2} and DAG while that of the novel isoforms is dependent on only DAG. Atypical PKC isoforms become activated independent of Ca^{+2} and DAG. These PKC isoforms perform distinct functions in platelet activation and thrombus formation. PKCα is important for granule secretion, platelet aggregation and thrombus formation (Konopatskaya et al., 2009); PKCδ is important for integrin-activation and TxA_2 generation (Yacoub et al., 2006); PKCβ and PKCθ are important for platelet spreading (Buensuceso et al., 2005; Hall et al., 2008); PKCδ and PKCθ seem to be important in TxA_2 generation in an agonist-specific manner (Chari et al., 2009; Nagy et al., 2009).

Following activation, PKC has been shown to phosphorylate a number of platelet proteins such as SNAREs, MARCKS, pleckstrin, etc. which are involved in agonist-induced platelet secretion. Hence we wanted to confirm the ability of PKC to phosphorylate its substrate proteins in platelets following its activation upon thrombin stimulation. We decided to address this using a phospho-specific antibody that can recognise an entire profile of proteins being phosphorylated at serine

residues by PKC specifically. The advantage of using this antibody was the ability to analyse several proteins at the same time, thus increasing the throughput of this particular experiment. To save time and avoid variation in the observed effects, we decided to study the set of platelet samples that were prepared during the analysis of MLC^{Ser19}, $MYPT^{Thr853}$ and $MYPT^{Thr696}$ phosphorylation. Phosphorylation levels of a number of proteins were shown to increase with thrombin stimulation of platelets in a concentration-dependent fashion (Figure 4.12), which confirmed that PKC activity in platelets was enhanced with thrombin stimulation. The same proteins appeared to be phosphorylated with thrombin stimulation across all the donors tested, confirming the specificity of the phospho-PKC substrate antibody.

Prednisolone was found to decrease phosphorylation of most of the PKC substrate proteins when platelets were stimulated with either 0.005 or 0.01 U/ml thrombin, indicating that prednisolone could reduce thrombin-induced PKC activation in platelets. PKC activation induced by thrombin concentrations lower than 0.005 U/ml remained unaffected by prednisolone, in line with MLC^{Ser19} phosphorylation. The reduction in PKC activation was rather variable across donors for samples stimulated with 0.005 U/ml thrombin, since prednisolone was able to reduce phosphorylation of most of the substrate proteins in only two out of the four donors tested. As examples, thrombin-induced increase in phosphorylation of two PKC-substrate proteins with molecular weights of approximately 80 and 45 KDa could be reduced by prednisolone in only two out of four donors. These two proteins might represent MARCKS and pleckstrin respectively, which are well-established substrates of PKC and have shown to get phosphorylated in platelets upon PKC activation. In the other two donors, phosphorylation of most of the PKC-substrate proteins remained unaffected, except the ones with molecular weights of approximately 120, 90 and 25 KDa. This implied that in such donors, when platelets were activated by thrombin at the indicated concentration, prednisolone might not be able to target PKC activation fully, due to which the ability of PKC phosphorylate some of its substrates was still retained. It is noteworthy that despite reduction of MLC^{Ser19} phosphorylation observed consistently with prednisolone in all these four donors, the reduction in PKC activity was variable. This observation seems

important as it could partly explain the underlying mechanisms of variability in prednisolone-mediated effects observed on thrombin-mediated platelet aggregation across donors, as mentioned in chapter 3 (section 3.7.4). Prednisolone-mediated reduction of phosphorylation of nearly all the PKC-substrate proteins was however observed in all the donors when 0.01 U/ml thrombin was used to stimulate platelets.

This study could have been improved by introducing a time-course of thrombin on PKC activation to understand its kinetics. Gαq-mediated Ca^{+2} signalling is known to trigger earlier than $G_{\alpha 12/13}$-mediated signalling following thrombin stimulation. Since platelets were stimulated with thrombin for 1 minute, these samples might have provided conditions for optimal activation of $G_{\alpha 12/13}$-mediated signalling events. But if PKC activation occurred earlier similar to Ca^{+2}-signaling, we might have missed conditions for optimal PKC activation, which could have been more robustly targeted by prednisolone. In most of the studies that have looked for PKC-mediated effects platelets have been stimulated with agonists for 1 minute, but these aimed to activate PKC signalling and hence required sustained PKC activation. Since our aim was the opposite, in that we wanted to determine inhibitory effects of prednisolone on PKC activation, hence conditions that caused moderate PKC activation would have been suitable, which might have been achieved by shorter thrombin stimulation of platelets.

MLC has known to be phosphorylated by the Ca^{+2}-calmodulin-dependent MLC kinase (MLCK), which itself gets phosphorylated by the cAMP-dependent kinase PKA as well as PKC (Nishikawa et al., 1985). Hence, MLCK phosphorylation could also be differentially modulated by prednisolone through reduction in PKC activity that in turn could affect the ability of MLCK to phosphorylate MLC. Thus prednisolone could potentially regulate platelet shape change through a second mechanism involving PKC-mediated regulation of MLC^{Ser19} phosphorylation by MLCK other than the RhoA/ROCK-mediated signalling. Since cytoskeletal reorganisation during platelet shape change is believed to be an important factor in regulating platelet secretion, prednisolone might indirectly affect platelet secretion through regulation of PKC-mediated MLCK activity, besides its direct effects on PKC-

mediated proteins crucial for secretion. Looking into MLCK phosphorylation in isolation downstream of thrombin-induced PKC activation in the presence of selective PKC inhibitors could assess this possibility.

The kinetics of prednisolone-mediated effects on thrombin-induced PKC activation was determined. Reduction of phosphorylation of some of the PKC-substrate proteins in response to thrombin (0.005 U/ml) stimulation peaked with only a minute of prednisolone incubation and was completely lost with 5 minutes incubation or longer (Figure 4.2 A), in line with MLC^{Ser19}, $MYPT^{Thr853}$ and $MYPT^{Thr696}$ phosphorylation events. This observed rapid inhibitory effects of prednisolone on thrombin-induced PKC activation again confirms its non-genomic mode of action, as observed in platelet aggregation.

4.14.6 Prednisolone reduced thrombin-induced ERK2 phosphorylation in platelets

Thrombin stimulation has been shown to provoke ERK2 phosphorylation in platelets (Papkoff et al., 1994), which is believed to be mediated by the tyrosine-threonine MAPK/ERK kinases 1 and 2 or MEK1/2 (Börsch-Haubold et al., 1995) and PKC (Aharonovitz and Granot, 1996). Thus, it was interesting to observe the effect of prednisolone treatment on thrombin-induced ERK2 phosphorylation. Thrombin was shown to cause ERK2 phosphorylation, although the level of phosphorylation did not increase with increasing thrombin concentrations. All the cited articles on thrombin-mediated ERK2 phosphorylation used a 100-fold higher thrombin concentration (1 U/ml) compared to the highest concentration (0.01 U/ml) used in our studies, because of which they detected a much stronger ERK2 phosphorylation signal than ours, which might have just been a threshold signal.

The effect of thrombin stimulation on ERK1 phosphorylation has been controversial. In our study, we too found that thrombin stimulation failed to increase ERK1 or p44 phosphorylation over basal levels in most donors. In some donors, ERK1 was very slightly phosphorylated. Thus ERK1 phosphorylation was not pursued any further.

Prednisolone was able to reduce ERK2 phosphorylation induced by 0.005 and 0.01 U/ml, but not by 0.001 or 0.002 U/ml thrombin (Figure 4.14). The reduction in phosphorylation was more evident in case of phosphorylation induced by 0.01 U/ml thrombin compared to 0.005 U/ml, similar to reduction of PKC activation. It would have been useful to test the effect of prednisolone on ERK2 phosphorylation stimulated by higher thrombin concentrations.

Prednisolone reduced thrombin-induced ERK2 phosphorylation in platelets following a different temporal pattern as that of PKC activation, where inhibition began with 5 minutes incubation and was maintained up to the longest incubation of 30 minutes. This observation was striking, as so far prednisolone-mediated inhibitory effects on platelet function or signalling mechanisms were transient. The sustained inhibition of thrombin-induced ERK2 phosphorylation possibly had no toxic effect on platelets, as they seemed to recover and aggregate normally in response to thrombin. Incubation of platelets with prednisolone might activate specific phosphatases, which participate in dephosphorylating ERK2 and these phosphatases might remain active throughout the length of these experiments. This could explain the sustained reduction of thrombin-induced ERK2 phosphorylation in the presence of prednisolone. Although ERK2 phosphorylation seems to be carried out mainly by MEK1/2 and PKC, there lies a possibility of the involvement of another kinase downstream of PKC that can also contribution to ERK2 phosphorylation (Nadal-Wollbold et al., 2002). Thus the effect of prednisolone on kinases upstream of ERK2 also needs to be determined.

Our finding that prednisolone reduced ERK2 phosphorylation in thrombin-stimulated platelets confirmed with the well-established interference of glucocorticoids with MAPK signalling pathways (Kharwanlang and Sharma, 2011). Hence it will be interesting to extend these findings by looking into thrombin-induced $p38^{MAPK}$ and JNK1 phosphorylation.

4.15 Conclusion

From this chapter we can conclude that prednisolone inhibited signalling events in platelets in response to thrombin stimulation and these effects were particularly

expressed in the RhoA/ROCK-mediated signalling cascades that led to an inhibition of the phosphorylation of the key contractile protein myosin. These effects were non-genomic in nature and are likely to be mediated by the GCR. Although the use of GCR antagonist RU486 was suggestive that the GCR may be important in the effects of prednisolone, one should be cautious while interpreting the data as it is unclear if the receptor targets the surface or intracellular receptor. Yet, it could be speculated that it targets both since in our data it blocks very short-term effects of prednisolone. We also presented considerable evidence that prednisolone inhibited $G_{\alpha q}$-mediated PKC activation and ERK2 phosphorylation in washed platelets in response to thrombin stimulation. However these data are preliminary and thus need to be confirmed by further detailed characterisation. It would also be interesting to determine if the inhibitory effects of prednisolone are exerted upon upstream components of $G_{\alpha q}$-mediated signalling cascades particularly $G_{\alpha q}$ activation, PLCβ activation, Ca^{+2} mobilisation and PKC phosphorylation.

Chapter 5

Characterisation of glucocorticoid receptor in human platelets and megakaryocytes and evaluation of the effect of prednisolone on megakaryocyte maturation

5.1 Introduction

In the previous chapters we demonstrated the inhibitory effects of the synthetic glucocorticoid prednisolone on platelet function and characterised the signalling mechanisms underlying these inhibitory effects. The biological effects of glucocorticoids are believed to be mediated by the classical glucocorticoid receptor (GCR), which belongs to the nuclear receptor superfamily and acts as a ligand-activated transcription factor. GCR is expressed in various cells and tissues including brain, heart, lungs, liver, kidney, skeletal muscles, blood leukocytes, and peripheral mononuclear blood cells (PBMCs) (Pujols et al., 2002). An earlier study also reported the presence of GCR in human platelets by the combined use of immunoblotting, immunofluorescence and flow cytometry (Moraes et al., 2005). GCR has been reported to undergo post-translational modification with phosphorylation of GCR at three serine residues (203, 211 and 226) within its amino-terminal region observed under basal conditions (Chen et al., 2008; Wang et al., 2002). Glucocorticoids are shown to induce hyper-phosphorylation of GCR (Bodwell et al., 1995; Hoeck and Groner, 1990; Ortí et al., 1989). Several kinases have been implicated in performing GCR phosphorylation such as cyclin-dependent kinases (Cdk1), mitogen-activated protein kinases (MAPKs) and glycogen-synthase kinase 3β (GSK3β) (Galliher-Beckley et al., 2008; Itoh et al., 2002; Krstic et al., 1997; Miller et al., 2005; Rogatsky et al., 1998b). Phosphorylation at ser^{203} and ser^{211} has been associated to enhanced transactivation potential of GCR (Krstic et al., 1997; Miller et al., 2005; Wang and Garabedian, 2003), while that at ser^{226} decreases to

GCR transcriptional activation (Rogatsky et al., 1998b). In this chapter we wanted to study the presence of GCR in human washed platelets and its phosphorylation status in basal and stimulated platelets.

Since the earlier chapters established the effects of the synthetic glucocorticoid prednisolone on platelet functions and biochemical pathways, it was of interest to determine if prednisolone could affect thrombopoeisis from Mks. Thus, in this chapter we addressed this by studying the effect of prednisolone on Mks *in vitro*. In order to study MK cell biology two main assays were routinely used. The first is the analysis of MK ploidy. It is a useful marker to understand how complete the differentiation is. In addition, although not proven it is assumed that increased ploidy is required to produce the maximum number of platelets. The second technique is *in vitro* spreading of Mks and proplatelet formation, which determines the ability of Mks to extend cytoplasmic arms or proplatelets that yield platelets.

5.2 Aims

The overall aim was to determine the presence and role of the GR in platelets. The specific objectives of this chapter were

- To confirm the expression of GCR in washed platelets by various methods
- To assess the phosphorylation status of GCR in washed platelets under basal conditions
- To evaluate changes in GCR phosphorylation upon treatment of platelets with prednisolone or platelet agonists
- To determine the presence of GCR in Mks and study the effect of prednisolone on ploidy of Mks

5.3 Identification of glucocorticoid receptor (GCR) in human platelets

5.3.1 GCR detection by flow cytometry

In advance of examining the phosphorylation of the GR it was important to confirm its presence. In the first instance we used flow cytometry to detect the GCR in washed platelets. Here we employed a commercial antibody (Santa Cruz Biotechnology) raised in rabbit against an epitope mapping at the N-terminus on the human GCRα in flow cytometry. This antibody had been used previously to identify GCR in mammalian cell-lines (Bachmann, 2004; Schmidt et al., 2006). Moraes et al. (2005) detected GCR in permeabilised platelets by flow cytometry. Hence, in our study too washed platelets were fixed with paraformaldehyde (PFA) and permeabilised to allow the antibodies to reach inside the cell. Since antibodies exhibit sensitivity towards different permeabilisation methods, hence this protocol requires optimisation for every single antibody using various reagents. Thus, we appointed three permeabilisation reagents: 90% methanol, 0.2% Triton X-100 and a commercial permeabilisation buffer (BD Biosciences) based on saponin (1X). Following permeabilisation, the hGCR antibodies were allowed to interact with platelets. Rabbit or mouse IgGs were used as negative controls to account for non-specific binding. Secondary antibodies conjugated to different fluorophores were appointed to bind to the primary antibodies and signal was detected on the flow cytometer.

The rabbit polyclonal anti-hGCR antibody was able to recognise and bind to GCR in human platelets, which was determined by a rightward shift in the signal/peak obtained for this antibody when compared to the negative IgG isotype control (Figure 5.1). The saponin-based permeabilisation technique seemed to be the most effective as it produced a 5.5 times greater signal compared to IgG, while methanol and Triton X-100 reagents gave only 2.5 and 1.7 times greater signal (Figure 5.1). Thus GCR could be detected in human platelets through flow cytometry.

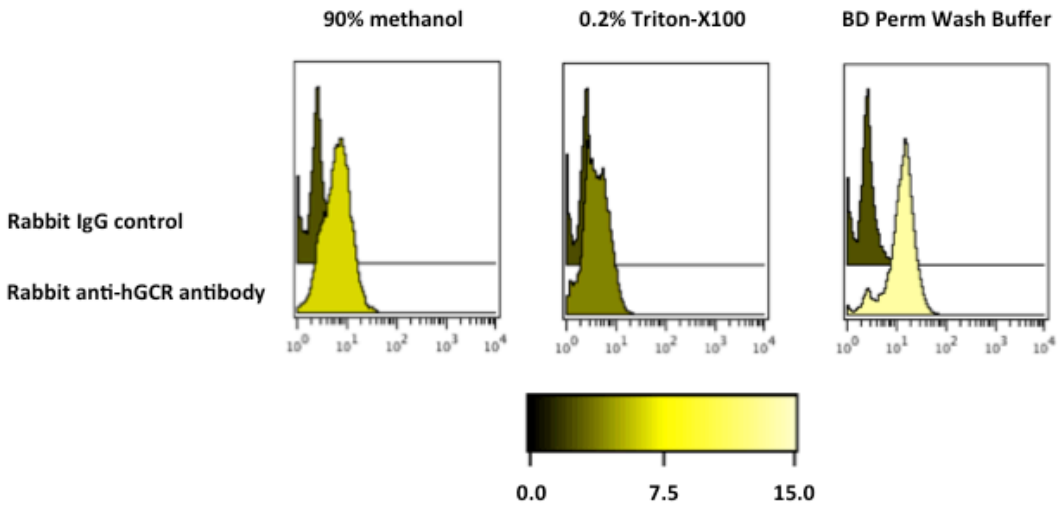


Figure 5.1 Detection of glucocorticoid receptor (GCR) in human washed platelets by flow cytometry. Washed platelets (1 × 10^8/ml) were fixed with 2% paraformaldehyde for 10 minutes and permeabilised using either 90% methanol, 0.2% Triton X-100 or BD Perm wash buffer (1X saponin) for another 10 minutes. Platelets were incubated for 30 minutes with 5 µg/ml of either rabbit IgG control or rabbit polyclonal anti-hGCR antibody (Santa Cruz Biotechnology). Platelets were next incubated for another 30 minutes with 1 µg/ml of a rabbit secondary antibody conjugated to Alexa647. Platelet samples were then analysed on a BD FACSCalibur™. Shown are representative histograms from a single experiment. For each panel, the top peak represents signal obtained from the IgG control and the bottom peak represents that from the GCR antibody.

	90% MeOH	0.2% TritonX-100	BD Perm wash buffer
-ve control (rabbit IgG)	2.55	2.35	2.46
hGCR (rabbit polyclonal)	6.44	3.89	13.58

Table 5.1 Median values of fluorescence obtained from binding of antibody to platelets when analysed by BD FACSCalibur™.

5.3.2 GCR detection by western blotting and immunoprecipitation techniques

When identifying a protein, data obtained from flow cytometry needs to be validated by immunoblotting in order to determine specificity of the antibody appointed for detection. Thus, the next objective was to confirm the presence of GCR in human washed platelets by a combination of immunoblotting and protein immunoprecipitation techniques using the antibodies as indicated for flow cytometry as well as another commercial mouse monoclonal antibody. The molecular weight of GCR has been reported earlier to be 94 or 97 kDa.

5.3.2.1 GCR immunodetection with a rabbit polyclonal anti-hGCR antibody

In the first instance, increasing amounts of platelet lysates were analysed on SDS-PAGE and immunoblotting for GCR was performed with the rabbit polyclonal anti-hGCR antibody (Santa Cruz Biotechnology). As monocytes are known to express GCR (Li et al., 2006; Werb et al., 1978), lysate from a human monocytic leukemic cell-line called THP-1 (Bo et al., 2006) was also analysed as a positive control for GCR detection. A weak protein band close to 100 kDa was observed in the platelet lysates (Figure 5.2 A, indicated by a black arrowhead), which could represent the GCR protein. The band appeared stronger with increasing amounts of lysates. THP-1 lysates also displayed a similar protein band (Figure 5.2 A), although its intensity was stronger than that in platelets. The antibody however recognised several other non-specific protein bands throughout the entire blot, most of which were stronger in intensity than the band representing GCR protein. In an attempt to reduce non-specific interaction of the antibody with platelet proteins, skimmed milk (5% solution in TBS-tween) was used to block the membrane instead of the usual blocking reagent BSA (10% solution). Under such conditions, although the occurrence of non-specific bands was reduced considerably making the blot much cleaner, the band corresponding to GCR protein was completely lost too (data not shown).

We then decided to pull-down or precipitate the GCR protein from platelet lysates using the above hGCR antibody applied in increasing amounts and sepharose beads conjugated to either protein A or protein G from *Staphylococcus aureus*.

Immunoblotting of the immunoprecipitated samples with the same hGCR antibody revealed a very faint band close to 100 kDa when platelet lysates were precipitated with either 2 or 5 μg of antibody using the protein A conjugated beads, but not protein G beads (Figure 5.2 B, black arrowhead). This band was not precipitated with a negative IgG isotype control antibody, indicating that the band may represent GCR protein. Despite potential identification of GCR, this antibody precipitated a large array of non-specific proteins in much larger amounts as compared to the GCR protein, as was apparent from their very strong band intensities. One possibility could be that the platelet lysates were added directly to the antibody-bead conjugated without a pre-clearing step. When protein immunoprecipitation is carried out with a polyclonal antibody that is prone to bind to a large number of non-specific proteins, cell lysates must be subjected to a pre-clearing protocol where lysates are incubated with a negative IgG isotype control antibody and beads so that most of the abundantly expressed proteins are precipitated out of the lysate by binding to the IgG antibody. This process minimises the precipitation of non-specific proteins and reduces background following immunoblotting of the immunoprecipitated protein. Thus we repeated the immunoprecipitation experiment using 5 μg of the rabbit antibody and protein A sepharose beads by incorporating the lysate pre-clearing protocol. Although GCR protein was detected very weakly, pre-clearing did not appear to reduce the appearance of non-specific proteins in the immunoprecipitated lysates (data not shown).

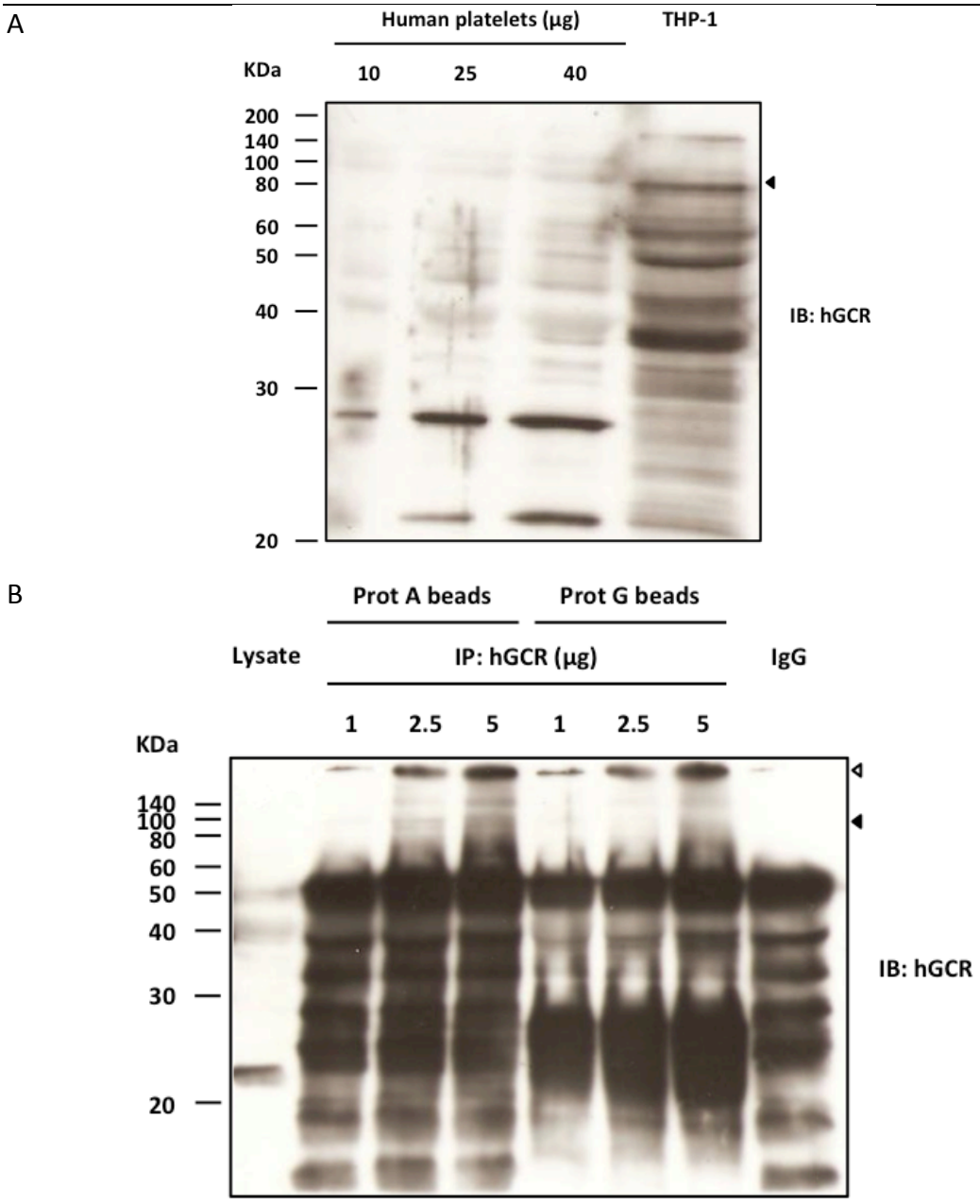


Figure 5.2 Detection of GCR in human washed platelets using the rabbit polyclonal anti-hGCR antibody. (A) Washed platelets (8×10^8/ml) were lysed using 2X Laemmli buffer. Platelets proteins were separated using SDS-PAGE and immunoblotted for GCR using the rabbit polyclonal antibody. Representative immunoblot from four independent experiments is shown. (B) Washed platelets (8×10^8/ml) were lysed using 1X lysis buffer followed by immunoprecipitation using sepharose beads conjugated to protein A or protein G and increasing amounts of the GCR antibody (1-5 µg) or a control rabbit IgG (1 µg). Immunoprecipitated samples were then resolved using SDS-PAGE and immunoblotted for GCR using the rabbit polyclonal antibody. Representative immunoblot from a single experiment is shown.

5.3.2.2 GCR immunodetection with a mouse monoclonal anti-hGCR antibody

Since immunodetection of GCR in human platelets using the above anti-hGCR antibodies faced a lot of challenges, we decided to appoint another commercial mouse monoclonal antibody (BD Biosciences) that was raised against an epitope spanning amino acid residues 176 to 289 on the hGCR. When immunoblotting was performed with this antibody, a band appeared around 100 KDa in platelet lysates, which could represent the hGCR protein (Figure 5.3, arrowhead). This antibody bound lesser non-specific proteins when compared to the rabbit polyclonal anti-hGCR antibody and the band intensity of the hGCR protein was relatively stronger too. Skimmed milk as a blocking reagent helped to slightly intensify the hGCR protein band as well as lower the intensity of the non-specific protein bands (Figure 5.3 Aii) when compared to BSA (Figure 5.3 Ai). Hence for further analyses skimmed milk was used as a blocking reagent.

Washed platelet and THP-1 lysates (500 µg) were next immunoprecipitated with the above mouse monoclonal anti-hGCR antibody followed by immunoblotting of the immunoprecipitates with the same antibody. A faint band representing GCR protein appeared in the platelet immunoprecipitate, which was absent in the negative IgG isotype control (Figure 5.3 B, arrowhead). The same protein was detected in the THP-1 immunoprecipitate and it appeared to be stronger than the platelet GCR. The THP-1 GCR was again absent in the negative IgG isotype control. Thus the GCR protein could be successfully detected in human washed platelets by combined immunoprecipitation and immunoblotting techniques using the second mouse monoclonal anti-hGCR antibody (BD Biosciences). Platelets however appeared to express GCR protein weakly when compared to the THP-1 monocytic cell-line.

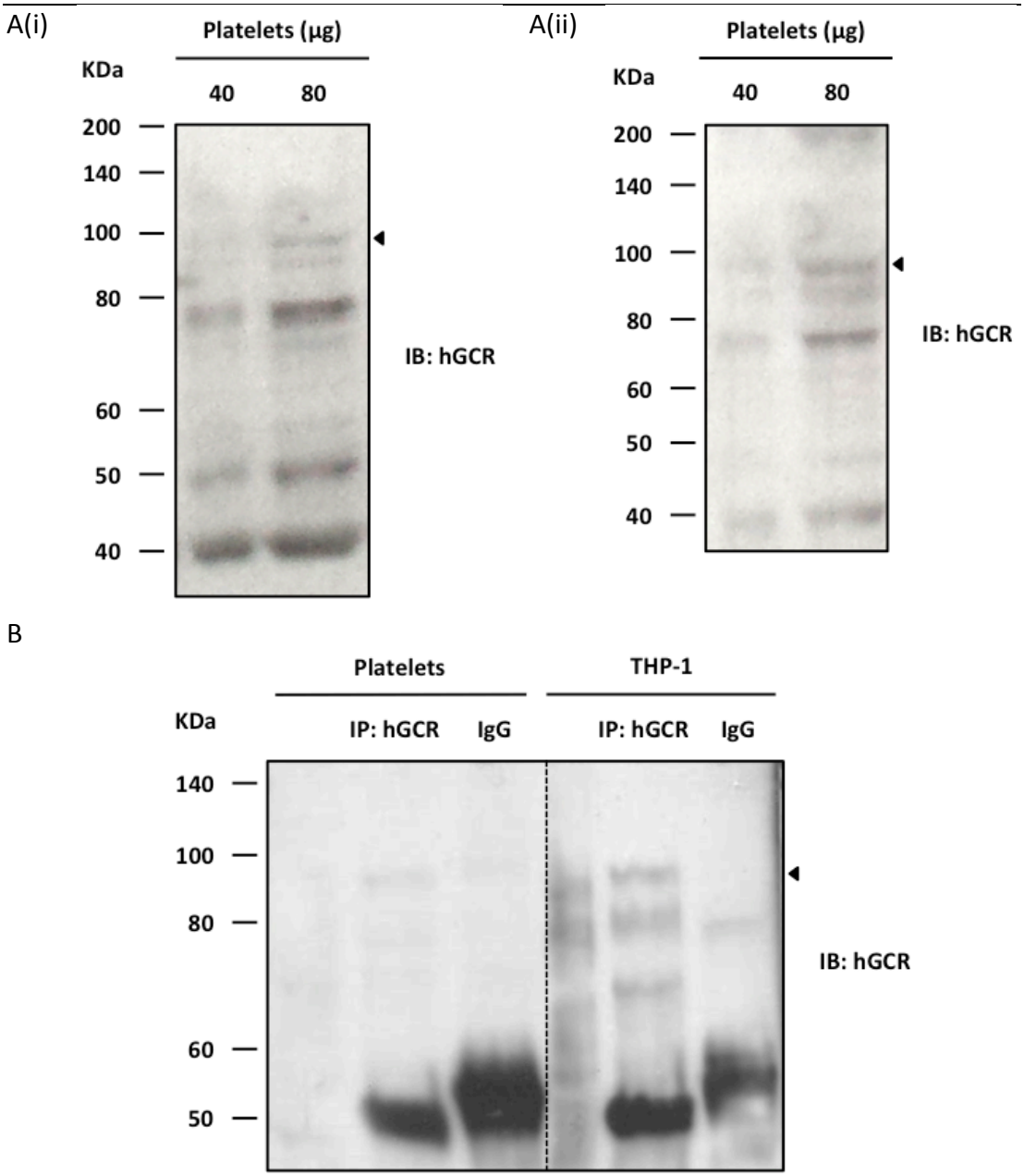


Figure 5.3 Detection of GCR in human washed platelets using another mouse monoclonal anti-hGCR antibody. (A) Washed platelets (8 × 10^8/ml) were lysed using 2X Laemmli buffer. Platelets proteins were separated using SDS-PAGE and immunoblotted for GCR using another mouse monoclonal antibody. Membranes were blocked with either 10% BSA (i) or 5% skimmed milk (ii). Representative immunoblot from a single experiment is shown. (B) Washed platelets (8 × 10^8/ml) were lysed using 1X lysis buffer followed by immunoprecipitation using protein A sepharose beads and either the GCR antibody (5 µg) or a control mouse IgG (1 µg). Immunoprecipitated samples were then resolved using SDS-PAGE and immunoblotted for GCR using the mouse monoclonal antibody. Representative immunoblot from a single experiment is shown.

5.4 Analysis of platelet GCR serine phosphorylation

Studies from various cell-lines reported basal phosphorylation of GCR at several serine residues, the most prominent ones being serines 203, 211 and 226 (Chen et al., 2008; Wang et al., 2002). Hence we wanted to investigate the phosphorylation status of GCR in human washed platelets. Here platelet lysates were subjected to immunoblotting using three phospho-specific antibodies that recognised GCR when it is phosphorylated at each of these epitopes. A band was detected by each of the phospho-antibodies between 80 and 100 KDa, which represented the phosphorylated forms of GCR (Figure 5.4 A). Unlike the antibodies, which were used to detect the GCR protein as detailed in the earlier section, these phospho-specific antibodies recognised very few non-specific proteins and thus the blots appeared clean. Using these phospho-specific antibodies bands were detected at the appropriate molecular weight as specified in the supplier datasheet (predicted molecular weights of 85 KDa for phospho-GCR-ser^{203} and phospho-GCR-ser^{226} and 95 KDa for phospho-GCR-ser^{211}). Under basal conditions platelet GCR was differentially phosphorylated on its serine residues with maximal phosphorylation detected at ser^{203} followed by ser^{211} and ser^{226}, respectively (Figure 5.4 A).

Immunoprecipitation of phosphorylated GCR was then attempted using the phospho-GCR-ser^{211} antibody followed by immunoblotting with the same antibody. No phosphorylated GCR protein was detected in the immunoprecipitated platelet lysate (Figure 5.4 Bi, lane 2), although the protein was present in platelet lysate (Figure 5.4 Bi, lane 1). When immunoblotting of the immunoprecipitated sample was carried out using the rabbit polyclonal anti-hGCR antibody, again no GCR protein was detected in the immunoprecipitated lysate (Figure 5.4 Bii, lane 2) despite the presence of the protein in platelet lysate (Figure 5.4 Bii, lane 1). Thus this particular phospho-GCR-ser^{211} antibody could not precipitate the phosphorylated form of GCR from platelets.

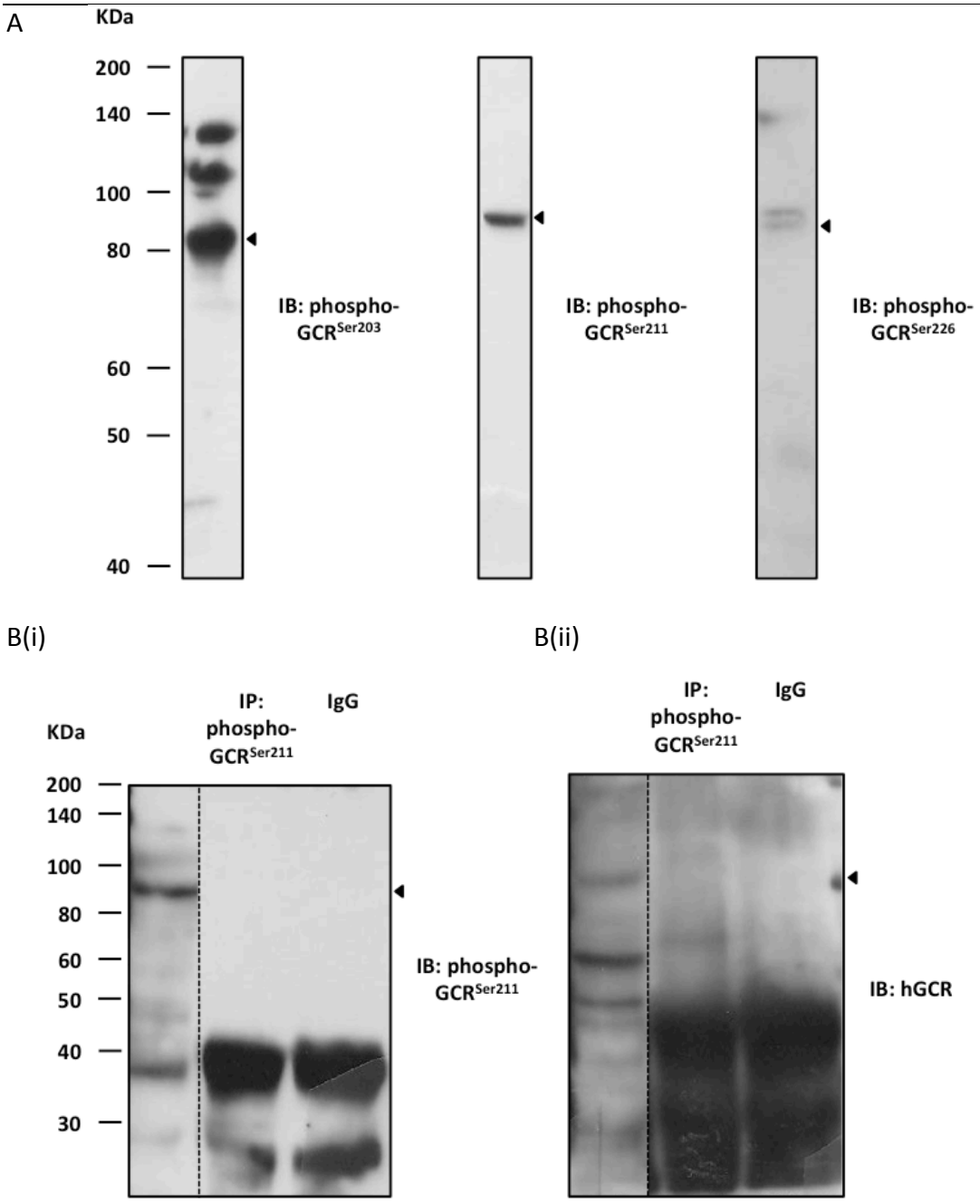


Figure 5.4. Detection of phosphorylation of GCR in human washed platelets. (A) Washed platelets (8 × 10^8/ml) were lysed using 2X Laemmli buffer. Platelets proteins were separated using SDS-PAGE and immunoblotted for phospho-GCR-ser^{203}, phospho-GCR-ser^{211} and phospho-GCR-ser^{226}. Representative immunoblot from a single experiment is shown. (B) Washed platelets (8 × 10^8/ml) were lysed using 1X lysis buffer followed by immunoprecipitation using protein A sepharose beads and the phospho-GCR-ser^{211} antibody (3 µg) or a control rabbit IgG (1 µg). Immunoprecipitated samples were then resolved using SDS-PAGE and immunoblotted for GCR using either phospho-GCR-ser^{211} antibody (i) or rabbit polyclonal anti-hGCR antibody (ii). Representative immunoblot from a single experiment is shown.

5.5 Platelet GCR phosphorylation following glucocorticoid (GC) treatment

Reports from various cell-lines have indicated that following interaction with the GC ligands, GCR phosphorylation status gets altered. Thus we decided to look into phosphorylation of platelet GCR at serine residues 203, 211 and 226 following treatment of washed platelets with various concentrations of prednisolone for 5 minutes. Basal phosphorylation of GCR at all these sites was found to be reduced following prednisolone treatment (Figure 5.5). Maximal reduction of basal phosphorylation at both Ser^{203} and Ser^{226} was seen with the lowest prednisolone concentration tested (0.1 μM), while minimal reduction was observed with the highest concentration (100 μM) (Figure 5.5 A and C). Basal GCR phosphorylation at Ser^{226} was completely abolished with all the prednisolone concentrations (Figure 5.5 C). Prednisolone reduced basal GCR phosphorylation at Ser^{211} too in a concentration-dependent manner with maximal reduction observed with 100 μM and minimal reduction with 0.1 μM prednisolone (Figure 5.5 B).

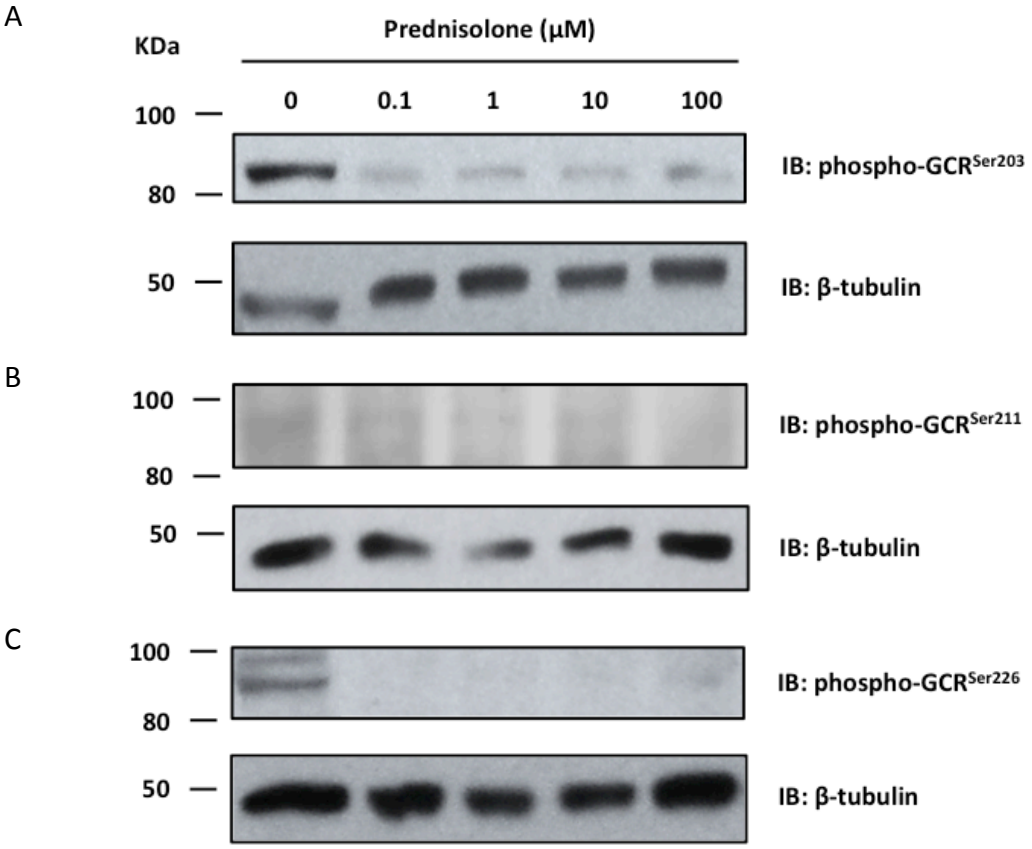


Figure 5.5 Changes in phosphorylation status of GCR in platelets upon prednisolone treatment. Washed platelets (× 10^8/ml) were incubated with apyrase (2 U/ml), indomethacin (10 μM) and EGTA (1 mM) for 20 minutes before performing further experiments. Platelets were then treated with various concentrations of prednisolone (0.1-100 μM) for 5 minutes. Samples were then lysed, resolved by SDS-PAGE and immunoblotted for phospho-GCR^{Ser203} (A), phospho-GCR^{Ser211} (B) and phospho-GCR^{Ser226} (C). Membranes were later re-probed for β-tubulin. Representative immunoblots from three independent experiments are shown.

5.6 Detection of glucocorticoid receptor (GCR) in the human MEG-01 cell line

Before studying the effects of prednisolone on maturation of Mks in culture, it was important to determine whether Mks expressed glucocorticoid receptor (GCR). To address this, we examined a megakaryoblastic cell-line called MEG-01. These cells were isolated from the bone marrow of a 55-year old male patient with blast crisis of chronic myelogenous leukemia (CML) and were shown to possess Mk-like properties after thorough characterisation (Ogura et al., 1985). Ever since, they have been used in several Mk-related studies and provide as a cell-line model of Mk function. We maintained MEG-01 in culture as per supplier's guidelines and prepared lysates that were analysed by immunoblotting for the expression of GCR using a mouse monoclonal anti-hGCR antibody (BD Biosciences). This antibody was also used for GCR detection in human washed platelets (chapter 6, section 5.3.2.2). Immunoblotting revealed a protein band just below 100 KDa in MEG-01 lysates, which appeared stronger with higher amount of lysates loaded. A similar protein occurred in washed platelets too (Figure 5.6 A). From chapter 6, this protein was confirmed to be GCR by immunoprecipitation and immunoblotting. Thus MEG-01 cells were shown to express GCR by immunoblotting. The GCR protein appeared at the expected molecular weight when membranes were blocked with milk (Figure 5.6 A, left panel). When BSA was used as a blocking reagent, a protein of slightly lower molecular weight than GCR was detected (Figure 5.6 A, right panel). Thus for future immunodetection of GCR in MEG-01 cells, milk was used the blocking reagent.

To confirm the identity of the protein band corresponding to GCR in MEG-01, immunoprecipitation of lysates were carried out using the same mouse monoclonal antibody followed by immunoblotting of immunoprecipitated samples. GCR protein appeared in immunoprecipitated samples similar to lysates, but not in the IgG isotype control (Figure 5.6 B). As controls, washed platelets and THP-1 cells were also appointed and GCR appeared in both cases in the immunoprecipitated samples and lysates, but not in IgG controls. GCR expression was found to be the highest in MEG-01 cells followed by THP-1 cells and platelets, respectively (Figure 5.6 B).

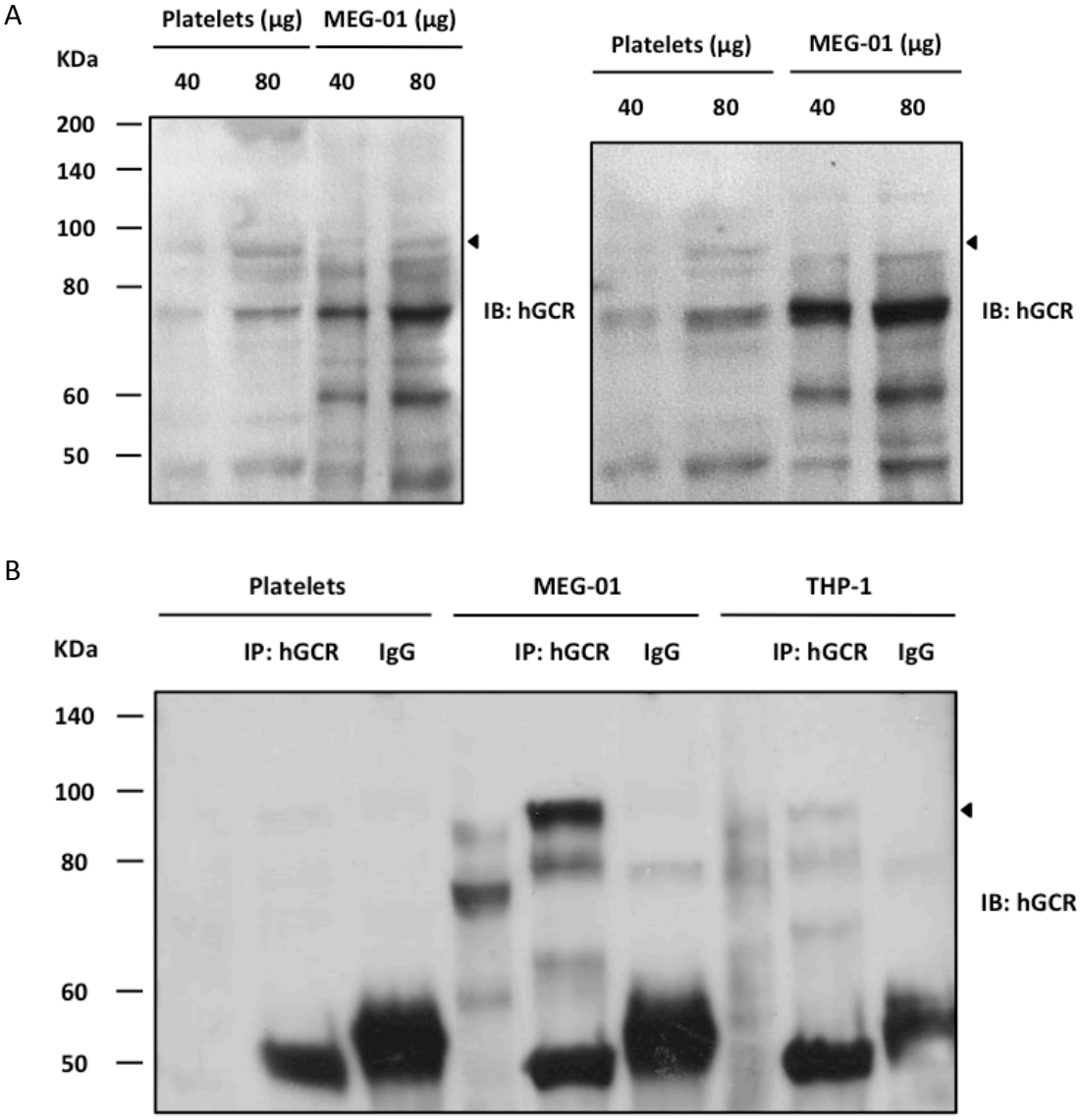


Figure 5.6 Detection of GCR in the human MEG-01 cell line. (A) MEG-01 cells and washed platelets were lysed using 2X Laemmli buffer. Lysates were separated using SDS-PAGE and immunoblotted for GCR using a mouse monoclonal antibody. Membranes were blocked with either 5% skimmed milk (left panel) or 10% BSA (right panel). Representative immunoblot from a single experiment is shown. (B) MEG-01 cells, washed platelets and THP-1 cells were lysed using 1X lysis buffer. Lysates (500 µg) were pre-cleared by incubation with protein A sepharose beads and mouse IgG for 2 hours at 4°C under rotation. Lysates were then incubated overnight with protein A sepharose beads and either the GCR antibody (5 µg) or a control mouse IgG (1 µg) at 4°C under rotation. Beads were pelleted by centrifugation, washed and eluted with 2X Laemmli buffer. Eluted samples containing immunoprecipitated proteins were then resolved using SDS-PAGE and immunoblotted for GCR using the mouse monoclonal antibody. Representative immunoblot from a single experiment is shown.

5.7 Effect of prednisolone on Mk differentiation from murine bone marrow derived Mk committed progenitors

The differentiation of Mks from committed bone marrow-derived Mk progenitors under *in vitro* culture conditions has been well established (Dumon et al., 2006; Mazharian et al., 2009; Schachtner et al., 2013; Senis et al., 2007). Hence we sought to pursue the possibility if prednisolone might affect Mk differentiation and maturation using these established methodologies.

5.7.1 Effect of prednisolone on the ploidy of murine bone marrow derived Mks

By day 5-6 of isolation from murine bone marrow, Mk progenitors propagated in the presence of the cytokines stem cell factor (SCF) and murine Tpo were shown to differentiate into Mks, visually characterised by their larger size in comparison to other cells in the culture medium. When analysed for their DNA content by flow cytometry, the entire Mk population appeared to consist of five distinct subpopulations displaying increased ploidy ranging from the usual diploid (2N) population up to its fifth exponential, i.e. 4N, 8N, 16N and 32N (Figure 5.7, Ai and ii). When prednisolone was added in the medium during differentiation, the progenitors appeared to give rise to the same five distinct Mk polyploid subpopulations (Figure 5.7, Bi and ii). No significant difference was observed in the percentage of Mks in each of these polyploid populations between the untreated and prednisolone treated Mk progenitors ($p=0.39$ for 2N, 0.43 for 4N, 0.36 for 8N, 0.29 for 16N and 0.39 for 32N) (Figure 5.7 C).

5.7.2 Effect of prednisolone on CD41 expression of murine bone marrow Mks

The effect of prednisolone on CD41 expression during Mk differentiation was then determined. Prednisolone treatment caused no significant difference ($p=0.35$) in the percentage of Mks that expressed CD41 (Figure 5.8 Ai). However, when analysed for the amount of CD41 expressed per Mk as determined by mean fluorescence intensity (MFI), prednisolone treatment appeared to reduce CD41 expression in the Mk population, albeit the change was non-significant ($p=0.14$) (Figure 5.8 Aii). Prednisolone also insignificantly reduced the MFI for CD41

expression within the different Mk polyploid subpopulations when compared to untreated Mks (p=0.19 for 2N, 0.22 for 4N, 0.17 for 8N, 0.19 for 16N and 0.25 for 32N) (Figure 5.8 B).

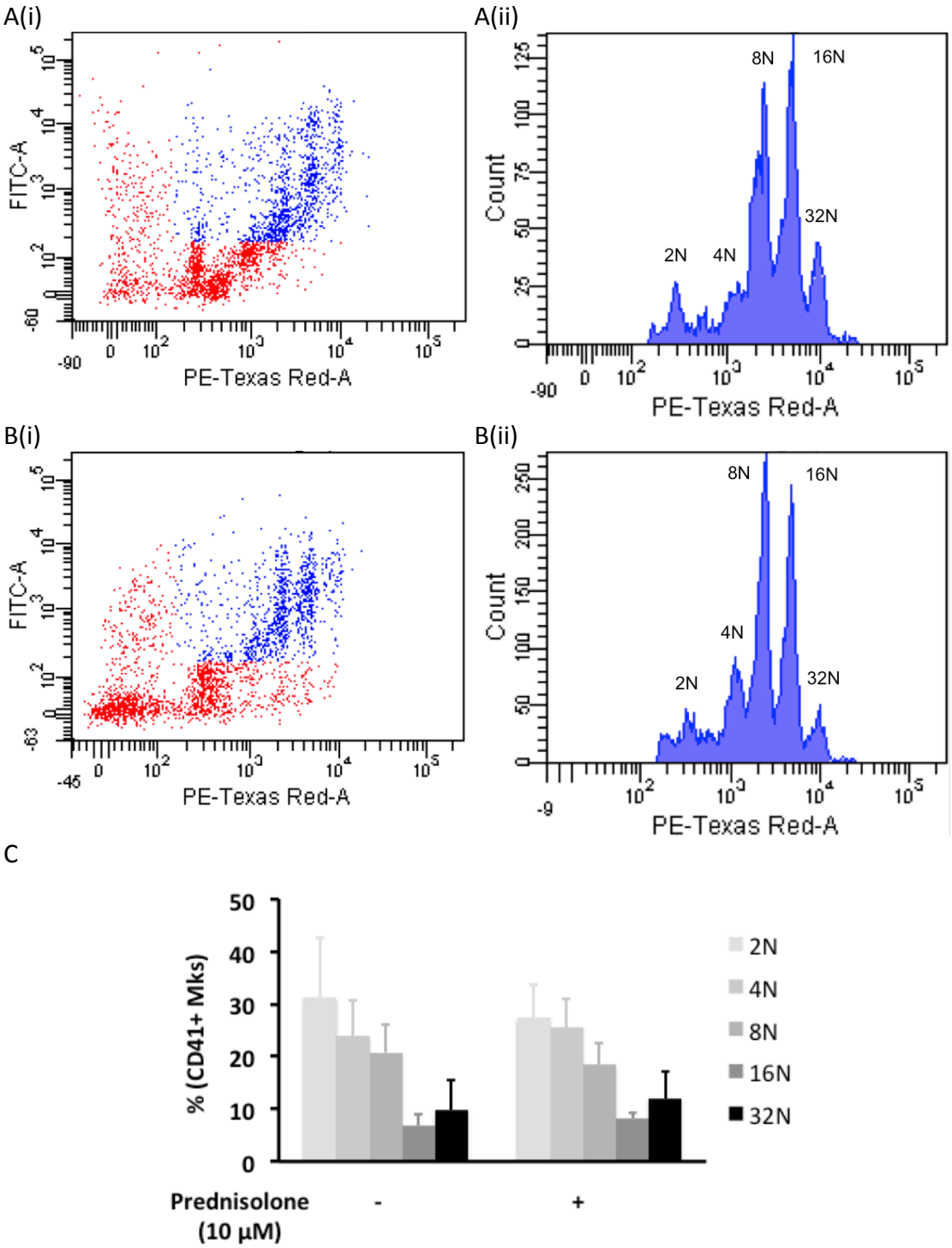


Figure 5.7 Effect of prednisolone on the ploidy of murine bone-marrow derived Mks. Mks were isolated from the bone marrow of C57BL/6 wild type mice and cultured for 6 days in DMEM containing 20 ng/ml stem cell factor (SCF) and 50 ng/ml murine thrombopoietin (TPO) either in the absence (A) or presence of 10 µM prednisolone (B). Mks were purified over a BSA gradient and incubated with an anti-CD41 mouse monoclonal antibody conjugated to FITC for 1 hour at 4°C. Cells were fixed in 0.5% paraformaldehyde (PFA) and permeabilised in 0.05% saponin. Mks were incubated with the DNA staining dye propidium iodide (PI) overnight at

4°C. Cells were then washed in phosphate buffer saline (PBS) and analysed in the FACSCalibur™ flow cytometer to determine their ploidy. An unlabelled Mk sample was first run and the Mk population (represented by red dots) was gated by adjusting the forward scatter (FSC) and side scatter (SSC). Thereafter labelled Mk samples were run and another gate was drawn within the Mk population around the cells positive for both CD41 (FITC) and PI (PE-Texas Red) labels (indicated by blue dots). Representative dot plots (Ai and Bi) and histograms (Aii and Bii) from eight different mice are indicated. The peaks on the histograms represent different subpopulations of Mk having DNA content that are exponentials of a normal diploid DNA content, i.e. 2N, 4N, 8N, 16N and 32N. Representative bar graph (C) indicates the percentage of Mks in each polyploid subpopulation.

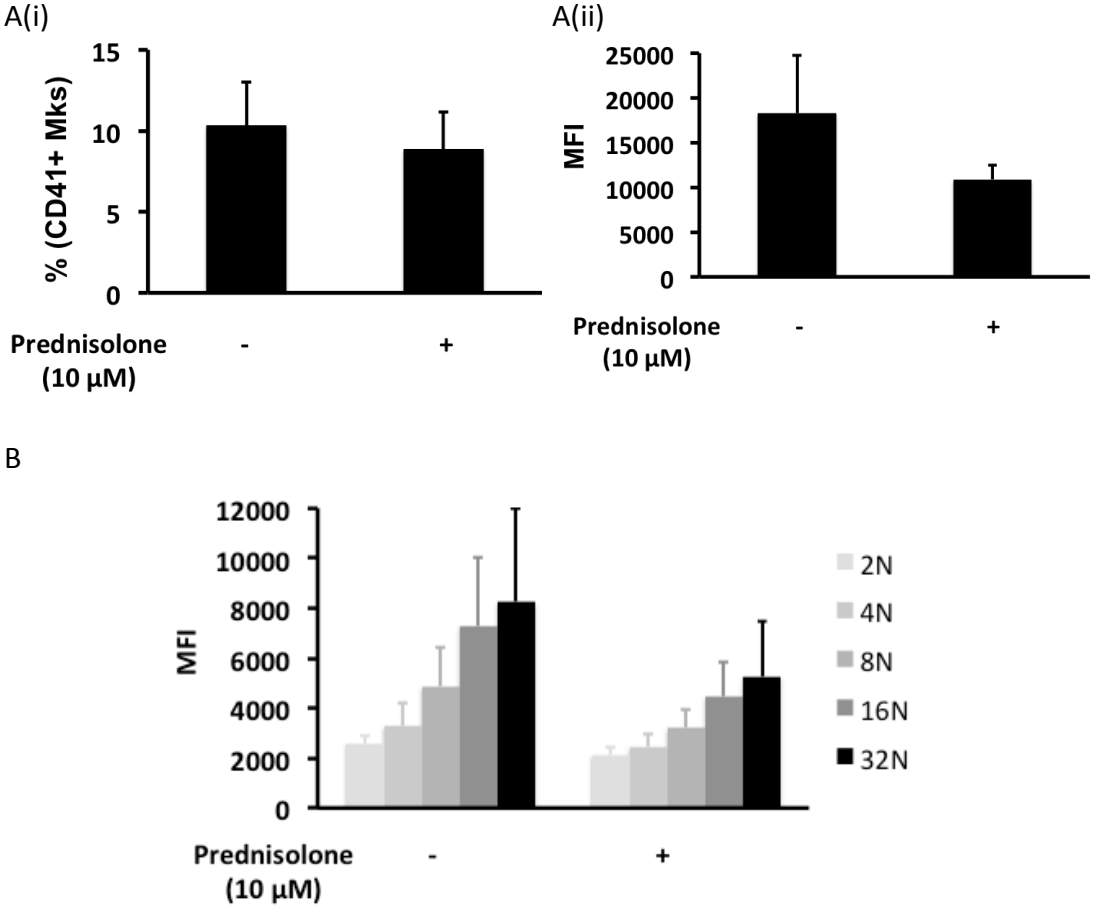


Figure 5.8 Effect of prednisolone on CD41 expression of murine primary bone-marrow derived Mks. Mks were isolated from the bone marrow of C57BL/6 wild type mice and cultured for 6 days in DMEM containing 20 ng/ml stem cell factor (SCF) and 50 ng/ml murine thrombopoietin (TPO) either in the absence or presence of 10 µM prednisolone. Mks were purified over a BSA gradient and incubated with an anti-CD41 mouse monoclonal antibody conjugated to FITC for 1 hour at 4°C. Mks were fixed in 0.5% paraformaldehyde (PFA) and permeabilised in 0.05% saponin. Mks were incubated with the DNA staining dye propidium iodide (PI) overnight at 4°C. Mks were then washed in phosphate buffer saline (PBS) and analysed in the FACSCalibur™ flow cytometer to determine their CD41 expression. The Mk population was identified and cells positive for both CD41 and PI labels were identified. Representative bar graphs indicate the percentage of CD41-positive cells (Ai) as well as the mean fluorescence intensity (MFI) for CD41 expression within the CD41-positive Mks (Aii) and the different polyploid subpopulations (B) from eight mice.

5.8 Discussion

This chapter presented data to confirm the potential presence of the glucocorticoid receptor (GCR) in human platelets and also presented evidence that GCR in platelets, like most proteins, underwent post-translational modifications in the form of protein phosphorylation. However, the investigation was hampered by poor quality antibodies.

5.8.1 Expression of the GCR protein in human platelets

Flow cytometry revealed the presence of hGCR protein in human washed platelets using a polyclonal antibody raised against an epitope on hGCR. A strong shift in signal compared to the negative IgG control encouraged the idea that the hGCR protein was present in abundance in platelets. However, using the same antibody and immunoblotting we could not validate the presence of hGCR protein. Although a band corresponding to hGCR protein appeared in platelet samples and the positive control THP-1 cell-line, an array of non-specific bands was also revealed during immunoblotting. This observation meant that the signal obtained from this antibody in flow cytometry could arise from greater binding to non-specific proteins. Thus, the specificity of this particular antibody was questioned. An alternative mouse monoclonal antibody from BD Biosciences recognised the protein and also solved some issues of non-specific binding, as it recognised fewer non-specific proteins through immunoblotting and immunoprecipitation. The hGCR protein however appeared to be expressed at very low levels in platelets when compared to the THP-1 cell line. Thus, our ability to definitively identify the presence of GCR in platelets was complicated by the quality of the antibodies available and it is noteworthy that many of the groups working with GCR generated antibodies “in house” rather than from commercial sources (Blind and Garabedian, 2008; Chen et al., 2008; Cidlowski et al., 1990; Galliher-Beckley et al., 2011; Wang et al., 2002). A useful and informative method to ascertain the presence of GCR in platelets at the mRNA level will be quantification using real-time PCR, which we could not perform due to time constraints. Such an analysis is essential to add an extra dimension to the field of nuclear receptors in platelets. The differential

expression of GCR mRNA levels across several donors could be investigated and quantified with more precision using this technique compared to immunoblotting.

An interesting observation that emerged during some of the GCR immunoprecipitation experiments was the appearance of an additional protein band around 200 KDa. GCR is known to dimerise before binding to specific sequences on its target genes. While the formation of GCR homodimers is quite established, some studies have shown the existence of GCR heterodimers with mineralocorticoid receptors (MCR) (Nishi et al., 2004; Savory et al., 2001). MCR, like GCR, also belongs to the family of nuclear receptors that behave as ligand-activated transcription factors. The presence of MCR in human platelets was reported earlier in the same study that identified the presence of GCR (Moraes et al., 2005). In the same study, MCR was shown to be immunoprecipitated by the GCR antibody indicating that these two proteins resided in a complex in human platelets. The molecular weight of MCR is reported to be 110 KDa, while the molecular weight of GCR was found to be around 100 KDa. Thus if GCR and MCR interact and form a complex, then the molecular weight of the complex should be around or just above 200 KDa. Thus the appearance of the protein around 200 KDa detected in the GCR immunoprecipitates could be the GCR-MCR complex, which was also precipitated by the antibody and thereby detected in the immunoblots. It might be informative to perform immunoprecipitation of platelet proteins with an antibody raised against human MCR and then immunoblot for GCR to verify the existence of GCR-MCR complex. Besides, if a specific MCR antibody were available, this approach could be an alternative strategy to validate the presence of GCR in platelets considering the non-specific nature of the GCR antibodies employed in this study for GCR detection.

5.8.2 Analysis of phosphorylation status of platelet GCR

Data from numerous cell-lines have revealed that, like most other proteins, GCR also undergoes several post-translational modifications such as phosphorylation, acetylation, ubiquitination and SUMOylation (Anbalagan et al., 2011; Bodwell et al., 1995; Ito et al., 2006; Tian et al., 2002; Wallace and Cidlowski, 2001). Of these

events, GCR phosphorylation has been studied extensively and GCR is found to be phosphorylated on several serine and threonine residues (Ismaili and Garabedian, 2004; Krstic et al., 1997). These residues are reportedly clustered mainly in the amino-terminal domain of GCR (Hoeck and Groner, 1990), which determines the transactivation function of GCR, although some have been found also within the central DNA-binding domain (DBD) and carboxyl-terminal ligand-binding domain (LBD) (Galliher-Beckley and Cidlowski, 2009; Rao and Fox, 1987).

The three best characterised serine residues (203, 211 and 226) are within the N-terminal transactivation domain of GCR. In the absence of GCs, maximal GCR phosphorylation in other cells has been observed on ser^{203} followed by ser^{226} and ser^{211}, respectively (Chen et al., 2008; Wang et al., 2002). In agreement to these findings, our data indicated that in the absence of GCs platelet GCR was also differentially phosphorylated on its serine residues with maximal phosphorylation detected on ser^{203} and this observation was consistent across the donors tested. The phosphorylation levels at ser^{211} and ser^{226} were lower than that on ser^{203} and these levels were variable across donors with some donors expressing higher phosphorylation at ser^{211} than ser^{226}, while others having the reverse. Differential levels of phosphorylation were observed for each of these residues across donors as determined by immunoblotting.

GCR in other cells has been reported to be hyperphosphorylated in response to stimulation with a battery of GCs with prednisolone and dexamethasone being the most potent of all (Chen et al., 2008; Wang et al., 2002). The most dramatic increase of phosphorylation has been noted in case of ser^{211} followed by that on ser^{226} (Chen et al., 2008). Although GCs can increase phosphorylation on ser^{203}, the change is modest probably due to the high level of basal phosphorylation detected on this residue (Blind and Garabedian, 2008; Chen et al., 2008; Wang et al., 2002). Similarly, mouse and rat GCRs have also been reported to undergo hyperphosphorylation on similar homologous residues in response to GC treatment (Bodwell et al., 1995; Hoeck and Groner, 1990; Ortí et al., 1989). Thus we aimed to study the phospho-status of platelet GCR following prednisolone treatment. Instead

of an expected increase, there was a sharp decrease in basal phosphorylation of GCR at all the serine residues with stronger effects observed at ser^{203} and ser^{226}. Prednisolone also reduced GCR phosphorylation at ser^{211}, albeit the reduction was modest compared to the other serine residues. Phosphorylation at ser^{226} was completely abolished with all prednisolone concentrations. Thus, these data not only confirmed the change in phospho-status of GCR in platelets following prednisolone treatment, but also revealed that the outcome of GC treatment on GCR phosphorylation might be cell or tissue-specific. However, these observations are preliminary and warrant further investigations. A time-course of prednisolone treatment needed to be performed in order to understand the kinetics of GCR phosphorylation changes.

Two scenarios could explain the decrease in phosphorylation at the serine residues on GCR. Firstly, prednisolone treatment might deactivate kinases responsible for phosphorylating these residues in platelets. Several kinases are involved in site-specific phosphorylation of GCR in other cells, the most notable ones being cyclin-dependent kinases (Cdks) and mitogen activated protein kinases (MAPKs) including p38 MAPK, extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK). The most widely reported phosphorylated events are Cdk-mediated ser^{203} and ser^{211} phosphorylation (Krstic et al., 1997), ERK-mediated ser^{203} phosphorylation (Takabe et al., 2008), p38 MAPK-mediated ser^{211} phosphorylation (Miller et al., 2005) and JNK-mediated ser^{226} phosphorylation (Itoh et al., 2002). The outcome of prednisolone treatment on the activation of MAPKs and Cdks in platelets was not examined in our study and hence could be pursued in future. The second possibility behind reduction of GCR phosphorylation by prednisolone could be the activation of certain phosphatases that can dephosphorylate the receptor. GCR has been shown to be associated to protein phosphatase 5 (PP5) at its C-terminal region, which modulates GCR activity by dephosphorylating residues in its N-terminal region under basal conditions (Wang et al., 2007). The way to address whether prednisolone reduced GCR phosphorylation by activating phosphatases is to treat platelets with prednisolone in the presence of phosphatase inhibitors calyculin A (PP1, PP2A, PP2B and PP5) or okadaic acid (PP2A, PP2B and PP1) and

analyse the phospho-status of GCR. If prednisolone failed to affect GCR phosphorylation in platelets in the presence of these phosphatase inhibitors, it would indicate that prednisolone-mediated decrease of GCR phosphorylation is carried out through activation of phosphatases.

GCR phosphorylation has been implicated in several aspects of GC signalling such as GCR subcellular localisation, protein stability and GCR-mediated transactivation of its target genes and the outcomes seem to be kinase-specific. For instance, GC-induced GCR phosphorylation on ser^{211} has been shown to encourage nuclear localisation of GCR (Wang et al., 2002) and is also indispensible for maximal GCR-mediated transactivation of its target genes. GCR transactivation is blunted in the presence of ser^{226} phosphorylation and thus suggests ser^{226}-mediated antagonism on GCR-mediated transactivation (Chen et al., 2008). Thus, the transactivation potential of GCR appears to be regulated by the relative phosphorylation levels at ser^{211} and ser^{226}. Besides, JNK-mediated ser^{226} phosphorylation antagonises nuclear retention of GCR by promoting its nuclear export of GCR after GC withdrawal (Itoh et al., 2002). The consequence of GCR phosphorylation on ser^{203} seems to be unclear. In rat GCR, phosphorylation on ser^{224} (homologous to human ser^{203}) by Cdk2 was shown to be important for maximal transactivation of target genes, while ERK-mediated phosphorylation on the same site inactivated the receptor (Krstic et al., 1997). In human intestinal GCR, ser^{203} phosphorylation prohibited GCR nuclear localisation as well as GCR-mediated transcriptional regulation (Takabe et al., 2008). GSK3β-mediated GCR phosphorylation at ser^{404} in response to GCs was shown to promote GCR nuclear export, reduce GCR protein stability and blunt GCR-mediated transcriptional regulation of target genes. Interestingly, ser^{404} phosphorylation encouraged GCR transcriptional regulation of a distinct set of genes (Galliher-Beckley et al., 2008). Taken together, all these studies highlight that GCR phosphorylation is a key determinant for overall GCR function. Hence, it will be interesting to understand whether the alteration of phospho-status of platelet GCR might have any functional implication on platelets. It might be an interesting approach to study the phospho-status of GCR in donors upon prednisolone administration simultaneously with the effect of prednisolone on platelet

aggregation and determine if these observations correlate with each other. Variability observed in prednisolone-mediated effects on thrombin-induced platelet aggregation across donors could be attributable to the sensitivity of GCR phospho-status in response to prednisolone.

Two different isoforms have been reported for the GCR, which are denoted as GCRα and GCRβ (Bamberger et al., 1995; Kino et al., 2009). Differential levels of GCRα and GCRβ mRNA have been detected in a tissue-specific manner with GCRα mRNA being expressed several fold higher than GCRβ mRNA. Eosinophils and PBMCs were shown to contain lower levels of GCRα and higher levels of GCRβ when compared to brain cortex (Pujols et al., 2002). Thus, it might be useful to study the expression of GCR α and β mRNA and protein isoforms in human platelets too using specific primers and antibodies that can distinguish between them.

5.8.3 GCR expression in Mks

The first step was to determine the expression of GCR in Mks. Murine Mks were obtained through *in-vitro* differentiation of murine bone marrow-derived progenitors in the presence of SCF and TPO. The amount of mature Mks obtained at the end of the differentiation process was too few to be sufficient for the analysis of GCR expression by immunoblotting. Hence we decided to study GCR expression in the widely used MEG-01 cell-line. GCR was detected in the whole cell lysates of MEG-01 by immunoprecipitation and immunoblotting (Figure 5.6). However, it is important to mention that the lysate was prepared from an entire population of MEG-01 cells that were not differentiated into mature Mks. MEG-01 cells consist of a heterogenous population of cells at various differentiation stages. Thus the presence of GCR in the MEG-01 whole cell lysate does not necessarily indicate that terminally differentiated Mks, or in other words, mature Mks would specifically express GCR. Hence, it would have been ideal to obtain mature Mks by differentiating the MEG-01 cells with the phorbol ester PMA, followed by flow cytometric analysis of CD41 expression in the mature Mks and immunoblotting of lysates from CD41-expressing Mks to determine GCR expression in mature Mks.

5.8.4 Effect of prednisolone on maturation of Mks

Although prednisolone and other GCs are largely popular in the clinic for their anti-inflammatory and immunosuppressive roles, these drugs are also commonly employed as therapeutic measures to treat idiopathic or immune thrombocytopenic purpura (ITP). This is a clinical condition associated with low platelet counts due to accelerated platelet destruction by autoantibodies that bind platelet proteins and target them for degradation by tissue macrophages. Both prednisolone and its drug precursor prednisone have proven to be fairly efficacious in promoting disease remission and increasing platelet counts to the normal range, although its long-term administration is often associated with disease relapse (Cines and Bussel, 2005; Stasi and Provan, 2004). In another study, prednisone was shown to increase the percentage of mature Mks and reduce those presented with ultrastructural features of apoptosis and para-apoptosis in the bone-marrow of an ITP patient (Houwerzijl et al., 2004). These reports suggested that prednisolone promotes platelet production and also supports Mks to retain their normal morphology. Thus, prednisolone could also possibly encourage Mk differentiation and maturation. Hence we sought to determine the effect of prednisolone on two key features of Mk maturation, ploidy and CD41 expression. To our knowledge, this is the first attempt to understand whether prednisolone might influence the processes of megakaryocytic differentiation and maturation.

5.8.4.1 Effect of prednisolone on polyploidisation of Mks

In the presence of the cytokines SCF and TPO bone-marrow derived murine megakaryocytic progenitors appeared to undergo terminal differentiation into Mks with a characteristic huge size that was easily distinguishable from the remaining cells in the medium under a light microscope. When analysed for their DNA content by staining with propidium iodide, Mks contained five subpopulations with vaying ploidy from 2N up to 32N. This observation is in line with previous studies (Mazharian et al., 2009; Senis et al., 2007). Prednisolone treatment did not affect either Mk differentiation into the five polyploid subpopulations or the percentage of Mks in each of the subpopulations when compared to control. This indicates that

prednisolone has no effect in murine Mk maturation, at least in terms of endomitosis and polyploidisation. Mk morphology under the light microscope also appeared to be unaffected (data not shown).

5.8.4.2 Effect of prednisolone on CD41 expression of Mks

Polyploidisation is one of the markers of Mk differentiation and maturation. Another useful determinant of Mk maturation is the expression of certain glycoproteins such as CD41 (GPIIIa), CD61 (GPIIb) and GPIb. CD41 associates with CD61 to form the GPIIb/IIIa or CD41/CD61 complex, which also appears in platelets as the integrin $\alpha_{IIb}\beta_3$ that acts as a receptor for adhesive proteins such as fibrinogen. Thus, it was also important to study CD41 expression in both primary murine Mks and determine if prednisolone can alter CD41 expression.

When murine bone marrow derived Mks were analysed for CD41, only 10% of the Mks appeared to be CD41 positive. This observation is very unusual considering the fact that CD41/CD61 appears quite early in megakaryopoiesis (Ogura et al., 1985) and is retained in platelets too at high levels. A possible explanation for the occurrence of only a few CD41-positive Mks could be the dual-staining technique. Mks were stained with CD41 followed by fixation and permeabilisation of cells to allow propidium iodide to enter the cells and stain the nucleus. Since CD41 is a surface molecule, the permeabilisation step could have damaged most of the interaction of the antibody with the surface molecules. It might have been ideal to analyse the Mks for CD41 expression straight after CD41 staining and fixation to minimise the loss of signal. An earlier work on murine primary Mks too analysed the expression of CD41 and other glycoproteins in isolation (Mazharian et al., 2009). It also documented a protocol for analysis of ploidy on CD61-positive Mks where Mks were stained for CD61, fixed and stained with propidium iodide. The permeabilisation step was omitted possibly to avoid loss of CD61 signal. In order to ensure that propidium iodide entered the cells, it was prepared in a solution of saponin (Mazharian et al., 2009). In this way, both the CD61 and PI signals could be maintained with equal strength. Hence for future work, the above protocol should be employed for ploidy analysis. Prednisolone reduced the amount of CD41

expressed per Mk in the total Mk population and also in all the polyploid subpopulations when compared to control, without affecting the percentage of CD41 expressing Mks. However, since the data was insignificant, it needs further validation.

Another glycoprotein GPIb or CD42b, appears on more mature Mks (Greenberg et al., 1988; Ogura et al., 1985). Its expression is retained in platelets, where it associates with GPV and GPIX to form a heterotrimeric complex GPIb/V/IX that acts as a receptor for the subendothelial matrix protein vWF. Thus, it appears that CD42b is a more accurate marker of Mk maturation. Hence, the assessment of the effect of prednisolone on CD42b expression in primary murine Mks seems a more logical approach in the context of assessing the effect of prednisolone on Mk maturation.

A few preliminary assays were performed to study the effect of prednisolone on the ability of Mks to undergo spreading and form proplatelets. The data indicated that prednisolone had no effect on these aspects of Mk differentiation. The data requires further validation.

5.9 Conclusion

Overall this chapter reveals the possible presence of GCR in human platelets and identifies basal phosphorylation of the receptor on three serine residues. It will be interesting to characterise receptor complexes and interaction of GCR with key regulators of platelet activation.

Chapter 6

General discussion

Glucocorticoids (GCs) are a group of hormones that contribute significantly to several processes such as growth, development, metabolism, cognition, immunity, reproduction, homeostasis and stress management. Under conditions of starvation and stress, GCs perform a number of functions that cumulatively result in the catabolism of carbohydrates, proteins and fats to release energy for the sustainment of various life processes (Ashmore, 1964; Macfarlane et al., 2008; Peckett et al., 2011). Another very important function performed by GCs is the suppression of inflammatory responses for which they have garnered an enormous recognition in the clinical field and have been actively administered as anti-inflammatory therapeutics over the past decades (Coutinho and Chapman, 2011). Atherothrombosis is a typical inflammatory condition involving blood platelets, leukocytes as well as immune cells in the blood such as monocytes/macrophages that often results in the development of cardiovascular diseases (CVD). Platelets, which are commonly held responsible for the thrombotic events post atherosclerotic plaque rupture, also are thought to contribute to endothelial dysfunction, release of inflammatory mediators and enhanced monocyte recruitment to the endothelium (Dalli et al., 2008; Huo et al., 2003; Norling et al., 2008; Schober et al., 2002), all of which are attributes for the development of atherothrombosis. Thus, being endowed with anti-inflammatory properties, the impact of GCs on platelet activation needs to be evaluated as a potential step in the search for molecules that can inhibit platelet function in disease states.

The discovery of possible non-genomic effects of glucocorticoids has revived interest in these important biological agents. Platelets, being anucleate, had been proposed to be an excellent model to study nuclear receptor-mediated non-genomic effects of hormones in isolation (Bishop-Bailey, 2010). A previous study established the presence of GCR in human platelets and GCR-mediated inhibitory effects of the synthetic GC prednisolone on platelet activation in human plasma

(Moraes et al., 2005). It also revealed the specificity of the nuclear receptor-ligand interaction in human platelets, as dexamethasone and other GCs failed to elicit similar responses. The findings of the study held a great deal of importance considering the potential clinical implications of prednisolone in the treatment of CVD arising from platelets. Thus it was essential to characterise the inhibitory properties of prednisolone in platelet function in greater detail and investigate the underlying mechanisms.

With the above aims set for this study, we were able to present the following key findings:

- Prednisolone exerted inhibitory effects on aggregation in washed human platelets following thrombin and ADP stimulation.
- Prednisolone suppressed platelet function in its physiological milieu, plasma.
- Prednisolone targeted the RhoA/ROCK-mediated signalling events in platelets following thrombin stimulation by suppressing biochemical reactions such as RhoA activation and phosphorylation of the myosin light chain phosphatase (MYPT1-thr^{696} and MYPT1-thr^{853}) and myosin light chain (MLC-ser^{19}).
- The inhibitory properties of prednisolone on platelets appeared to be non-genomic owing to the rapid onset (1-5 minutes), and the effects were reversed in less than 30 minutes of treatment.
- Prednisolone mediated its effects through the GCR since it effects were reversed in the presence of the classical GCR antagonist, mifepristone (RU486).

Despite the above important observations, there were significant challenges encountered during the study. Prednisolone-mediated inhibition of platelet aggregation induced by thrombin was dependent on the concentration of thrombin used to stimulate platelets. This observation was quite similar to the dependence of the platelet inhibitor GSNO on the concentration of thrombin to inhibit platelet

aggregation (chapter 3). At higher thrombin concentrations prednisolone completely failed to inhibit platelet aggregation, in contrast to GSNO that could still exert a modest inhibitory effect on aggregation. Thrombin response in terms of platelet aggregation is highly variable throughout donors. Hence for these experiments, we used an approach of examining the effects of prednisolone at consistent levels of aggregation, to achieve this we used variable thrombin concentrations that induced approximately 60% aggregation rather than a set concentration of the agonist. Using this approach we were able to demonstrate that prednisolone could inhibit thrombin-induced aggregation. However, the major issue we faced was the variability in the ability of prednisolone to inhibit platelet between different donors. Prednisolone-mediated suppression of platelet aggregation appeared to be donor-specific with platelets from a number of donors being absolutely non-responsive to prednisolone treatment. Prednisolone-mediated reduction of signalling events in platelets in response to thrombin stimulation was also found to be donor-specific. Platelets from donors that responded to prednisolone were shown to have reduced RhoA/ROCK-mediated signalling that was manifested in every marker of this pathway tested from RhoA activation to MLC-ser^{19} phosphorylation. Similarly, non-responsiveness to prednisolone in certain donors was maintained in each step of this signalling cascade, negating the possibility of any false-positive results. Thus, non-responsiveness to prednisolone observed in a group of donors was not only manifested in platelet functional responses, but was also present in signalling events underlying these responses. Prednisolone non-responsiveness has been noted in several patients suffering from inflammatory disorders (Adcock, 2000; Barnes, 2001), who are on prednisolone treatment. This phenomenon is popularly known as GC resistance, which is the outcome of several biochemical mechanisms that can explain this condition (discussed in Chapter 1). It was interesting to observe insensitivity to prednisolone in platelets from a group of healthy volunteers too.

Response of a cell to any biochemical molecule normally relies on the series of biochemical signals that are generated upon ligation of the molecule to its cellular

receptor. Thus receptor expression acts as a crucial determinant to the overall physiological response. A published study revealed that variable expression of macrophage scavenger receptors across individuals was intimately associated with the variability observed in their inflammatory response to oxidised low-density lipoproteins (ox-LDL) (Martín-Fuentes et al., 2007). Hence, it is possible that differential platelet response to prednisolone observed across the donors recruited in our study could be attributed to the GCR expression in these donors. In order to address this possibility, it was essential to perform both aggregation and signalling experiments on each donor as well as analyse GCR expression in platelets. Unfortunately, it was not feasible to retrieve the desired amount of platelets sufficient for the all the above experiments from each donor. However, we were able to isolate sufficient platelets from 6 donors to examine GCR expression and prednisolone responses. When GCR expression in platelets of these 6 donors was evaluated by immunoblotting, all the donors tested were shown to express GCR in their platelets, albeit in donor 1 the protein appeared as a faint band that was barely detectable (Figure 6.1). Interestingly differential amounts of GCR protein were observed across the donors with donor 6 showing the highest expression (Figure 6.1). Platelets from all these donors responded to prednisolone in signalling experiments, that is, for all these donors thrombin-induced phosphorylation of myosin light chain (MLC-ser^{19}) and myosin light chain phosphatase (MLCP) was suppressed significantly by prednisolone (10 μM). Although there was a logical correlation of GCR expression with signalling data in these donors, it could not prove conclusive as it lacked the functional (aggregation) data. Moreover, as much as we liked to, we could not analyse platelet GCR expression from some of the donors who were non-responsive to prednisolone. Thus, it could not be established with certainty whether GCR expression in individuals can account for their prednisolone responsiveness.

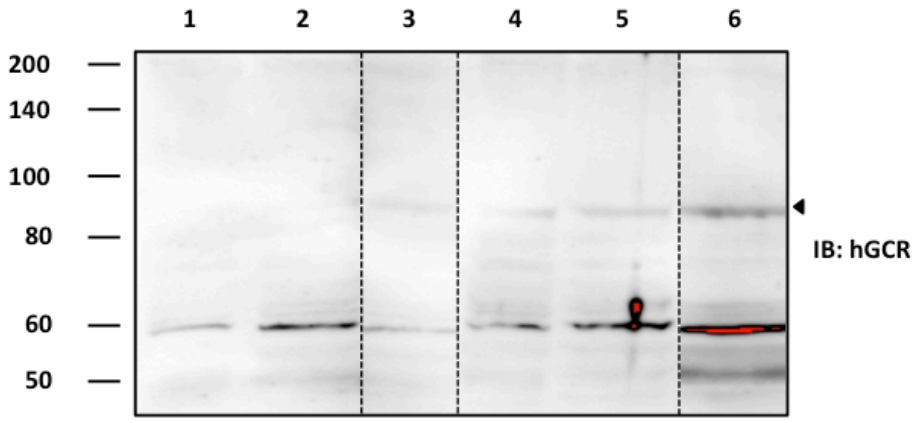


Figure 6.1 GCR expression in platelets from different individuals. Washed platelets (1×10^9/ml) were lysed using 1X lysis buffer and protein concentration of the lysates were determined. 100 µg of lysates from each donor was mixed with an equal volume of 2X Laemmli buffer. Platelets proteins were separated using SDS-PAGE and immunoblotted for GCR using a rabbit polyclonal antibody. Membranes were blocked with 10% BSA. Representative immunoblot from a single experiment is shown.

Future work

Through the key findings of this study we established that the synthetic GC prednisolone inhibited platelet aggregation and RhoA/ROCK-mediated signalling events downstream of thrombin stimulation. It will be interesting to address some additional questions to extend our findings, which are as follows:

- Although our data suggested the involvement of GCR in prednisolone-mediated suppression of RhoA/ROCK-mediated signalling events downstream of thrombin stimulation, the exact mechanism needs to be demonstrated. Our data showed that prednisolone inhibited RhoA activation. This process is mediated by a class of proteins called guanine nucleotide exchange factors (GEFs) such as p115RhoGEF, which is in turn activated by the active G-protein, $G_{\alpha13}$ ($G_{\alpha13}$-GTP) (Aslan and McCarty, 2013). Activated p115RhoGEF catalyses dissociation of GDP from and association of GTP with RhoA. It will be interesting to explore the possibility whether GCR prevents p115RhoGEF from facilitating RhoA activation. It might achieve this by physically interacting with either p115RhoGEF directly or $G_{\alpha13}$, thereby preventing activation of p115RhoGEF. Both the possibilities can be verified by immunoprecipitation studies. We demonstrated that prednisolone reduced MLCP phosphorylation. The formation of a ternary complex between RhoA, ROCK2 and MYPT1 subunit of MLCP has been very recently shown to be responsible for MLCP phosphorylation and its subsequent inactivation (Aburima et al., 2013). Thus, it could be interesting to perform immunoprecipitation experiments to determine if prednisolone can disassemble this complex through the recruitment of GCR.

- The expression of GCR in platelets needs to be confirmed by other methods. A comprehensive genome-wide transcriptome analysis indicated the presence of GCR mRNA in human platelets (Rowley et al., 2011). Thus it will be valuable to perform reverse transcriptase polymerase chain reaction (RT-PCR) or real-time PCR reactions that should provide an indication of the presence of the receptor mRNA transcripts.

- Although a synthetic GC, prednisolone administration should bear physiological relevance, as it resembles cortisol, the physiological GC, in many ways. Besides having comparable half-lives, prednisolone and cortisol have similar binding affinities for both GCR and MCR and can activate both GCR and MCR with comparable efficacies. Dexamethasone, the more widely studied synthetic GC, however differs from cortisol considerably, as it binds GCR with much higher affinity and MCR with much lower affinity (Juruena et al., 2009; Rupprecht et al., 1993). Thus the observed effects of prednisolone could be mediated through either GCR or MCR or both. This can be an interesting avenue to explore as it could possibly address the issue of prednisolone insensitivity observed in a group of the donors recruited in our study. Thus, in each donor, expressions of both GCR and MCR need to be determined and antagonists to both these receptors should be employed either alone or in combination to look for reversal of prednisolone-mediated effects in functional assays. This could be extremely challenging and increasingly complex to achieve, especially because of the study of a number of variables across several donors.

- It has been suggested that existence of receptor heterodimers can result in the variability of a biological response and this hypothesis has been shown to hold relevance in the context of nuclear receptors. GCR/MCR heterodimers have been shown to participate in the regulation of a distinct set of target genes when compared to GCR/GCR homodimers (Trapp and Holsboer, 1996). Having confirmed the existence of MCR and GCR/MCR heterodimers in platelets (Moraes et al., 2005), the comparison of functional outcomes mediated by GCR, MCR and/or GCR/MCR heterodimer remains a prospective area of investigation. This can be achieved by performing ligand-binding assays to be able to relate the type of prednisolone-mediated response with the nature of ligand-receptor complex. This analysis might again pose several challenges owing to the involvement of several variables under study.

Conclusion

The findings from our platelet functional and biochemical experiments in this study clearly demonstrate that prednisolone bears the potential to blunt platelet activation. These findings should add to the list of clinical applications of this synthetic GC, which is otherwise popular as an anti-inflammatory drug. In conclusion, this study presents evidence for prednisolone as an agent with promising anti-platelet properties, which when combined with its established anti-inflammatory attributes could potentially be employed as therapeutics in the treatment of inflammatory cardiovascular diseases.

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