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REGULATION OF BLOOD PLATELET FUNCTION BY NITRIC OXIDE

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Abstract

Upon vascular injury, platelets instantly adhere to the exposed extracellular matrix resulting in platelet activation and aggregation to form a haemostatic plug. This self-amplifying mechanism requires a tight control to prevent uncontrolled platelet aggregate formation that could occlude the vessel. Endothelial-derived nitric oxide (NO) and prostacyclin (PGI_2) are strong negative regulators that modulate platelet adhesion, activation, aggregation, secretion and shape change. In this study the effects of NO on Ca^{2+} dependent and independent pathways of activation were investigated. The data produced during the course of this study reveals new insights into the mechanisms by which NO regulates platelet responses via the activation of the AGC family of Ser/Thr protein kinases. NO inhibited platelet shape change in a concentration dependent manner. Platelet shape change is driven by phosphorylation of myosin light chain (MLC) and the experimental data shows that NO blocked this critical phosphorylation event. Phospho-MLC generated in response to platelet agonists occurs through a Ca^{2+} dependent and RhoA kinase (ROCK)-dependent mechanisms and NO differentially inhibits both pathways. Activation of the ROCK pathway via RhoA leads to the phosphorylation MLC phosphatase Threonine$^{696/853}$, which inhibits enzyme activity. Experimental evidence in this thesis indicates that NO, acting through cGMP and protein kinase G, prevents this inhibitory phosphorylation of MLCP by at least two mechanisms, (i) inhibiting the ROCK pathway that phosphorylates MLCP, and (ii) directly phosphorylating MLCP at an independent site, Serine^{695}. These original observations hint at a novel mechanism for platelet regulation by the NO-cGMP-signalling pathway.

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Abbreviations

αIIbβ3	Integrin alpha IIb beta 3
Ab	Antibody
ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
2-APB	2-Aminoethoxydiphenyl borate
ATP	Adenosine triphosphate
BAPTA	1, 2-Bis (2-aminophenoxy) ethane-N, N, N, N-tetraacetic acid tetra (acetoxymethylester)
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DAG	1,2-diacyl-glycerol
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DNA	Deoxyribonucleic acid
DTS	Dense tubular system
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid
ER	Endoplasmic reticulum
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
Fe^{2+}	Ferrous ion

GDP	Guanosine diphosphate
GMP	Guanosine 5’-monophosphate
GP	Glycoprotein
GP VI	Glycoprotein VI
GPIb-IX-V	Glycoprotein Ib-IX-V
GPCR	G-Protein Coupled Receptor
GSNO	S-Nitrosoglutathione
GTP	Guanosine triphosphate
Hb	Haemoglobin
HRP	Horseradish peroxidase
HSC	Human stem cells
Ig	Immunoglobulin
IL	Interleukins
IP_3	Inositol-1, 4, 5-trisphosphate
IP_3R	IP_3 receptor
IRAG	IP_3 receptor associated PKG I substrate
Mg^{2+}	Magnesium
ML-7	1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepinehydrochloride
MLC	Myosin light chain
MLCK	MLC kinase
MLCP	MLC phosphatase
MYPT1	Myosin phosphatase target subunit 1
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)

nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
$O_2^{\cdot -}$	Superoxide radical
$OH^{\cdot -}$	Hydroxyl ion
OC	Ovarian cancer
ODQ	1H-1, 2, 4 Oxadiazolo4, 3-quinoxalin-1-one
ORAI	Calcium release-activated calcium channel protein
PAR	Protease activated receptors
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PGI_2	Prostacyclin I_2
PIP_2	Phosphatidyl-1, 4-bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PLCβ	Phospholipase C beta
PLCγ2	Phospholipase C gamma 2
PRP	Platelet rich plasma
PVDF	Polyvinylidene difluoride
ROC	Receptor-operated Ca^{2+} channels
ROCK	Rho-associated coiled-coil containing protein kinase

RSNO	S-nitrosothiols
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SERCA	Endoplasmic reticulum calcium ATPase
sGC	Soluble guanylyl cyclase
siRNA	Small interfering RNA
SOC	Store-operated channels
SOCE	Store-operated calcium entry
STIM	Stromal interaction molecule
TBS	Tris buffered saline
TEMED	N, N, N', N'-Tetra methyl ethylenediamine
TF	Tissue factor
TPO	Thrombopoietin
TRPC	The transient receptor potential canonical
TxA_2	Thromboxane A_2
VASP	Vasodilator stimulated phosphoprotein
vWF	von Willebrand factor
WP	Washed platelets
Y-27632	Trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, dihydrochloride

CHAPTER 1 INTRODUCTION

1.1 Overview

Platelets are critical components of cardiovascular medicine(Heemskerk *et al.*, 2002). They have roles in the maintenance of haemostasis and also are highly involved mediators in pathophysiological processes in cardiovascular disease. This includes arterial thrombosis that is the key to the pathogenesis of acute ischemic syndromes. Blood vessels that have been affected by metabolic disturbances, smoking or high blood pressure develop lipid rich plaques in the walls that on rupture leads to activation of the platelets and precipitates clot formation that may occlude the lumen resulting in a heart attack (Gregg & Goldschmidt-Clermont, 2003). Unregulated platelet aggregation plays a fundamental role in development of cardiovascular events and hence controlling the function of these cells is important in prevention and treatment of these events(Kumar & Kao, 2009). Anti-platelet drugs such as aspirin, di-pyridamole and clopidogrel have been used successfully in secondary stroke prevention(Bednar & Gross, 1999) and improving mortality, though yielding disappointing results in the long term treatment of chronic stable cardiovascular disease (Coccheri, 2010). A possible basis of future treatment is centred on pre-existing biological inhibitors, such as novel nitric oxide releasing derivatives and combining them successfully with existing therapies (Li *et al.*, ; Saavedra *et al.*, 2000).

1.2 Platelet formation

Blood platelets are small sub-cellular fragments that are derived from the bone marrow cells megakaryocytes, which then circulate in blood in the discoid shape. Megakaryocytes arise from pluripotent human stem cells (HSCs) that develop into

two types of precursors, burst-forming cells and colony-forming cells, both of which express the cluster designation CD34 antigen (Briddell *et al.*, 1989). Development of both cell types continues along an increasingly restricted lineage culminating in the formation of megakaryocyte precursors that develop into megakaryocytes (Ogawa, 1993). The major function of the megakaryocytes is to produce platelets, a process termed thrombopoiesis, which are critical for haemostasis. During its lifespan, a mature megakaryocyte can produce up to 10,000 platelets (Long, 1998). Thrombopoietin (TPO), the primary regulator of thrombopoiesis, is currently the only known cytokine required for megakaryocytes to maintain a constant platelet mass (Kaushansky, 2005). TPO is thought to act in conjunction with other factors, including interleukins IL-3, IL-6, and IL-11, although these cytokines are not essential for megakaryocyte maturation (Kaushansky & Drachman, 2002). Cells committed to the megakaryocytic lineage begin to expressCD41 and CD61 (Integrin αIIbβ3), CD42 (glycoprotein Ib) and glycoprotein V (Roth *et al.*, 1996; Hodohara *et al.*, 2000), providing cell surface markers. Megakaryocytes tailor their cytoplasm and membrane systems for platelet biogenesis. Before a megakaryocyte has the capacity to release platelets, it enlarges considerably to an approximate diameter of 100μm and fills with high concentrations of ribosomes that facilitate the production of platelet-specific proteins (Long *et al.*, 1982) and undergoes endomitosis to increase in size and ploid to a deoxyribonucleic acid (DNA) content in excess of 16n and amassing reserves of membrane(Radley & Haller, 1982),granules and cytoskeletal proteins (Schulze & Shivdasani, 2004). Upon completion to mature megakaryocytes, they are polyploid and no longer proliferate but generate platelets by remodelling their cytoplasm (Italiano *et al.*, 1999) resulting in the formation of cytoskeletal tracks, termed

proplatelets, which serve as assembly lines for platelet production (Becker & De Bruyn, 1976).

Components essential for platelets such as granules, organelles and ribosomes are transported from the megakaryocyte body to the protrusions where synthesis and release of the platelet occurs (Italiano *et al.*, 1999). Platelet release occurs as these proplatelets extrude into the marrow sinusoids (De Botton *et al.*, 2002) or the entire megakaryocyte enters the circulation for release (Tavassoli & Aoki, 1981).

1.3 Blood platelets

As a product of evolution, mammals have evolved the use of sub-cellular fragments called platelets as specialised components within the blood to prevent excessive blood loss, a process termed haemostasis. However platelets can also perform important roles in wound healing and inflammation (Szalai *et al.*, 2006). They are present with in the blood at concentration as high as $2\text{-}3x10^{8}$ml; in circulating blood platelets are small discs of the dimensions 0.5 x 3.0µm (Bessis *et al*., 1973). Platelets are generated continually and release into the blood stream, then circulate for approximately seven days, and can leave the circulation by two major mechanisms: (1) consumption at common sites of minor vascular injury and (2) phagocytosis by macrophages, predominantly in the spleen and liver (Keith R. McCrae, 2006). Generation of small, discoid platelets allows efficient dispersal of clot promoting cells within circulation. As consequence of their small size and the complex motion of red blood cells, which constitute a large fraction of the blood's volume, platelets are pushed to the outer area of the lumen thereby resulting in an

intimate relationship with the endothelium and rapid responses to potential sites of vascular injury (Crowl & Fogelson, 2010).

1.4 Platelet Structure

Platelets are anucleate cells, but contain a number of organelles that enable them to perform their functions. The outer surface of the platelet is the thicker exterior coat or glycocalyx (White, 1971), which is covered with glycoprotein's (GP) necessary receptors for cell-cell and cell-vessel wall interactions to trigger full activation of the platelet, principally GP Ib-IX-V complex and integrin $\alpha_{IIb}\beta_3$ (the GPIIb-IIIa complex), but also a wide range of other receptors.

The lipid bilayer on which the glycocalyx rests is a typical membrane (White & Conard, 1973), though it serves an extremely important role in the acceleration of clotting, specific to platelets. The bilayer consists of cholesterol-rich lipid islands in the resting platelet surface membrane (Del Conde *et al.*, 2005) which following platelet activation become associated with the outer layer of the unit membrane, bringing anionic phosphatidylserine to the exposed surface and converting pro-thrombin to thrombin.

The surface connected Open Canalicular System (OCS) is derived from the plasma membrane and demarcation membrane system of the megakaryocytes, though is not only connected to the platelet surface membrane, it is an extension of the surface membrane (Behnke, 1968). The OCS channels are an extension of the surface membrane weaving through the cytoplasm. It provides two distinct functions; it greatly expands the surface area of the platelet exposed to circulating

plasma(Frojmovic *et al.*, 1992) and provide a means for substances to reach the deepest recesses of the cell(White, 1972), and serve as channels for the release of products stored in secretory organelles during the platelet release reaction(White & Krumwiede, 1987). After adhesion to a damaged vascular surface, the platelet extends filopodia to bind firmly to the injured area. This is followed by the assembly of cytoplasmic actin and spreading of the platelet to cover as much area as possible, resulting in a 420% increase in exposed surface area, only possible due to the reserve of densely compacted OCS allowing for the expanded surface area of the spreading platelet (Escolar *et al.*, 1989).

The Dense Tubular System (DTS) has been indicated to haveCa^{2+} binding sites and enzymes involved in prostaglandin synthesis (Gerrard *et al.*, 1976). Like the OCS, channels of the DTS are randomly dispersed in the platelet cytoplasm, though in close association with the circumferential band of microtubules, the DTS representing residual smooth endoplasmic reticulum of the parent cell.

The area lying just under the membrane is the platelet cytoskeleton, critically important to platelet function. The cytoplasmic domains of all transmembrane receptors interact in the sub-membrane area, with numerous protein constituents regulating the signalling processes of platelet activation. A number of these proteins, such as the actin-binding protein filamin, are linked to the GPIb-IX-V complex and the cytoplasamic tails of GPIIb and GPIIIa, are associated with calmodulin, myosin, and the short actin filaments making up the membrane contractile cytoskeleton. The contractile system is involved in the translocation of receptor complexes, including GPIb-IXV and GPIIb-IIIa, on the platelet exterior surface.

There are two filament systems in the platelet cytoplasm. One is the circumferential coil of microtubules, the loss of which is linked to the loss of discoid form (White & Krivit, 1967). The other, the actomyosin filament system, is involved in shape change (Escolar *et al.*, 1986). The cytoplasmic actin filament cytoskeleton has a function in platelet physiology separate from that of the submembrane actin cytoskeleton. In the resting cell, it serves as the matrix on which all organelles and other structural components are suspended and maintained separate from each other and the cell wall (Escolar *et al.*, 1986).

Following platelet activation in suspension or on surfaces, the cytoplasamic actomyosin cytoskeleton has a unique role in contractile physiology. It constricts the circumferential microtubule coils and drives the α-granules and dense bodies into close association in platelet centres (White, 1968) and prolonged stimulation resulting in the secretion of granule and dense body contents via channels of the OCS (Escolar *et al.*, 1986) leaving behind a dense, central mass of actomyosin. The constriction of the activated platelet thus influences the release of secondary mediators from internal organelles; α-granules, dense bodies and lysosomes, of which α-granules are the most numerous of the platelet organelles (Reed, 2004). There are usually 40 to 80 α-granules per platelet; they are round to oval in shape and 200 to 500nm in diameter, with their interior substructure divided into zones.

Human platelet dense bodies are smaller than the α-granules and fewer (Berger *et al.*, 1996), there being four to eight dense bodies per platelet which are rich in adenine nucleotides, including adenosine triphosphate (ATP) and adenosine

diphosphate (ADP), and serotonin. The last of the main storage granules are lysosomes, which are spherical in form and slightly smaller than α-granules.

Their contents, together with products stored in α-granules and dense bodies, can be released when platelets are exposed to strong, sustained stimulation.

1.5 Platelet activation and thrombus formation

Platelets activation is critical in thrombus formation and can be summarised into three overlapping stages; initiation, extension, and perpetuation.

1.6 Platelet adhesion to vessel wall

At the sites of vascular injury, the sub-endothelial extracellular matrix (ECM) is exposed to constituents within the circulating blood, triggering activation. The ECM contains a number of proteins that can contribute to the capturing of platelets including collagen, collagen-bound von Willebrand factor (vWF), laminin and fibronectin. The most important of these are collagen and vWF. The mechanisms of platelet adhesion at the sites of injury are determined by the rheological conditions. Under low shear conditions, collagen alone is able to immobilise and activate platelets, though under the conditions of high shear found in the arterial circulation, vWF plays an essential role in adhesion and activation of platelets. Blood flows with a greater velocity in the centre of the vessel then near the wall, thereby generating shear forces between layers of fluid becoming maximal at the wall (Eskin et al., 2006). The drag which opposes platelet adhesion increases with the shear rates depending on the vessels. Under high shear rates present in small arteries the initial tethering to the site of injury is mediated by GPIb-IX complex on the platelet surface and VWF bound to collagen(Savage *et al.*, 1998). The binding of GPIb to VWF is insufficient to mediate strong or stable adhesion; though it enables the maintenance

of platelet contact with the damaged area enabling the characteristic ‘rolling’ along damaged surfaces **(Figure.1.1)**. This allows the platelet to establish contact with the thrombogenic collagen via its GPVI. The GPVI receptor binds directly to collagen through the specific Gly-Pro-Hyp peptide repeat sequence triggering the clustering of receptors and an activatory signalling cascade through tyrosine kinases(Knight *et al.*, 1999). These signalling events termed inside-out signalling leads to the conversion of platelet integrins from low to a high affinity state, allowing them to form stable adhesion with the ECM. Inside-out signalling can be initiated through platelet adhesion or via the different G-protein subfamilies. Upon damage to the sub-endothelial layers, collagen and VWF are exposed leading to the engagement of the GPVI and the GPIb-IX-V complex upon the platelet triggering inside-out signalling, with collagen been able to induce shape change, aggregation and secretion (Packham *et al.*, 1977). Binding of collagen to GPVI, the major platelet collagen receptor to mediate cellular activation (Nieswandt & Watson, 2003) allows the clustering of the receptor and its associated Fc receptor γ chain. This leads to the activation of PLCγ2 via Syk(Moroi & Jung, 2004) and subsequently inducing the formation of secondary messengers DAG and IP_3, activating PKC and elevating cytosolic Ca^{2+} levels (Grosse *et al.*, 2007), while been supported by collagen binding to $\alpha_2\beta_1$ simultaneously (Holtkotter *et al.*, 2002). Together this results in strong integrin activation and release of stored mediators (Nieswandt & Watson, 2003) VWF binding to the GPIb-IX-V complex can also activate the $\alpha_{IIb}\beta_3$ integrin.

In addition, several members of the integrin family facilitate platelet interactions with adhesive proteins. Integrins are heterodimeric cell surface molecules which consist ofα and β subunit. Eight β-type subunits are known and have been shown to be

highly homologous to one another. Further, 14 α-type subunits have been identified, which also demonstrate similarities. Each β-subunit can form a non-covalent complex with anα-subunit to form a functional adhesive protein receptor. The most widely expressed integrin on platelets is the integrin $\alpha_{IIb}\beta_3$ with up to 40,000 copies expressed on the membrane of one resting platelet. The numbers of $\alpha_{IIb}\beta_3$ integrins are increased during platelet activation to up to 80,000 copies per cell. The integrin $\alpha_{IIb}\beta_3$ primarily binds to the plasma protein fibrinogen. In the context of platelet adhesion inside-out signalling leads to the activation of $\alpha_2\beta_1$, which can then forma high affinity interactions with collagen(Moroi *et al.*, 2000).

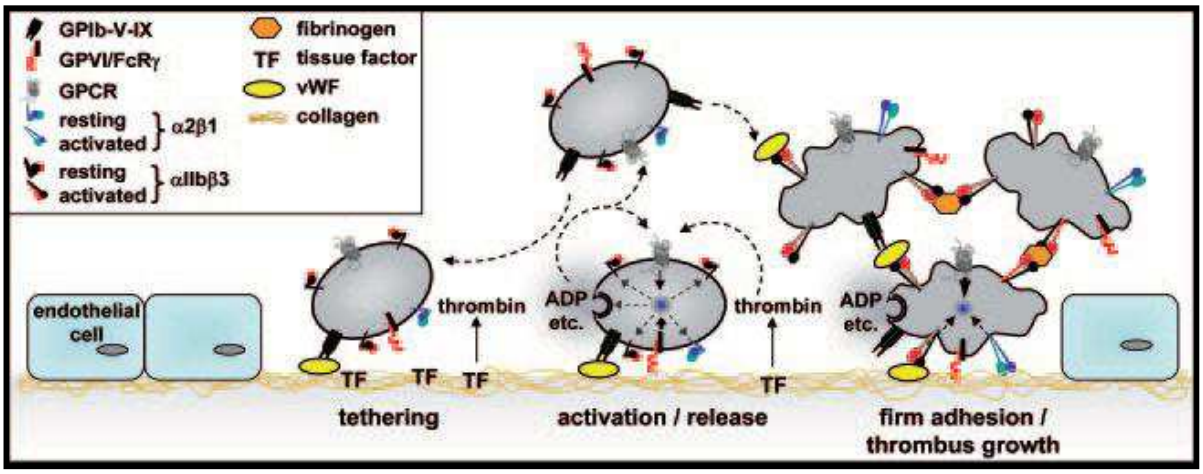

Figure.1.1 Platelet adhesion and aggregation on the ECM

The GPIb-vWF interaction mediates platelet tethering thereby enabling GPVI interaction with collagen. This triggers the shift of integrins to a high-affinity state and release of ADP and TXA2. In parallel, tissue factor (TF) locally triggers thrombin formation which also contributes to platelet activation (Varga-Szabo *et al.*, 2008b).

1.7 Extension of the platelet plug

Following the formation of the platelet monolayer upon exposure to collagen and vWF further platelets are recruited to this monolayer. This is the recruitment of circulating platelets to the adhered platelets at the wound site, termed aggregation. Key to the extension of the platelet plug beyond the monolayer adhered directly to collagen is the presence of local accumulations of soluble agonists that are secreted from platelets, such as ADP and $TXA_2$and by the local generation of thrombin upon the surface of activated platelets. In order for platelet aggregation to occur fibrinogens as well as divalent cations are required. Platelet activation by one or more of the above agonists leads to the rapid activation of the integrin $\alpha_{IIb}\beta_3$. Once activated, each integrin heterodimer is able to bind one fibrinogen molecule in the presence of divalent cations, such as Ca^{2+}. In the absence of Ca^{2+} fibrinogen no longer binds to its receptor and platelet aggregation does not occur. Further, it has been demonstrated that integrin clustering can lead to integrin activation and fibrinogen binding (Hato *et al.*, 1998; Li *et al.*, 2001; Li *et al.*, 2004). Initial fibrinogen binding to the integrin $\alpha_{IIb}\beta_3$ is reversible, which can lead to the disaggregation of platelets, as would be the case if Ca^{2+} were removed. However, fibrinogen binding undergoes a time dependent stabilisation which results in irreversible fibrinogen binding and irreversible aggregation (Bennett & Vilaire, 1979). $\alpha_{IIb}\beta_3$can also bind with multimeric VWF, fibronectin and thrombospondin(Ma *et al.*, 2007). Once bound to its ligand, fibrinogen,$\alpha_{IIb}\beta_3$ generate intracellular signals, achieved through a short β-subunit cytoplasmic tail (Tadokoro *et al.*, 2003).This type of signalling refers to intracellular signalling events that do occur downstream of activated integrins (Shattil & Newman, 2004).

1.7.1 Platelet activation by soluble agonists

As highlighted in the previous section recruitment of platelets into the growing aggregate requires the use of potent autocrine and paracrine signalling pathways though secretion of the soluble mediators **(Figure.1.2)** once platelet adhesion has been initiated. The receptors involved in their detection are typically members of the G-Protein Coupled Receptor (GPCR) family, which are ideally suited to the process as they are able to bind their ligands with a high affinity. Agonist binding to the surface-accessible domains of GPCR cause a conformational change that activates the G proteins constitutively associated with the intracellular surface of the receptor(Hamm, 2001). The amplification of the original signal triggered by the agonist upon the receptor is another key feature due in part to its use of the guanine nucleotide exchange factors, allowing the receptor to activate multiple G-proteins and also other classes of G-protein and potentially other effector pathways ensuring the rapid activation and recruitment of platelets into the growing thrombus.

GPCR are membrane proteins that have an extracellular N-terminus, an intracellular C-terminus and seven transmembrane domains(Palczewski *et al.*, 2000). G proteins interact with the cytoplasmic domains of the receptor with a specificity determined by the receptor domain itself and by the sequence of the α-subunit of the G protein. The G proteins are heterotrimers comprising α, β, and γ subunits. The α-subunit contains a guanine nucleotide binding site, which is present in the off state by the occupation of the site with guanosine diphosphate (GDP). Upon receptor activation GDP is exchanged for guanosine triphosphate (GTP), thus altering the conformation of the α-subunit and leading to the exposure of the sites on both the G_{α} and $G_{\beta\gamma}$ for interaction with downstream effectors(Ford *et al.*, 1998). The duration of activation of

the G protein is intrinsically regulated by the action of the GTPase upon the GTP loaded α-subunit. Hydrolysis is accelerated by the presence of a regulator of G protein signalling (RGS) with the acylation of G_{α} and prenylation of $G_{\beta\gamma}$ allowing the subunits to remain bound to the membrane until the GTP from the G_{α} has been hydrolysed and ultimately return the heterotrimer to the inactive state awaiting further activation (Ross & Wilkie, 2000).

Human platelets express four families of G proteins that are referred to by the designation of the α-subunit, and there are ten forms of the G_{α} that fall within the $G_{s\alpha}$, $G_{i\alpha}$, $G_{12\alpha}$ and the $G_{q\alpha}$ families. These include at least one member of the G_s family, four members of the G_i family (G_{il}, G_{i2}, G_{i3} and G_z) which stimulate and inhibit cAMP formation by adenylyl cyclase. Three further of the G_q family (G_q, G_{11}, and G_{16}) which stimulate β isoforms of phospholipase C(Offermanns *et al.*, 1997), and two G_{12} members (G_{12} and G_{13}) which regulate the platelet cytoskeleton(Offermanns *et al.*, 1994; Klages *et al.*, 1999). The $G_{\beta\gamma}$ isoforms are less well understood than the α-subunit, and their contribution to selective activation of platelets, though the abundance of G protein types is necessary to support the differing actions of numerous agonists (Yang *et al.*, 2002).

GPCR responding to platelet agonists differ in the strength of their response and their preferences for intracellular effector pathways, using a distinct mechanism to achieve full activation. Receptors for thrombin, protease activated receptors (PAR-1 and PAR-4), ADP (P2Y1 andP2Y12) and TxA_2 (TP) cause activation of phospholipase C (PLC) leading to hydrolysis of phosphatidylinositol-4-5-bisphosphate (PIP_2) in the membrane and subsequent secondary messengers

elevate cytosolicCa^{2+} concentration by activating G_q(Offermanns *et al.*, 1997). The ADP and epinephrine receptors, P2Y12 and α_{2A}-adrenergic receptors are coupled to G_{i2} or G_z which can regulate the inhibition of adenylyl cyclase(Yang *et al.*, 2000; Yang *et al.*, 2002) and activation of phosphatidylinositol 3-kinase (PI3K) and downstream effector the serine/threonine kinase Akt(Vanhaesebroeck *et al.*, 2001; Cantley, 2002). Thrombin and TxA_2 activation can also cause the rearrangement of the actin cytoskeleton defining platelet shape change through the RhoA/Rho-kinase mediated pathway regulated by the G_{12}/G_{13} family, in which activated G_{13} binds and activates Rho-specific guanine nucleotide exchange factors (Offermanns *et al.*, 1994; Hart *et al.*, 1998).

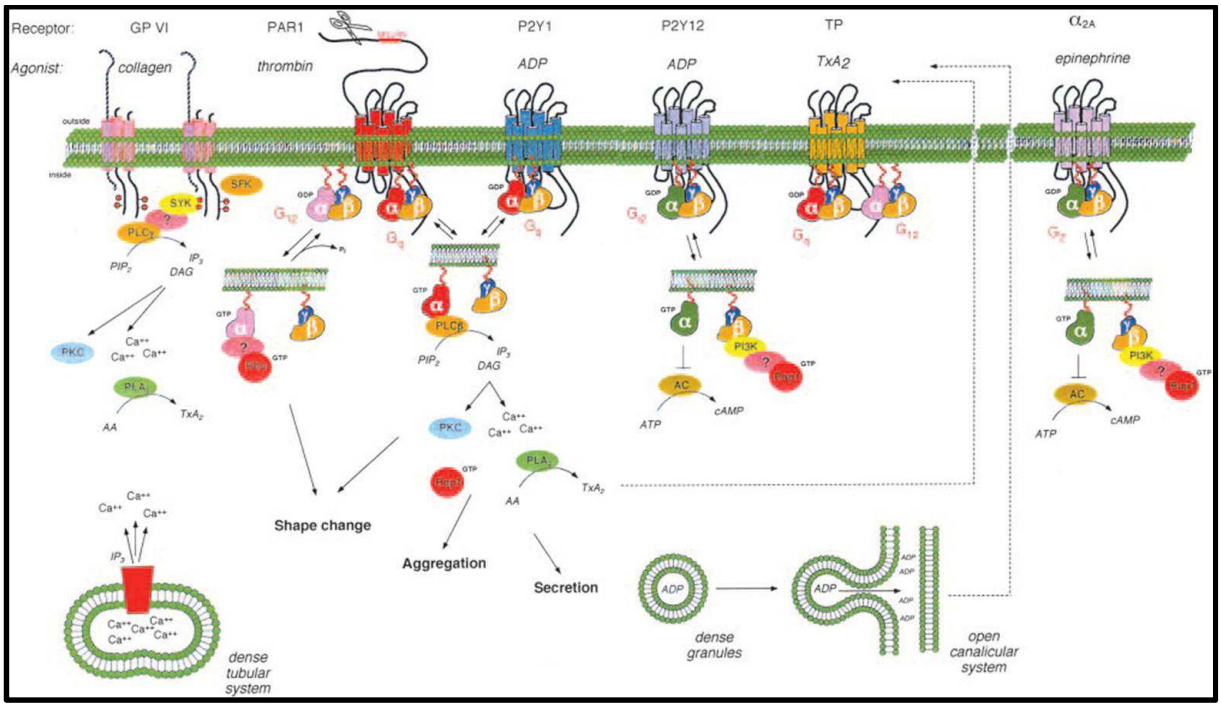

Figure.1.2 Overview of platelet activation

Most platelet agonists activate platelets via G protein coupled receptors on the platelet surface. Critical responses include G_q mediated activation of phospholipase Cβ isoforms to allow an increase in cytosolic Ca^{2+}, activation of phospholipase A_2 and protein kinase C, and G_{12} mediated activation of Rho family members to support rearrangement of the platelet cytoskeleton (shape change). The increase in cytosolic Ca^{2+} is initially caused by the IP_3triggered release of Ca^{2+} from within the dense tubular system of the platelet, which in turn triggers Ca^{2+} influx across the platelet plasma membrane. TP = thromboxane receptor (Brass, 2003).

1.8 Platelet Aggregation

The accumulation of platelets into a thrombus is based on multiple ligands (fibrinogen, fibronectin and VWF), receptors (GPIbα and $\alpha_{IIb}\beta_3$) and platelets in different activation states (Jackson, 2007). The major integrin mediating platelet aggregation is αIIbβ3, binding to fibrinogen and VWF. The structural arrangement of these ligands, fibrinogen being dimeric and the multimeric structure of VWF allows the formation of cross-bridges with other platelets and generation of a platelet aggregate. In the quiescent platelet the $\alpha_{IIb}\beta_3$ integrin is in an off state, though upon activation and intracellular signalling process the cytoplasamic part of $\alpha_{IIb}\beta_3$ is converted into an active state (inside-out signalling) resulting in the binding of fibrinogen and VWF thus mediating platelet aggregation (Sehgal & Storrie, 2007).

The formation of reversible platelet aggregates results in the platelet activation and formation of stable aggregates as the space between aggregating platelets provides an enclosed environment for accumulation of soluble agonists such as thrombin, ADP and TxA_2. These agonists inducing activation, shape change and secretion of granule products act via GPCR, resulting in inside-out signalling of $\alpha_{IIb}\beta_3$.

The requirement of Ca^{2+} elevation via the G_q mediated signalling for agonist induced $\alpha_{IIb}\beta_3$ has been demonstrated on G_q deficient platelets, which fail to aggregate in response to thrombin, ADP and TxA_2 due to the lack of PLC activation (Offermanns *et al.*, 1997) though not solely based on G_q activation. A role for G_i mediated signalling in $\alpha_{IIb}\beta_3$ activation is shown by its deficiency in platelets, with a reduced response to thrombin and ADP (Jantzen *et al.*, 2001). Thus G_q and G_i are required in order to induce platelet activation.

Through the activation of G_q and G_i, CalDAG-GEFI, guanine nucleotide exchange factor activates Rap1 (Dupuy *et al.*, 2001), Rap1 absence results in decreased $\alpha_{IIb}\beta_3$ activation and decreased platelet activation (Crittenden *et al.*, 2004). Studies on platelets lacking the G_{13} coupled protein confirm its mediation over the activation of $\alpha_{IIb}\beta_3$ and RhoA/Rho-kinase pathway, indicate their role in platelet aggregation (Missy *et al.*, 2001; Schoenwaelder *et al.*, 2002).

Although the precise signalling mechanisms are as yet unclear, it has been demonstrated that rapid platelet aggregation requires the heterotrimeric G-proteins G_q, $G_{12/13}$ and G_i to achieve efficient $\alpha_{IIb}\beta_3$ activation.

1.9 Perpetuation

Further signalling events are necessary to stabilise the platelet plug, preventing disaggregation and break-up of the clot. Perpetuation occurs at this point when platelets are in close enough contact and for a sufficient duration allowing contact dependent signalling possible, which can only occur once platelet aggregation begins. Estimations of the width required to allow molecules on the platelet surface to bind to each another range from zero to 50nm(Skaer *et al.*, 1979). This takes the form of contact dependent signalling mechanisms. When multivalent adhesive proteins bind activated $\alpha_{IIb}\beta_3$ on adjacent platelets, this involves outside-in signalling through the integrin (Phillips *et al.*, 2001), which can associate with cytoskeletal structures in thrombin aggregated platelets (Phillips *et al.*, 1980). The β_3 domain of activated $\alpha_{IIb}\beta_3$ is essential for any thrombus formation (Hodivala-Dilke *et al.*, 1999) and deficiency or absence of its ligands fibrinogen, VWF and fibronectin result in

slow and unstable thrombus formation. They are necessary for anchoring the platelet mass, thus preventing the release of the thrombus further downstream and embolism forming (Ni *et al.*, 2000; Ni *et al.*, 2003). The gaps between platelets within the thrombus also allow the accumulation of platelet derived molecules. In addition to the continual release of ADP and TXA_2, platelets also shed surface proteins. This exodomain shedding of CD40 ligand and thereby the accumulation in the inter-platelet gaps promotes thrombus stability by binding to activated $\alpha_{IIb}\beta_3$(Andre *et al.*, 2002). The stability of the thrombus is also mediated by other adhesion and signalling receptors besides $\alpha_{IIb}\beta_3$, such as platelet endothelial cell adhesion molecule-1 (PECAM-1), junctional adhesion molecules (JAM), endothelial cell specific adhesion molecule ESAM, CD226 and Eph kinases/ephrins and Gas 6 on the platelet surface (Brass *et al.*, 2005). The close contact of the platelets enables these interactions, allowing the ligands of one platelet to interact with the receptor of an adjacent platelet regulating the growth of the platelet thrombus and stability.

1.10 Platelet Pro-coagulant Activity

Thrombus formation is linked to the activation of the coagulation cascade resulting in its final stabilisation by a fibrin network, platelet adherence and aggregation at the site of injury localise subsequent pro-coagulant events.

The coagulation cascade is composed of the contact activation pathway (intrinsic), and the tissue factor (TF) pathway (extrinsic); both activate a final common pathway of factor X, thrombin and fibrin formation **(Figure.1.3)**.

The blood coagulation cascade is propagated through the formation of enzymatic complexes composed of a vitamin K-dependent serine protease and a non-enzymatic cofactor protein that are assembled on the membrane surface in a Ca^{2+} dependent manner (Mann *et al.*, 1990).

Coagulation is initiated upon damage to the endothelium lining, when sub endothelial TF, an integral membrane protein, is exposed to blood flow (Weiss *et al.*, 1989). TF can also be expressed under pathological conditions on the surface of activated monocytes or endothelial cells (Carlsen *et al.*, 1988; Camera *et al.*, 1999). The TF pathway can be considered the fuse that ignites coagulation with a small amount of thrombin (Orfeo *et al.*, 2005).

The serine protease factor VIIa (activated factor VII), circulating in blood at sub-nanomolar concentrations (Eichinger *et al.*, 1995), binds to tissue factor to form extrinsic Xase, which activates the zymogens, factor IX and factor X, to their corresponding serine proteases factor IXaβ and factor Xa(Bom & Bertina, 1990). Subsequent to formation of a TF/factor VIIa complex, the limited amounts of factor Xa produced assemble into prothrombinase via a Ca^{2+} dependent interaction with membrane bound factor Va initially to generate picomolar amounts of thrombin (Kalafatis *et al.*, 1994). Upon formation, thrombin significantly amplifies and also sustains the coagulant response. Thrombin also recruits more platelets to the growing thrombus and sustains coagulation. Thrombin activates additional factor V to factor Va, as well as factor VIII to factor VIIIa to thereby allow continued prothrombinase and intrinsic Xase formation and function(Butenas *et al.*, 1997).

Thus, membrane-bound factor VIIIa binds factor IXaβ in a Ca^{2+}-dependent manner forming intrinsic Xase(Tans *et al.*, 1991), which activates factor X 50 to 100 times faster than the factor VIIa-TF complex (extrinsic Xase)(Ahmad *et al.*, 1992). The thrombin, continuously produced via prothrombinase, amplifies its own generation by activating factor XI (Gailani & Broze, 1991) and continuing to activate additional platelets and factors V and VIII (Pieters *et al.*, 1989). Further activation of platelets leads to secretion of α-granule contents and coagulation factors leading to platelet pro-coagulant activity by the surface exposure of phosphatidylserine supporting the formation of thrombin and by facilitating the assembly of prothrombinase, tenase and coagulation factors on the surface of the activated platelet (Heemskerk *et al.*, 2002).

Thrombin now converts fibrinogen to fibrin(Mosesson, 1992) and factor XIII (Naski *et al.*, 1991), forming a loose mesh, the building block of the haemostatic plug, with thrombin acting upon factor XIII to form covalent bonds that cross link the fibrin polymers that form from the activated monomers to stabilising the dense mesh and allowing the formation of a stable thrombus(Brummel *et al.*, 1999).

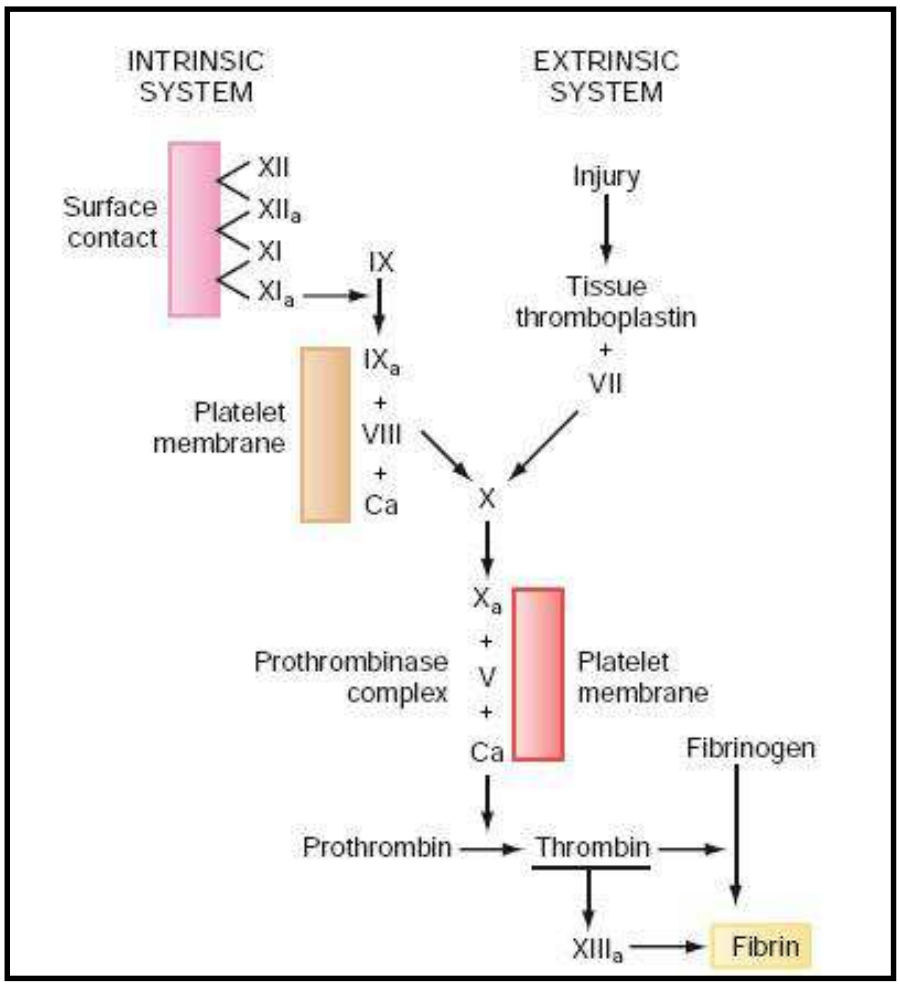

Figure.1.3 Intrinsic and extrinsic systems of the coagulation cascade

Interaction between clotting factors (XII, XIIa, XI, XIa, IX, IXa, VII, VIII, X, Xa, and XIIIa) and the platelet membrane. (Modified from Fuster V, Stein B, Ambrose JA, et al: Atherosclerotic plaque rupture and thrombosis: evolving concepts. Circulation 1990; 82[Suppl II]: II-47-II-59.)

1.10.1 Calcium signalling in platelets

The activation pathway for all agonists converge downstream to increase the intracellular Ca^{2+} concentration (Rink & Sage, 1990). The increase in cytosolic Ca^{2+} upon platelet activation derives from two sources; namely from intracellular stores, such as the endoplasmic reticulum (ER)/ dense tubular system (DTS) and acidic stores, though also from extracellular compartment. The major Ca^{2+} store in platelets is the DTS; its Ca^{2+} concentration is in the micromolar range and approximately ten thousand times higher than in the cytosol. This results in the leakage of Ca^{2+} though it is continually pumped back into the ER/DTS via the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Papp *et al.*, 1991). Cytosolic Ca^{2+} elevation occurs as a result of the release of the Ca^{2+} cation from the intracellular stores and influx from the outside medium**(Figure.1.4)**. The key element involved in this elevation is the activation of cell surface receptors coupled to G_q that lead to the stimulation of phospholipase C (PLC), leading to the hydrolysis of phosphatidyl-1,4-bisphosphate(PIP_2) into 1,2-diacyl-glycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), causing Ca^{2+} release and activating protein kinase C (PKC), together implicating G_q in both processes (Somlyo & Somlyo, 1994). IP_3 in turn induces Ca^{2+} release from the ER/DTS by binding to its channel pore receptor, the IP_3 receptor (IP_3R) in the stores. As the internal store becomes depleted specific plasma membrane Ca^{2+} channels open allowing extracellular Ca^{2+} to enter the cytosol further increasing the Ca^{2+} concentration and also to refill the now depleted internal stores. The mechanisms and channels for Ca^{2+} entry are poorly understood though are critical for full activation of the platelet (Nesbitt *et al.*, 2003). This process is termed store-operated Ca^{2+} entry (SOCE) and is a major pathway for Ca^{2+} entry after store depletion (Putney *et al.*, 2001).

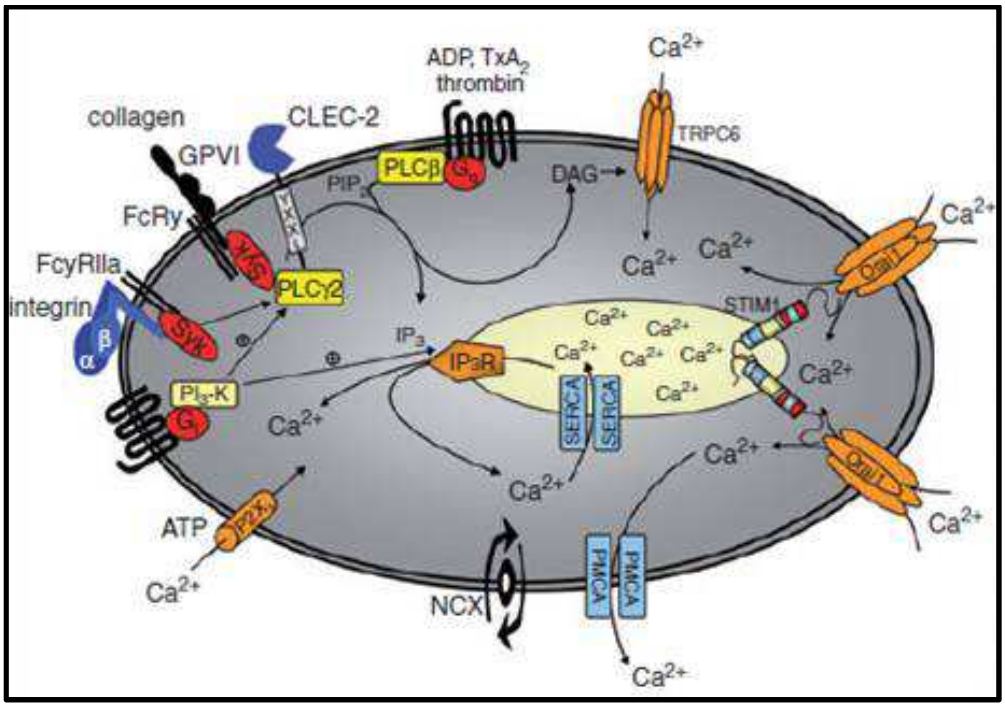

Figure.1.4 Platelet calcium

Upon receptor activation different phospholipase (PL) C isoforms hydrolyze phosphatidilinositol-4, 5-bisphosphate (PIP_2) to inositol-1, 4, 5-trisphosphate (IP_3) and diacyl-glycerol (DAG). IP_3 releases Ca^{2+} from the intracellular stores and in turn STIM1 opens Orai1 channels in the plasma membrane, a process called store-operated calcium entry (SOCE), whereas DAG mediates non-SOCE through canonical transient receptor potential channel 6 (TRPC6). Additionally, a direct receptor-operated calcium (ROC) channel, P2X1, and a Na^+/ Ca^{2+} exchanger (NCX)contribute to the elevation in $[Ca^{2+}]i$. The counteracting mechanisms involve sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCAs) and plasma membrane Ca^{2+} ATPases (PMCAs), which pump Ca^{2+} back into the stores or through the plasma membrane out of the cell, respectively. IP_3R, IP_3receptor; ATP, adenosine triphosphate; ADP, adenosine diphosphate; GPVI, glycoprotein VI; FcRγ, Fc receptor γ chain; FcγRIIa, Fc γ receptor IIa;CLEC-2, C-type lectin-like receptor 2; PI3-K, phosphatidylinositol 3-kinase; Syk, spleen tyrosine kinase. Due to controversies about the localization and role of TRPC1 in the literature, this protein is not depicted in the figure (Varga-Szabo, 2009).

1.10.2 Store-Operated Calcium Entry

In non-excitable cells, as seen in platelets, the IP_3 mediated Ca^{2+} release from the internal stores and their depletion triggering Ca^{2+} entry from the extracellular environment is referred to as store-operated calcium entry (SOCE)(Parekh & Putney, 2005). This itself does not represent a distinct channel type or mechanism (Guo & Huang, 2008), though numerous potential mechanisms**(Figure.1.5)** have evolved on the molecular link between the Ca^{2+} stores release and SOCE (Putney, 2007).

1.10.3 Role of STIM

Grosse e*t al* (Grosse *et al.*, 2007) were the first to demonstrate that the stromal interaction molecule 1 (STIM 1) is key to Ca^{2+} signalling. Stim 1 is a single transmembrane spanning protein containing two N-terminal EF hand (Ca^{2+}-binding motifs composed of two helixes (E and F) joined by a loop, Ca^{2+} is bound by the loop region) domains situated in the ER lumen and bindsCa^{2+}. In resting platelets these EF hands are occupied by Ca^{2+}, though upon activation and depletion of Ca^{2+} stores, the EF hand are no longer occupied and STIM 1 redistributes to puncta to interact with channels in the plasma membrane (Dziadek & Johnstone, 2007). Mice expressing the mutant EF domain in the Stim 1 protein displayed increased bleeding and pre-activation of platelets in circulation due to elevated basal Ca^{2+} levels (Grosse *et al.*, 2007), while genetic deletion of STIM 1 impaired platelet activation and adhesion (Varga-Szabo *et al.*, 2008a).

1.10.4 Role of ORAI

Human and mouse platelets were shown to express all three isoforms of the Orai channel family (Orai 1-3), with Orai 1 being the predominant isoform. Orai 1 is a four membrane spanning channel with intracellular N and C termini and was identified as an essential component of SOCE by the analysis of T cells from severe combined immuno deficiency patients (Feske *et al.*, 2006) and is impaired in platelets lacking Orai 1 (Braun *et al.*, 2009) and from mice expressing the loss of function mutation of Orai 1 ($Orai^{R93W}$) (Bergmeier *et al.*, 2009). Sustained increases in cytosolic Ca^{2+} are critical for the ability of platelets to switch from an adhesive state to a coagulant, phosphatidylserine (PS) state (Bevers *et al.*, 1982; Heemskerk *et al.*, 2002); in the absence of Orai 1, platelets fail to maintain elevated Ca^{2+} levels necessary for PS exposure.

1.10.5 Role of TRPC

The transient receptor potential (TRP) proteins are proposed as components of SOCE and non-SOCE channels, which assemble into tetramers to form a channel. The TRP family can be divided into seven subfamilies, of which the canonical TRPs (TRPC) are the best described. TRPC members comprise seven channel proteins (TRPC 1-7), which are divided on the basis of their amino acid similarity. TRPC 1 and 2 are almost unique, TRPC 4 and 5 have about 64% similarity and TRPC 3, 6 and 7 are the most closely related members, having 75% sequence identity. Heterotetrameric association is known to occur within the TRPC 3, 6 and 7 subgroup and TRPC 1 is able to complex with -4 and -5 (Hofmann *et al.*, 2002) and may associate with TRPC 3 and -7 also (Zagranichnaya *et al.*, 2005). The strongest evidence of TRPCs been a component of SOCE is TRPC 1, as it is known to

contribute to SOCE in many cell types, such as smooth muscle, endothelial cells and platelets upon agonist stimulation (Rosado *et al.*, 2002; Venkatachalam *et al.*, 2003). Further studies indicate that Stim 1 proteins can interact and cause the activation of TRPC (Huang *et al.*, 2006). TRPC channels are also reported to interact with Orai proteins (Liao *et al.*, 2007), both Stim 1 and Orai 1 appear to be associated within a complex that contains the TRPC1 channel (Ong *et al.*, 2007), suggesting that all three proteins interact to form a SOC.

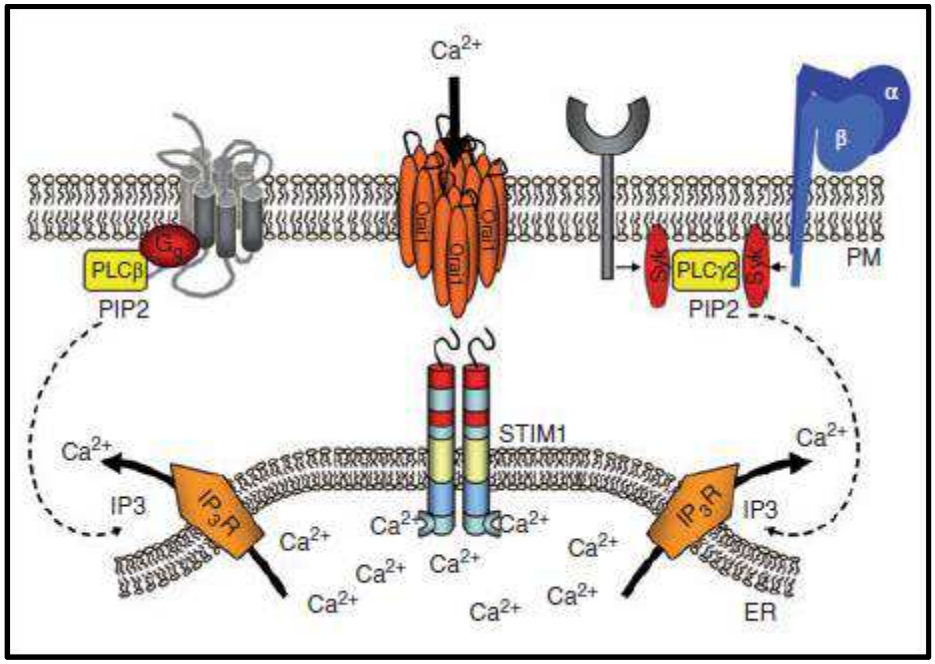

Figure.1.5 Store-operated calcium entry (SOCE) in platelets

In platelets, distinct subtypes of surface receptors activate two major isoforms of PLC; G-protein coupled receptors (GPCRs) activate PLCβ through G_q, whereas integrins and receptors coupled to an immunoreceptor tyrosine-based activation motif (ITAM) activate PLCγ2 through Syk. Receptor agonist binding results in calcium store release through inositol 1, 4, 5-trisphosphate receptors (IP_3R) in the endoplasmic reticulum (ER) membrane. This disrupts the calcium binding of the EF hand domain of STIM1 in the ER lumen and leads to the activation and redistribution of STIM1 to plasma membrane (PM) near puncta where it opens Orai1, the major store-operated calcium (SOC) channel in the PM, to allow calcium entry (Varga-Szabo, 2009).

1.11 Shape change

Platelet shape change is one of the earliest responses induced by a physiological agonist, as it this precedes spreading, aggregation and secretion. Resting, discoid platelets are transformed upon encountering the ECM, which is characterised morphologically by spheration, rearrangement of the cytoskeleton, the folding of the membrane and extension of lamellipods and pseudopods or filopods(Fox, 1993). Shape change depends on the cytoplasmic dynamics of the actin polymer (Fox & Phillips, 1981) and actin accessory proteins, which mediate severing, capping and cross-linking into a gel by filamin, while others are bundled by α-actinin and fimbrin generate morphological changes**(Figure.1.6)**.

In the resting platelet the short actin filaments are capped by CapZ and surrounded by a large pool of actin monomers bound to profilin. Upon activation by either direct contact with the damaged area or soluble agonist, a rapid intracellular signal transduction cascade occurs and resulting in a large influx of Ca^{2+} into the cytosol. This Ca^{2+} activates gelsolin to remodel the cytoskeleton by cleaving the capped filaments into fragments, now capped by gelsolin(Fox *et al.*, 1987; Sun *et al.*, 1999). The same pathway leading to the elevation of cytosolic Ca^{2+} also leads to a rise in PIP_2 levels though at a slower rate, which then inactivates gelsolin and CapZ thus removing them from actin and exposing the barbed filament ends(Weber *et al.*, 1991).

The freely exposed barbed filament ends generated by severing and uncapping are rapidly lengthened by the monomeric actin pool with recruitment of the actin monomers and the assembly of actin filament at the barbed ends with profilin,

providing the force for protrusive activity in platelets and driven by the generation of barbed ends(Hartwig, 1992). Some of these elongated actin filaments are cross-linked into a gel by filamin, while others are bundled by α-actinin and fimbrin, thereby allowing the extension of lamellipodia and filopodia enabling spreading across the damaged area, and platelet adhesion via integrins. The length of the actin filament is also heavily influenced by the vasodilator stimulated phosphoprotein (VASP) regulating the formation of filopods(Mejillano *et al.*, 2004).

With the decline of PIP_2 signalling CapZ returns to the ends of the actin filaments, rendering them stable against depolymerization and locking the platelet into its spreading form. Once tethered to actin, cytoplasamic myosin II using ATP is the molecular motor that applies the contractile force allowing sliding the long actin filaments relative to one another, causing contraction of the platelet and pulling the edges of the wound together. During the platelet shape change, new actin filaments are formed thus leading to the formation of the submembranous actin filament network and the extension of the filopodia. Simultaneously actomyosin contractile processes are stimulated resulting in the organelles, namely the dense and α-granules dispersed throughout the cell being centralised, termed internal contraction (White & Burris, 1984) and plays a role in driving granule secretion.

Finally with the depolymerization of the circumferential microtubule coil the characteristic change from discoid to a spherical form platelet is achieved.

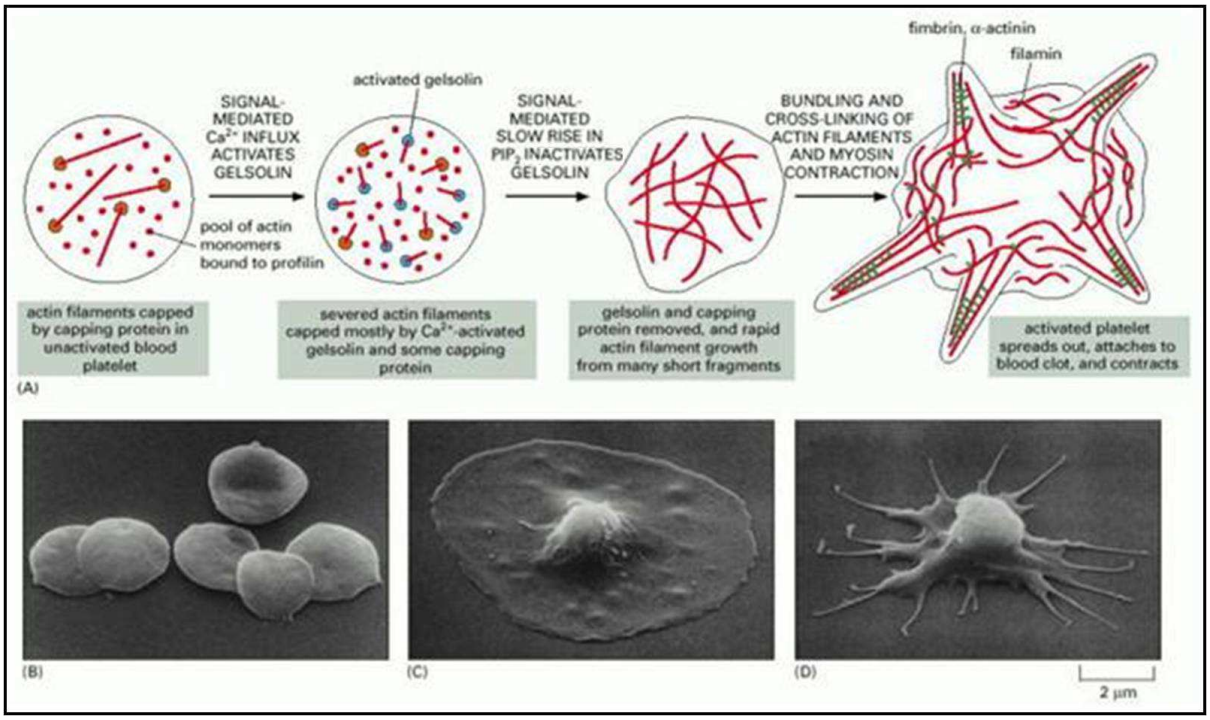

Figure.1.6 Platelet activation and subsequent shape change

(A) Platelet activation is a controlled sequence of actin filament severing, uncapping, elongation, recapping, and cross-linking that creates a dramatic shape change in the platelet. **(B)** Scanning electron micrograph of platelets prior to activation. **(C)** An activated platelet with its large spread lamellipodium. **(D)** An activated platelet at a later stage than the one shown in C, after myosin II-mediated contraction (Alberts *et al.,* 2002).

1.12 Platelet Secretion

Following platelet binding to the damaged sub-endothelial matrix and subsequent internal signalling, a controlled release reaction takes place. Platelet activation causes shape change, secretion of their intracellular granules and subsequent aggregation with one another (Gordon, 1976). Internal granules fuse with the outer membrane, thereby ejecting their contents via exocytosis. Bio-active molecules, acting in a para- and auto-crine manner quickly feed back, increasing activation of nearby platelets, magnifying secondary secretion and resulting in the amplification of the platelet activation process **(Figure.1.7)**.

Platelets contain at least three types of granules, distinguished as alpha (α), dense (δ) and lysosomes (λ) granules (White JG. 1994), which upon activation are secreted into the external medium (Ciferri *et al.*, 2000). The α-granules contain chemokines, growth factors and both pro- and anti-thrombotic molecules (Reed, 2004), and contain over two hundred and eighty different proteins (Maynard *et al.*, 2007). Dense granules contain fewer proteins, though an increased composition of membrane anchored proteins, histamine and serotonin. Lysosomes are composed mainly of enzymes such as serine peptidases, carbohydrases and phosphatases (McNicol & Israels, 1999). The controlled release of different granular contents can selectively modulate the microenvironment of the damaged area though switchable release reaction functions to choose between pro- and anti-regulatory cargo secretion (Italiano & Battinelli, 2009). The mediation of granule secretion is dependent upon the syntaxin family of membrane integrated soluble NSF attachment protein receptors (SNARE) cargo regulatory proteins, which are mobilised upon cytosolic

Ca^{2+} elevation and PKC concentration changes (Flaumenhaft, 2003) and their interaction and activation with the Munc18 family proteins (Houng *et al.*, 2003). SNARE syntaxin 2 acts on dense granules (Chen *et al.*, 2000) and syntaxin 4 and 2 act on α-granules (Flaumenhaft *et al.*, 1999). These syntaxin molecules are t-SNAREs, been bound to the plasma membrane an function as a target for the vesicle specific or v-SNARE (Ren *et al.*, 2007).

Platelet shape change, which precedes platelet secretion, generates the contractile force necessary to allow for the centralisation of granules, necessary for the fusion of granules with each other, the OCS and the plasma membrane. The contractile force and cytoskeletal rearrangement required for the secretion is supported by the phosphorylation of the MLC mediated by the Ca^{2+} and Rho dependent pathways (Suzuki *et al.*, 1999). Platelet secretion in response to thrombin is severely impaired upon disruption of the above pathway in platelets lacking G_{13}(Moers *et al.*, 2003).

1.12.1 Role of Adenosine Diphosphate

ADP is stored in dense granules at high concentration, which upon release activates platelets in an auto and paracrine manner. ADP activates platelets by the G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$(Murugappa & Kunapuli, 2006). $P2Y_1$ couples to G_q(Savi *et al.*, 1998), while $P2Y_{12}$ is coupled to G_i(Jantzen *et al.*, 2001) and both receptors are required for full platelet activation upon exposure to ADP (Jantzen *et al.*, 1999). In the absence of $P2Y_1$, platelets do not undergo shape change (Leon *et al.*, 1999) and in the absence of $P2Y_{12}$ an impaired aggregation response in mice (Fabre *et al.*, 1999). The deficiency of these receptors results in significantly prolonged bleeding times and the formation of unstable thrombi (Andre *et al.*, 2003).

1.12.2 Role of Thromboxane A_2

TxA_2 acts in an autocrine manner only, due to its short half-life and thereby its effectiveness is restricted locally. $TxA_2$is synthesised from arachidonic acid by the conversion by cyclooxygenase-1, which is targeted by aspirin and thromboxane synthase. The TxA_2 receptor (TP) which can also be activated by prostaglandin endoperoxides couples to G_q and $G_{12/13}$(Knezevic *et al.*, 1993; Offermanns *et al.*, 1994). Absence of the receptor results in prolonged bleeding times and non-stable thrombi (Thomas *et al.*, 1998).

1.12.3 Role of Thrombin in Platelet Activation

Thrombin formation is initiated by the exposure of tissue factor to plasma coagulation factors following injury to the endothelium. Thrombin formation also takes place upon the surface of the activated platelets (Heemskerk *et al.*, 2002), providing a mechanism in which platelets can activate the coagulation cascade. In addition, the presence of thrombin facilitates the activation of platelet, which is rapidly inactivated after its formation. Activation of platelets by thrombin is mediated via cleavage and activation of protease activated receptors (PAR) (Coughlin, 2005). In turn this receptor activate G_q, G_{12} and in some cases the G_i family, leading to the activation of PLCβ, PI3K and the monomeric GTP binding proteins Rho, Rac and Rab1, also leading to the elevation of cytosolic Ca^{2+}(Offermanns *et al.*, 1994; Klages *et al.*, 1999). The action of thrombin in this process is supported by the released ADP and TxA_2 binding to their respective receptors.

Four members of the PAR family have been identified; PAR 1 and PAR 4 are present on the human platelet (Kahn *et al.*, 1999). Receptor activation begins with the cleavage of the N-terminus of the receptor, which then exposes a new N-terminus that serves as a tethered ligand activating the receptor. This conformational change in the PAR receptor leads to the initiation of signalling across the plasma membrane to promote the exchange of GDP for GTP on the associated G proteins. PAR1 mediates platelet activation at low thrombin concentrations, though PAR4 requires 10 to 100 fold higher concentrations due to the lack of a hirudin-like sequence close to the C-terminal cleavage site that facilitates receptor cleavage by thrombin (Kahn *et al.*, 1998), though PAR4 activation maybe more sustained (Covic *et al.*, 2000).

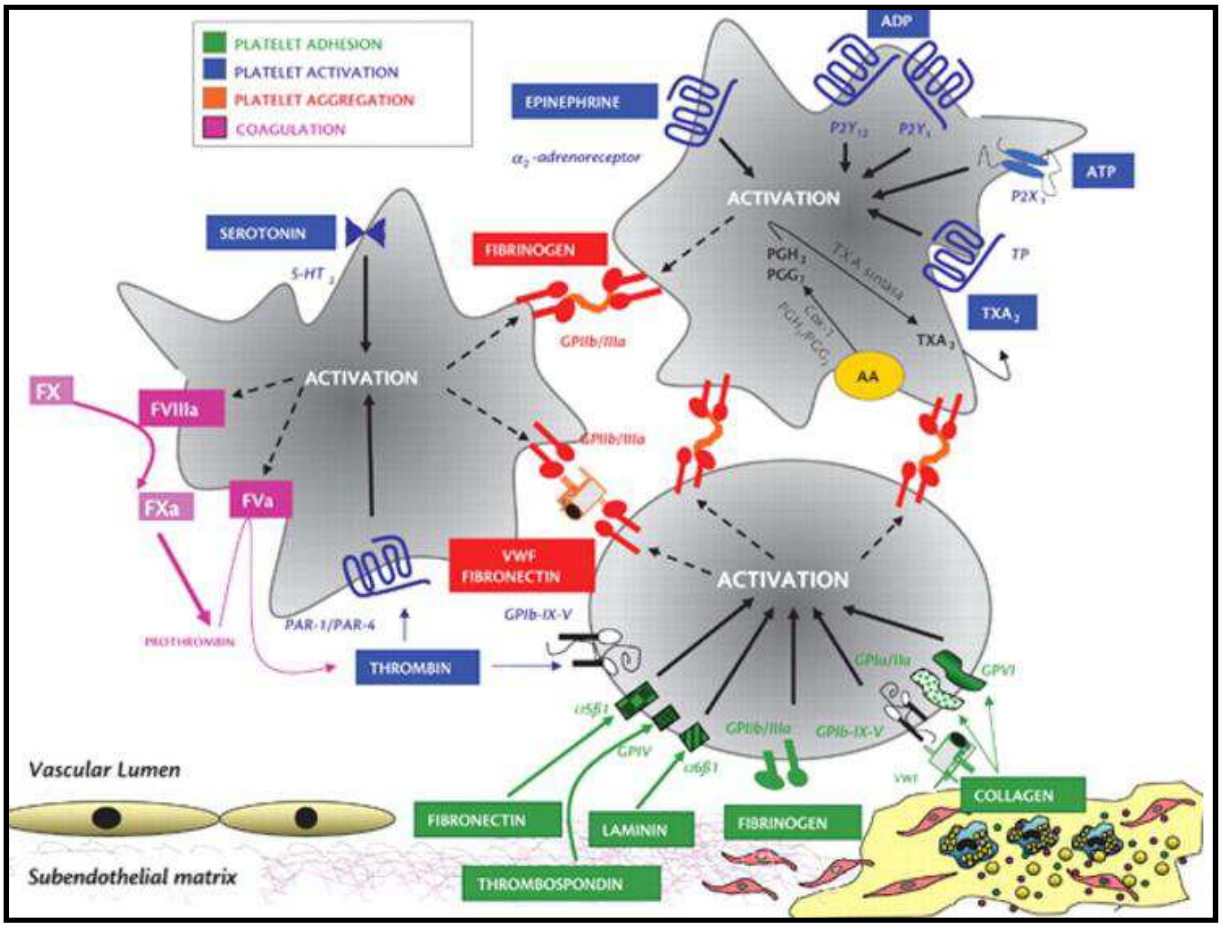

Figure.1.7 Various stages of platelet activation

Schematic representation of the main platelet activation pathways, soluble mediators and vessel-related mechanisms involved in platelet adhesion, activation, and further aggregation. AA indicates arachidonic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; vWF, von Willebrand factor; PAR, protease-activated receptor; PG, prostaglandin; TP, thromboxane receptor; TXA2, thromboxane A2 (Tubaro *et al.,* 2011).

1.13 Role of Myosin IIA

Platelets contain only one type of non-muscle myosin, the heavy chain isoform, myosin IIA (Maupin *et al.*, 1994), which interacts with the actin to develop the contractile unit. Myosin II molecules are composed of three pairs of peptides; two heavy chains of 220kDa and two 20kDa regulatory light chains that regulate myosin II activity and two 15kDa essential light chains that stabilize the heavy chain structure. The functional action of the myosin IIA is controlled by the phosphorylation of the heavy and light chains, which upon phosphorylation assemble into filaments required for the interaction with actin (Scholey *et al.*, 1980). The phosphorylation of the 20kDa regulatory Myosin Light Chain (MLC) is essential to allow myosin to move along the actin, thus providing the contractile force (Sellers *et al.*, 1985) and develop the actin activated adenosine triphosphatase (ATPase) activity (Adelstein & Conti, 1975), whilst phosphorylation of the heavy chain would prevent the myosin IIA filament formation (Dulyaninova *et al.*, 2005). The myosin necessary for contractile force is distributed throughout the platelet; though upon agonist stimulation via thrombin it relocates to the centre (Painter & Ginsberg, 1984), binding to cytoskeletal structures, and assembles into filaments (Fox & Phillips, 1982; Fox, 1993). Thereby it facilitates cytoskeletal rearrangements, folding of the surface membrane and internal contraction centralising the secretory granules and GPIb-IX whilst driving granule secretion (McNicol & Israels, 1999), all observed during shape change (Siess, 1989; Kovacsovics & Hartwig, 1996).

1.13.1 Role of Myosin Light Chain in Platelet Shape Change

The regulation of the myosin light chain (MLC) phosphorylation at serine 19 on the 20kDa proteins increases the ATPase activity of myosin in the presence of actin (Somlyo & Somlyo, 2003) by controlling the conformation of the myosin heads (Wendt *et al.*, 2001) and assembly the myosin into filaments (Scholey *et al.*, 1980).

Platelet stimuli such as TXA_2 and thrombin have been shown to activate G_q and $G_{12/13}$ pathways(Shenker *et al.*, 1991; Offermanns *et al.*, 1994; Ushikubi *et al.*, 1994). MLC phosphorylation is thereby mediated by a Ca^{2+} dependent (G_q)and, aCa^{2+} independent pathway ($G_{12/13}$) (Bauer *et al.*, 1999; Paul *et al.*, 1999). The Ca^{2+} dependent pathway effects MLC phosphorylation via the Ca^{2+}/calmodulin dependent MLC-kinase (MLCK), while the Ca^{2+} independent pathway is mediated by the Rho-kinase (activated by the small GTP binding protein RhoA), which can directly phosphorylate the MLC and the regulator of MLC phosphorylation MLC-phosphatase (MLCP)**(Figure.1.8)**. This thereby inhibits the catalytic subunit of the enzyme responsible for regulation of the phosphorylation state of the MLC leading to further increases in phosphorylation and ultimately shape change (Kimura *et al.*, 1996).

1.13.2 Regulation of Myosin in blood platelets

Contractile tension necessary for the changes in the actin cytoskeleton is regulated by myosin and the phosphorylation of the MLC. The phosphorylation state of MLC is under the control of two enzymes, MLC kinase (MLCK) and MLC phosphatase (MLCP), and the relative activity of these enzymes will control phosphorylation of the light chain. Phosphorylation of the MLC on Ser^{19} leading to the contractile response is induced by agonists that signal through G_q coupled receptors. Activation of the

receptor leads to an increase in cytosolicCa^{2+} and subsequent formation a of Ca^{2+}-calmodulin complex that then activates the MLCK (Nairn & Picciotto, 1994). The principal mediator of cytoskeletal tension is the GTPase RhoA and its downstream effector Rho associated kinase (ROCK). GTP binding and hydrolysis switches the RhoA between its GTP bound active state and GDP bound inactive state(Etienne-Manneville & Hall, 2002). RhoA has been shown to regulate smooth muscle contraction through modulating MLCP activity, via its downstream effector ROCK. The ROCK inhibitor Y27632 suppressed force activated by TXA_2 analog U46619 with parallel decreases in MLC phosphorylation(Tsai & Jiang, 2006). In endothelial cells through the phosphorylation and deactivation of the myosin binding subunit of myosin phosphatase (MYPT1) at the equivalent platelet inhibitory sites Thr^{696} and Thr^{853} results in the loss of MLCP ability to dephosphorylate MLC and along with the direct phosphorylation of the MLC, leads to force generation and shape change (Ruiz-Loredo *et al.*, ; Kimura *et al.*, 1996). Particularly thrombin has been shown to inactivate MLCP via ROCK in endothelial cells (Essler *et al.*, 1998).

1.13.3 Role of RhoA/Rho-Kinase

Agonists that can activate G_q also activate $G_{12/13}$, both capable of activating RhoA. Three main classes of Rho GTPase, Rho, Rac1 and Cdc42 regulate actin cytoskeleton dynamics (Leung *et al.*, 1996). The RhoA inactive form (RhoA.GDP) is present in the cytosol when bound to guanine nucleotide dissociation inhibitor (GDI). Activation of RhoA by either G_q or $G_{12/13}$ is mediated by Rho specific guanine nucleotide exchange factors (Rho GEF) promoting the exchange of inactive form (GDP bound) for the active form (GTP bound). The GEF proteins share a common structural motif that includes the RGS (regulator of G protein signalling), Dbl homology (DH) responsible for the exchange of GDP for GTP, and pleckstrin

homology (PH) domains (Kaibuchi *et al.*, 1999). When in the GTP bound conformation, RhoA interacts and activates the downstream effector Rho-kinase. The effector of RhoA, Rho-kinase(Matsui *et al.*, 1996)is a serine/threonine kinase, which possesses an amino terminal kinase domain followed by a coiled-coil region and other functional motifs at the carboxyl terminus. In the inactive form of Rho-kinase, the PH and Rho binding domains of Rho kinase bind to the catalytic amino region, thus leading to an autoinhibitory loop. Activated RhoA (Rho GTP) binds to the Rho binding domain, thus leading to the unfolding of the enzyme, freeing its catalytic activity(Riento & Ridley, 2003) and resulting in the spatial regulation of MLC phosphorylation in fibroblasts(Totsukawa *et al.*, 2000), which in platelets can occur independently of Ca^{2+}(Getz *et al.*).

1.13.4 Inhibition of Myosin Phosphatase via Rho-Kinase mediated phosphorylation

Myosin light chain phosphatase (MLCP) regulates the phosphorylation of MLC by active dephosphorylation of the Ser^{19} residue, thereby negative regulating actin myosin based contractility. Rho kinase phosphorylates the MYPT1 regulatory subunit of MLCP and inhibits catalytic activity(Fukata *et al.*, 2001). It is a heterotrimer, consisting of a 37-KDa catalytic subunit (protein phosphatase type 1, PP1c), a 130KDa regulatory subunit (myosin phosphatase target subunit 1, MYPT1) and a 20 KDa subunit of unknown function (Ito *et al.*, 2004). At the N terminus of the MYPT1 is a PP1c binding motif (K/R-I/V-X-F/W) adjacent to seven ankyrin repeats, and upon interaction of PP1c with the motif, substrate specificity is altered thus enhancing catalytic activity. In smooth muscle and cerebral arterial tone addition of U-46619 agonist led to the phosphorylation of $Thr^{696}$and Thr^{853} on MYPT1 by Rho-kinase leading to dissociation and inhibition of the activity of the catalytic subunit and

reducing the affinity of MYPT1/MLCP towards myosin, thus allowing phosphorylation of MLC Ser^{19}(Velasco *et al.*, 2002; Neppl *et al.*, 2009).

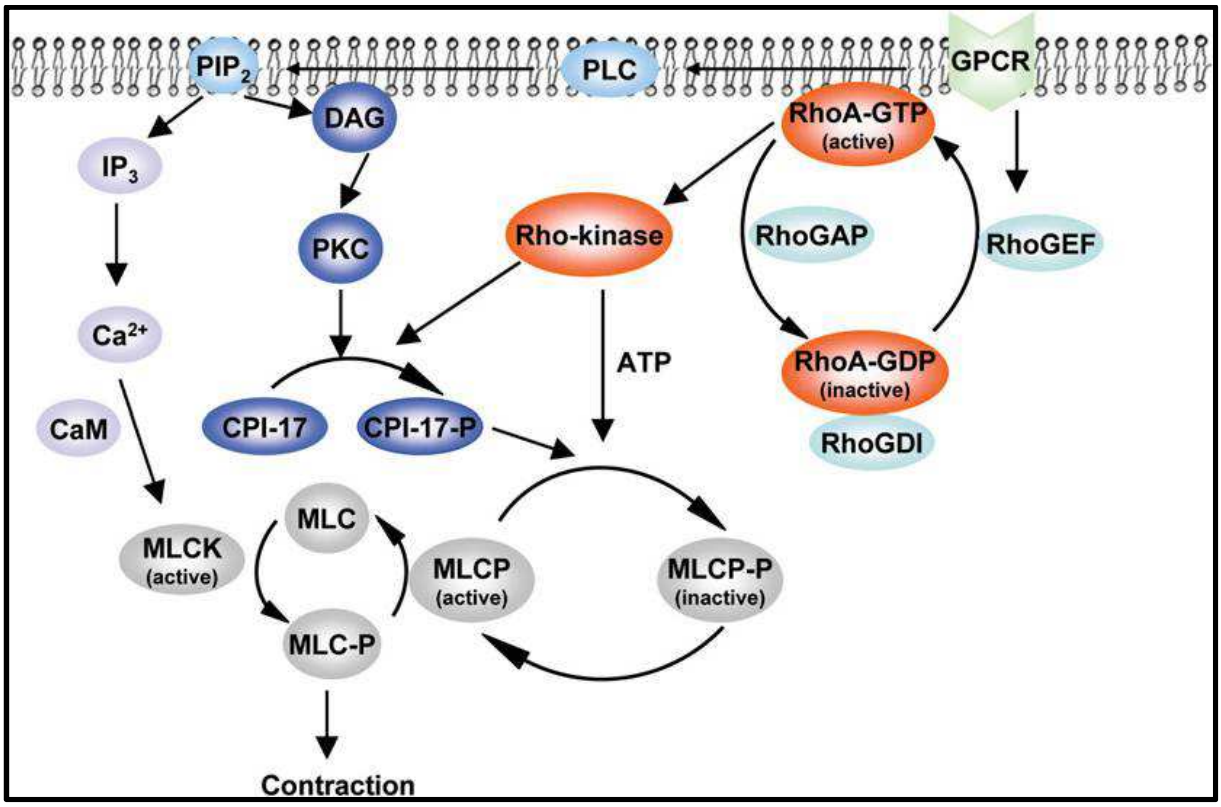

Figure.1.8 Ca^{2+} sensitization and Ca^{2+}dependent pathways

Stimulation of GPCRs leads to the activation of both Ca^{2+} sensitization and Ca^{2+} dependent pathways. PLC catalyses PIP_2 into IP_3 and DAG. IP_3 increases intracellularCa^{2+} levels and, together with CaM (calmodulin), Ca^{2+} activates MLCK. MLCK then phosphorylates MLC, resulting in smooth muscle contraction. After activation by DAG, PKC phosphorylates CPI-17. Phosphorylated CPI-17 has a high affinity for the catalytic subunit of MLCP and decreases MLCP activity through phosphorylation. Activation of GPCRs also stimulates RhoGEF activity, which facilitates the exchange of GTP for GDP on RhoA and dissociates RhoA from RhoGDI. The active RhoA-GTP translocates from the cytosol to the plasma membrane and activates Rho-kinase. Subsequently, Rho-kinase phosphorylates the targeting subunit of MLCP, leading to increased MLC phosphorylation. In addition, Rho-kinase has been shown to phosphorylate CPI-17. Meanwhile, RhoGAP accelerates the intrinsic GTPase activity of RhoA and promotes hydrolysis of GTP; thus, inactive RhoA-GDP re-associates with RhoGDI and relocates to the cytosol (Jin &Burnett, 2006).

1.14 Regulation of platelet functions

During normal circulation platelets are in a quiescent state as long as the vasculature remains intact in vessels, thereby platelets undergo no significant interaction or activation during their lifetime. However, upon trauma the main function of platelets is to assist in the stopping of haemorrhaging following vascular injury.

Upon exposure to the damaged endothelial cell, this lining triggers platelet adhesion via VWF, collagen and fibronectin. Initial tethering occurs via the GP Ib-IX-V on the platelet **(Figure.1.9)**and triggering 'inside-out' signalling, activating the high affinity state of integrin $\alpha_{IIb}\beta_3$allowing binding to divalent fibrinogen or multivalent VWF (Savage *et al.*, 2001). In addition exposed collagen is bound via the key GPVI, with the state of the damaged area dictating how these receptors interact with each other and platelet activity as a whole. The subsequent activation of the platelet causes release of additional agonist, such as ADP and thromboxane A_2 (TXA_2), recruiting further platelets to the growing thrombus and aided by the formation of thrombin.

Spontaneous or sustained platelet activation must be limited at all times, preventing undesirable obstruction of the lumen of the vessel and limiting the activation to the site of injury. There are three primary independent pathways by which the endothelium regulates platelet activity; nitric oxide (NO) and prostacyclin (PGI_2) are continually perfused into the vessels, driven by the pulsatile action of blood flow, their inhibitory action occurs through the secondary messengers, cyclic nucleotides like cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) that play a critical role in platelet inhibition (Schwarz et al., 2001). The third

pathway is the endothelial CD39, which reacts with the ADP released from activated platelets, hydrolyzing ADP to adenosine monophosphate (AMP) preventing further pro-thrombotic platelet activation and mitigating against excessive platelet recruitment (Marcus *et al.*, 1997) induced by collagen, ADP, arachidonate, and thrombin receptor agonists (Marcus *et al.*, 2001a; Marcus *et al.*, 2001b). Taken together the mentioned agents are strong negative regulators of platelet adhesion, activation and aggregation.

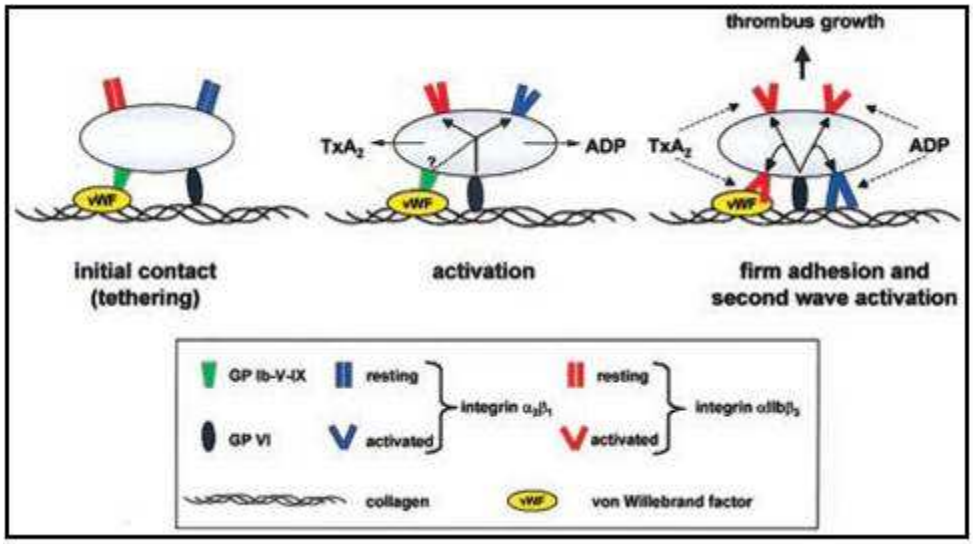

Figure.1.9 Model for platelet-endothelial interactions

The initial contact (tethering) to the ECM is mediated predominantly by GPIb-VWF and GPVI-collagen interactions. The GPIb-VWF interaction is essential at high shear rates (>500s^{-1}) but may not be required at lower shear rates. In a second step, GPVI-collagen interactions initiate cellular activation followed by shifting of integrins to high-affinity state and the release of second-wave agonists, most importantly ADP and TxA_2.GPIb-mediated signalling may amplify GPVI-induced activation pathways. Cellular activation and up-regulation of integrin affinity is proposed to be a strict prerequisite for adhesion. Finally, firm adhesion of platelets to collagen through activated $\alpha_2\beta_1$ (directly) and $\alpha_{IIb}\beta_3$ (indirectly via VWF or other ligands) results in sustained GPVI signalling, enhanced release, and procoagulant activity. In this process, $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ have partially redundant roles. Released ADP and TxA_2 amplify integrin activation on adherent platelets and mediate thrombus growth by activating additional platelets (Nieswandt & Watson, 2003).

1.15 Regulation of platelet activation and aggregation

In circulation platelets remain in a quiescent state while the endothelium lining the blood vessel remains intact. Platelets are key mediators of thrombosis (Libby, 2002)therefore tight regulation of platelet activation is essential, as excessive activation can lead to thrombosis, myocardial infarction and ultimately a stroke (Ruggeri, 2002). Therefore there are strong inhibitory signals preventing platelets from undesirable activation and limiting it and thrombus formation to the site of the damaged sub-endothelial layer. There are three predominant negative regulators, through which the endothelium controls platelet reactivity. The endothelial cell surface ectonucleotidase CD39interacts with the platelet releasate, neutralising a source of prothrombotic platelet by rapidly converting ATP and ADP to AMP, thus functioning as a cell-bound ecto-ADPase. It limits the recruitment of additional platelets into the growing platelet plug by removing ADP released from the dense granules of activated platelets and from damaged erythrocytes and endothelial cells(Gayle *et al.*, 1998; Marcus *et al.*, 2001a). Nitric Oxide (NO)(Furchgott & Zawadzki, 1980)and prostacyclin (PGI_2) (Moncada *et al.*, 1976)are released into the lumen of the vessels to inhibit platelet adhesion to the endothelium, activation and aggregation as well as having a vasodilatory effect upon the vessel. These agents act as global modulators of platelet activity, inhibiting multiple aspects of platelet function, although the detailed mechanisms of action remain obscure.

1.15.1 Nitric Oxide

Nitric Oxide (NO) is a gaseous lipophilic free radical messenger that has been implicated in the regulation of blood flow in the vessels (Ignarro, 1999),

neurotransmission in synaptic function (Garthwaite, 2008) and anti-thrombotic processes in the vasculature (Palmer *et al.*, 1987). The bioavailability of NO is a central mediator in maintaining homeostasis via regulating platelet activity and preventing the onset of thrombosis.

NO achieves this by preventing platelet adhesion and aggregation to the sub-endothelium (de Graaf *et al.*, 1992), and upon platelet activation releasing NO, thereby limiting excessive recruitment of platelets into the growing thrombus (Freedman *et al.*, 1997).

1.15.2 Nitric oxide synthesis

Nitric oxide biosynthesis is due to the activity of a class of enzymes known as NO synthases (NOS) using the amino acid L-arginine(Palmer *et al.*, 1988), present in high concentrations in the blood (60-80μM) and in extracellular fluid while been present in higher concentrations within cells. NOS first converts L-arginine to an intermediate, L-hydroxyarginine and subsequently to NO and L-citrulline which can be converted back to L-arginine. The nitrogen for NO is derived from the guanidino nitrogen atoms of L-arginine and the oxygen from molecular oxygen. The reaction requires a number of cofactors including NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (H_4B)(Ignarro *et al.*, 2001).NO has different physiological functions and therefore the different isoforms are specifically tailored to locations and production where NO is required (Bruckdorfer, 2005).

NOS exists in at least three different isoforms(Papapetropoulos *et al.*, 1999); neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) and there is some

evidence that another form of NOS exists in mitochondria (mNOS) (Schild *et al.*, 2003). The presence of NOS isoforms (eNOS and iNOS) in platelets has yielded conflicting reports (Naseem, 2008), though a recent study by Gambaryan *et al* using newly developed techniques demonstrate that the afore mentioned isoforms of NOS as proteins are not present in human platelets(Gambaryan *et al.*, 2008).

1.15.3 S-Nitrosothiols

NO can react with molecular oxygen, haemoglobin, redox metals and the super oxide anion. The reaction with oxygen yields a reactive nitrogen oxide species; S-nitrosothiols (RSNO), which exist as major redox forms in the plasma (Stamler *et al.*, 1992a)and are formed when oxidised form of NO (N_2O_3 or $ONOO^-$) reacts with thiols and result in the extension of NO half-life allowing time for NO to exert its effects on distant targets (Rassaf *et al.*, 2002). RSNO are present in the vasculature and in both high molecular weight, S-nitrosohemoglobin (HbSNO) and S-nitrosoalbumin (AlbSNO), the predominant form of RSNO in plasma (Marley *et al.*, 2001), and low molecular weight, S-nitrosoglutathione (GSNO)(Stamler *et al.*, 1992a), the most abundant intracellular RSNO (Stamler *et al.*, 1992b). Both the high and low molecular weight RSNO have the ability to inhibit platelet function, as NO stimulates guanylyl cyclase thereby producing intracellular levels of cGMP and attenuate platelet function primarily via this mechanism. Release of NO from GSNO occurs via enzymatic GSNO reductase activity, by catalytic redox effects of transition metals (Cu^+, Cu^{2+}, Fe^{2+}) or protein disulphide isomerase(Xiao & Gordge, ; Hogg, 2002).

1.16 Role of nitric oxide dependent guanylyl cyclase

The pulses of NO formed via eNOS diffuse across endothelial layers and into the lumen of the vessel, coming first into contact with platelets at the periphery of blood flow. NO diffuses into the platelet via the lipid-bilayer into the cytosol whereupon it interacts with the enzyme soluble guanylyl cyclase (sGC).The binding of NO to sGC leads to the production of cyclic guanosine monophosphate (cGMP) from the nucleotide guanosine triphosphate (GTP) substrate, this being the NO dependent mechanism of platelet regulation. Cyclic GMP is present in almost all cells(Hardman & Sutherland, 1969), and is a secondary messenger which regulates many signalling events like activation of protein kinases, ion channels and phosphodiesterases (PDEs).

The sGC is a heterodimer composed of α- and β-subunits, of which there are differing isometric forms: α_1,α_2 and β_1, β_2, though the sGCα_1 and $\beta_1$subunits are the ubiquitously expressed and exist in a 1:1 stoichiometry of an α_1 and β_1 subunits (Katsuki *et al.*, 1977). Each sGC subunit is composed of three distinct regions; an N-terminal haem binding domain, a dimerisation domain and a C-terminal catalytic domain.

The catalytic domain is responsible for the conversion of GTP to cGMP(Koesling, 1999). The dimerisation domain is located in the middle of each subunit allowing binding between the individual subunits(Wilson & Chinkers, 1995). The N-terminus contains the haem binding domain which mediates the sensitivity of the enzyme to NO(Ignarro *et al.*, 1982). Upon binding of NO to the sixth coordinate position of the

haem, the bond between a histidine in the β_1 subunit (His_{105}) and the iron is broken, resulting in a five coordinated nitrosyl-haem complex (Stone & Marletta, 1994). sGC is activated by the binding of the free radical form of $NO^{\cdot}$, which has a high affinity for haem moiety/functional group of haem containing proteins (Ignarro, 1989). The binding of NO to, and activation of sGC, results in a 200-fold increase in enzyme activity from basal, though the half-life of the haem-NO complex is approximately 60-120 seconds in smooth muscle(Margulis & Sitaramayya, 2000) and as low as 0.2seconds in platelets and cerebellar cells, thereby allowing precise control over stimulation and NO production(Bellamy & Garthwaite, 2001; Russwurm *et al.*, 2002).

1.17 Mechanisms of cGMP regulation of platelet function

Increases in NO/sGC-mediated cGMP levels lead to the activation of a variety of signalling cascades which result in the general inhibition of platelet function (Schwarz *et al.*, 2001). Cyclic GMP increases the activity of the cGMP-dependent protein kinase (PKG) leading to the phosphorylation and inhibition of a number of target proteins **(Figure.1.10)**. NO-signalling through sGC seems to primarily affect the mobilisation of intracellular Ca^{2+}, and various studies have demonstrated that NO exerts it functions by inhibiting Ca^{2+}-dependent platelet signalling (Morgan & Newby, 1989; Nguyen *et al.*, 1991; Geiger *et al.*, 1994; Le Quan Sang *et al.*, 1996; Pernollet *et al.*, 1996). Phosphorylation of the IP_3 receptor by PKG results in decreased cytosolic Ca^{2+}(Cavallini *et al.*, 1996) and phosphorylation-dependent inhibition of the TxA_2 receptor causes a reduction in TxA_2-depedent platelet activatory signalling(Wang *et al.*, 1998). One of the major substrates of PKG is the cytoskeleton associated vasodilator sensitive phosphoprotein (VASP), a key protein required for the formation of focal adhesions, needed to allow for the polymerisation of actin and

formation of lamellipodia(Reinhard *et al.*, 2001), and present in platelets at high concentrations (Eigenthaler *et al.*, 1992). It interacts with other cytoskeletal proteins to permit shape change in platelets, namely vinculin at the N terminus, profilin in the centre and F actin at the C terminus. On VASP there are three phosphorylation sites; two serine and one threonine. Upon phosphorylation of Ser^{157} the apparent molecular weight changes from 47 to 50kDa, though the 157 site is most sensitive to PKA, the Ser^{239} site is more sensitive to PKG, while the Thr^{278} site is less sensitive to either of the kinases(Abel *et al.*, 1995), though it can be potentially phosphorylated by cAMP-dependent protein kinase as demonstrated in endothelial cells(Blume *et al.*, 2007). Depending on the phosphorylation status of these sites, VASP is able to regulate actin polymerisation and actin filament bundling(Reinhard *et al.*, 2001), thereby preventing full platelet activation and serving as a marker for platelet inhibition.

However VASP phosphorylation is not the only effect of PKG on the cytoskeleton, another is the reduction of MLC phosphorylation status, due to its crucial regulation of the cytoskeleton during platelet shape change(Daniel *et al.*, 1984). PKG interferes with the elevation of intracellular Ca^{2+} critical for the Ca^{2+}/calmodulin dependent MLCK phosphorylation of the MLC by inhibiting release from internal stores and extracellular Ca^{2+} entry via phosphorylation of the IP_3 receptors(Cavallini *et al.*, 1996), TRPC, ORAI and STIM pore forming proteins (Hassock *et al.*, 2002; Kwan *et al.*, 2004; Authi, 2007; Varga-Szabo *et al.*, 2009) and stimulating the sequestering of Ca^{2+} via PKG specific phosphorylation of the SERCA into the stores (Lincoln *et al.*, 2001).

PKG can reduce the phosphorylation status of the MLC by acting on one or more targets in the RhoA pathway, including RhoGEF and RhoA, as well as other downstream targets of RhoA such as Rho-kinase and MYPT1. PKG can phosphorylate activated, membrane bound RhoA at Ser^{188}(Sauzeau *et al.*, 2000). This phosphorylation, as shown in platelets stimulates its translocation back to the cytosol thereby inhibiting the activity of its membrane bound targets, such as Rho-kinase and thereby MLC phosphorylation (Murthy *et al.*, 2003).

Demonstrated in smooth muscle is the action of PKG on MYPT1,on both the inhibitory sites ($Thr^{696/853}$) and the Ser^{695} site(Wooldridge *et al.*, 2004; Nakamura *et al.*, 2007). Phosphorylation by PKG of Ser^{695} prevents MYPT1 phosphorylation at the adjacent Thr^{696} site via Rho-kinase, thereby blocking Rho-kinase dependent inhibition of MLCP activity (Wooldridge *et al.*, 2004). The inhibition of Rho-kinase dependent phosphorylation of MYPT1 on Thr^{853} site returns the affinity of MYTP1 to MLC and allows redemption of MLCP activity (Velasco *et al.*, 2002).

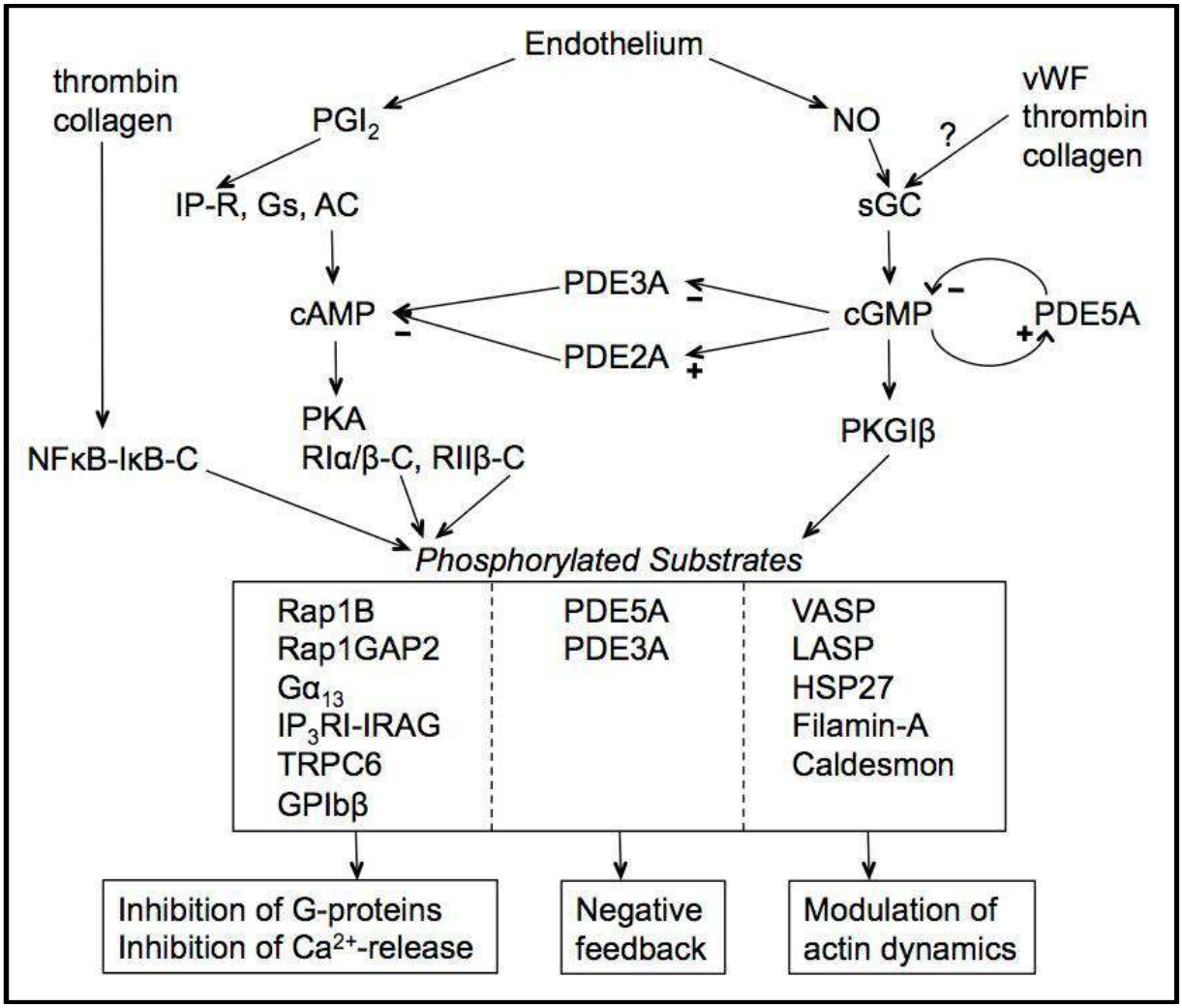

Figure.1.10 Schematic diagram of cAMP/cGMP signalling network in platelets

The intact endothelium releases prostacyclin (PGI2) and nitric oxide (NO) which bind to the prostacyclin receptor (IP-R) and the soluble guanylyl cyclase (sGC), respectively. The IP-R stimulates cAMP synthesis by adenylyl cyclase (AC) via heterotrimeric Gs protein. NO activates sGC resulting in the synthesis of cGMP.
Von Willebrand Factor (vWF), thrombin and collagen are probably also able to activate sGC, although to a much lesser extent than NO. Cyclic GMP activates phosphodiesterases 2A and 5A resulting in the degradation of cAMP and cGMP, whereas cGMP inhibits PDE3A. Cyclic AMP stimulates cAMP-dependent protein kinase (PKA) which is expressed in two isoforms composed of regulatory subunits RIα or RIβ together with catalytic C subunits, or RIIβ and C subunits. C subunit of PKA has also been found in association with an NFκB-IκB complex from which it can be released by thrombin and collagen signalling. Only the PKGIβ isoform of cGMP-dependent protein kinase (PKG) is expressed in platelets. C subunits of PKA and PKGIβ phosphorylate common substrates which have been grouped according to function. Substrate phosphorylation results in an inhibition of platelet activation, granule release, adhesion and aggregation (Smolenski, 2012).

1.18 AGC Protein Kinases

Protein kinases are key regulatory enzymes which change the properties of a substrate via attaching a phosphate group to the Ser, Thr or Tyr residues(Pearce *et al,.*2010). The subgroup of Ser/Thr protein kinases based on their catalytic kinase domains, PKA, PKG and PKC were coined as AGC kinases by(Hanks & Hunter, 1995). The AGC family contains 60 of the 518 human protein kinases(Manning *et al.*, 2002) which have been highly conserved in eukaryotic evolution. To date fourteen AGC kinase domain structures have been determined which all show the typical bilobal kinase fold that was first described for PKA(Knighton *et al.*, 1991). The bilobal kinase fold contains an amino-terminal small lobe (known as N-lobe) and a carboxy-terminal large lobe (known as C-lobe) which sandwiches one molecule of ATP to serve as the phosphate donor during phosphorylation.

1.18.1 PKA

The structure of PKA was the first protein kinase structure to be determined(Taylor *et al.*, 1990). Human platelets contain micromolar concentrations of PKA(Eigenthaler *et al.*, 1992). In its inactive state PKA is a heterotetramer composed of 2 catalytic subunits and 2 regulatory subunits. When cAMP binds to the regulatory subunits it induces a conformational change in which the catalytic subunits dissociate from the complex and phosphorylate their substrates thereby suppressing platelet activation(Taylor *et al.*, 1990). The main isoforms of regulatory and catalytic subunits expressed in human platelets are RIα, RIβ, RIIβ and Cα, Cβ resulting in the formation of PKAI and PKAII holoenzymes(Rowley *et al.*, 2011; Dittrich *et al.*, 2008).

1.18.2 PKG

PKG is a cyclic nucleotide-dependent kinase. Elevation of cGMP induces a binding-dependent activation of PKG leading to the catalytic transfer of the γ-phosphate from ATP to a serine or threonine residue on the target protein. This phosphorylated protein then mediates the translation of the extracellular stimulus into a specific biological function. In contrast to PKA, PKG forms homodimers, the regulatory cGMP-binding and catalytic domains of PKG are combined within one molecule that dimerises via its N-terminal regulatory region(Hofmann, 2005). PKGs are composed of N-terminal, regulatory, and C-terminal, catalytic domains. The N-terminal domain contains five regulatory sites:

(1) The subunit dimerisation site, consisting of an α-helix with a conserved leucine/isoleucine heptad repeat
(2) Autoinhibitory sites, involved in the inhibition of the catalytic domain in the absence of cGMP
(3) Auto-phosphorylation sites, which in the presence of cGMP may increase the basal catalytic activity and the affinity of PKGs for cAMP
(4) A site regulating the affinity and the co-operative behaviour of the cGMP binding sites
(5) The intracellular localization site, which determines the interaction of the enzyme with specific subcellular structures

The regulatory domain contains two cyclic nucleotide binding sites (termed A and B) that allow for full activation of the enzyme after specific binding of two molecules of cGMP. The catalytic domain is located at the C-terminus of PKGs, contains the

binding sites for Mg^{2+} -ATP and the target protein (Lincoln *et al.*, 1995; Lohmann *et al.*, 1997; Pfeifer *et al.*, 1999).

Two different isoforms for soluble PKG have been identified in mammals. The PKG I gene is located on human chromosome 10 and codes for the Iα and Iβ isoforms of PKG I, which arise by alternative splicing of the N-terminal region (Tamura *et al.*, 1996). The main isoform expressed in human platelets is PKGIβ. Knockout of the PKGI gene in mice revealed a pro-thrombotic phenotype, the inhibitory effects of NO-donors on fibrinogen binding, platelet shape change, granule release and aggregation were abolished, whereas effects of cAMP-analog where maintained indicating that most effects of endogenous cGMP are mediated by PKGI(Massberg *et al.*, 1999). A broad range of proteins are phosphorylated by PKG Iβ which are involved in the regulation of vascular remodelling, neoangiogenesis(Eigenthaler *et al.*, 1999; Lincoln *et al.*, 2006) and mediate smooth muscle cell (SMC) relaxation, vasodilatation and platelet shape change(Dangel *et al.*, 2010; Surks *et al.*, 1999).PKG is also thought to have overlapping effects with PKA and might also be involved in regulating long-term potentiation (Zhuo *et al.*, 1994).

1.18.3PKC

Protein kinase C (PKC) is a family of serine/threonine protein kinases that play a critical role in platelet activation as pharmacological inhibitors of PKC inhibit aggregation and secretion by the majority of agonists (Atkinson *et al.*, 2001). Many PKC substrates have been identified in platelets including components of the secretory machinery and signalling molecules (Wentworth *et al.*, 2006). The family is classified into three groups based on mechanism of activation and structural similarities (Parker *et al.*, 2004). Conventional isoforms (α, β, γ) contain a diacylglycerol (DAG)/phorbol ester-binding C1 domain and a Ca^{2+} binding C2 domain, novel isoforms (δ, ε, η, θ) also contain C1 domains, but their C2 domains are unable to bind Ca^{2+} and atypical isoforms (ζ and ι/λ) lack a C2 domain and have an atypical C1 domain, and are therefore regulated independently of Ca^{2+} or DAG (Konopatskaya *et al.*, 2010).

1.19 Regulation of cyclic nucleotide levels

Regulation of cyclic nucleotides by synthesis via adenylyl cyclase and guanylyl cyclase is controlled by degradation through phosphodiesterases (PDE) **(Figure.1.11)**. PDEs are a group of enzymes composed of several isoform families which hydrolyse the 3' phosphoester bond on cAMP and cGMP, thus converting them to inert 5' nucleotide metabolites. In platelets there are three types of PDEs; the cGMP stimulated PDE2, cGMP inhibited PDE3 and the cGMP specific PDE5 (Haslam *et al.*, 1999). PDE2 hydrolyses both cAMP and cGMP similarly, though it is stimulated by cGMP. PDE3 preferentially hydrolyses cAMP, though it is inhibited by cGMP and stimulated via PKA (Macphee *et al.*, 1988). Cyclic GMP can potentially

decrease cAMP via PDE2 or enhance via PDE3 a cAMP response. PDE5 is highly specific for cGMP hydrolysis, promoted by the cyclic nucleotide and PKG, indicating a negative feedback regulation of cGMP levels (Corbin *et al.*, 2000). The overall regulatory activity of PDE degradation of cGMP is higher than the PDE activity on cAMP(Haslam *et al.*, 1999).

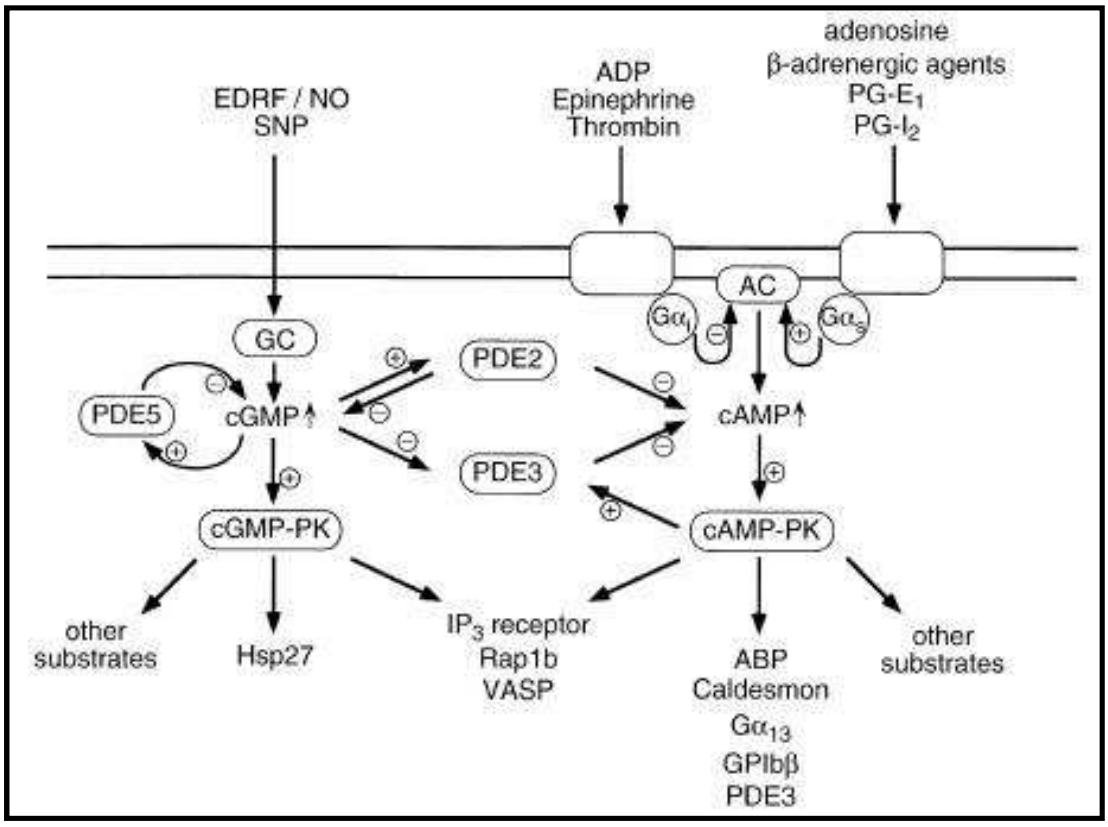

Figure.1.11 Regulation and known effector sites of cyclic nucleotides in platelets

PKA and PKG mediated inhibition of platelet activation by phosphorylation of substrate proteins (heat shock protein (Hsp27), IP_3 receptor, small GTPase Rap 1b, vasodilator stimulated protein (VASP), actin binding protein (ABP)). Also by cGMP mediated inhibition of cAMP hydrolysis by PDE3 (Schwarz *et al.*, 2001).

1.20 Aims of the study

Arterial thrombosis resulting from atheroma associated vascular injury is a major cause of death and morbidity in the industrialised world. The critical role of platelets in this process is signified by the success of antiplatelet drugs diminishing the occurrence of secondary thrombotic events. Primary inhibitory mechanisms are mediated by endothelial derived NO, which stimulates soluble guanylyl cyclase leading to activation of cGMP dependent pathways; however the mechanisms that inhibit platelet function have remained unclear. A critical event in thrombosis is the rapid platelet shape change, a process that requires rearrangement of the platelet cytoskeleton. Recently identified is MLCP, an enzyme that blocks cytoskeleton rearrangement, as a possible target for NO/cGMP signalling in platelets (Roberts *et al.,* 2009). This observation may be a critical signalling event that allows NO/cGMP to inhibit platelet shape change at the sites of vascular damage. The identification of mechanisms that modulate signalling cascades activated by NO may represent a novel target for the development of new anti-thrombotic strategies.

We hypothesise that NO modulates the MLC phosphorylation state through the MLCP; this study aims to investigate the mechanism that regulate MLC activity.

The aims of this study were;

- To dissect the regulation of the MLC phosphorylation state
- To determine the modulation of RhoA/Rho Kinase mediated inhibition of MLCP by NO/PKG
- To determine the role of phosphorylation in the regulation of platelet MYPT1 activity by NO/PKG
- To investigate the influence of NO on CPI-17 mediated inhibition of MLCP

CHAPTER 2 METHODS

2. Methods

2.1 Methods for the study of platelet function

2.1.1 Isolation and preparation of human platelets

Venous blood was drawn from patients using a 21-gauge butterfly needle. The initial draw of the blood was collected in a 5ml syringe and then discarded due to the possible containment of activated platelets from the site of venous intrusion. Whole blood was collected from the patient into syringes containing acid-citric dextrose (ACD) **(Appendix I-1)** to act as an anticoagulant at a ratio of 1:5. Whole blood was then transferred to centrifuge tubes and spun at 200g for 20minutes, allowing the separation of the platelet rich plasma in to a distinct layer from other blood constituents. Platelet rich plasma (PRP) was transferred to new centrifuge tubes with the addition of 0.3M citric acid resulting in 20µl/ml of PRP and spun at 800g for 12 minutes. The supernatant, platelet poor plasma (PPP) in these tubes was discarded and the pellet was resuspended in wash buffer **(Appendix I-1)**. These washed platelets were transferred to another tube and centrifuged for a further 12 minutes at 800g; the remaining wash buffer was discarded and the pellet resuspended in 1ml of Tyrodes buffer **(Appendix I-1)**producing the washed platelets (WP). In various experiments it was necessary the addition of apyrase 1Units/ml, indomethacin 10µl/ml, acting via inhibition of ADP and $TXA_{2,}$ blocking the secondary aggregation and secretory pathway of platelets. When looking specifically at platelets under non-aggregatory conditions ethylene glycol tetra-acetic acid (EGTA) was added at a final concentration of 1mM.

2.1.2 Calculation of platelet count

Prepared WP count was analysed via haemocytometer; 5µl of WP were diluted 1:100 in ammonium oxalate (1% w/v) and subjected to a gentle vortex, and the suspension was then pipetted into the haemocytometer and left for 5minutes for the platelets to settle. After counting on both sides of the chamber the platelet count was corrected for dilutions and expressed as platelets/ml **(Appendix I-2)**.

2.1.3 Determination of protein content

Quantification of protein in samples was achieved through the use the Bio-Rad DC Protein Assay. This reaction was based upon the Lowry assay, with the reaction between the protein and the copper in an alkaline medium, leading to reduction of Folin by the copper treated protein. The absorbance of the samples, each performed in triplicate, was compared to those generated by serial dilutions of known bovine serum albumin (BSA) concentrations, and using a wavelength of 750nm in a plate reader we determined the protein concentrations present in the samples **(Appendix I-3)**.

2.1.4 Analysis of platelet function via light transmission aggregometry

Optical aggregometry permits the production of reliable reproducible and rapid measurements of platelet aggregation (Born, 1962). In this method, a light beam was passed through a platelet suspension kept there by a miniature stirrer bar and detected by a photometer. When the platelets were induced to aggregate via the addition of an agonist light transmission through the photometer increases, and this was directly proportional to the degree of aggregation achieved.

Calibration of the aggregometer was achieved using untreated WP as base line and a tube containing Tyrodes buffer alone as 100%, and platelet aggregation assessed upon addition of various agonists. WP are suspended in Tyrodes buffer and used at a concentration of $2.5x10^8$platelets/ml **(Appendix I-4)**.

2.2 Analysis of protein phosphorylation

2.2.1 SDS-PAGE

In Sodium Dodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE), the proteins are denatured and coated in an overall negative charge, due to the bound SDS molecules and thus the basis for their separation was their mass. SDS is a strong anionic surfactant and the detergent disrupts all noncovalent interactions in the protein, unfolding the polypeptide chain. One molecule of SDS binds through its hydrophobic alkyl chain to the polypeptide backbone for every two amino acid residues; this then gives the protein a net negative charge that is proportional to its mass. The molecular separation of the protein was based on the size of the protein and its relative charge since the electrophoretic separation was carried out in polyacrylamide gel. This serves as a molecule sieve where small molecules more readily move through the pores in the gel then larger ones. Polyacrylamide was chemically inert and was formed by the polymerisation of acrylamide; the pore size in these gels was controlled by the concentration of acrylamide used.

The uses of SDS-polyacrylamide gels that contain a gradient of increasing acrylamide concentration and decreasing pore size have advantages over uniform concentration gels. Gradient gels contain two resolving acrylamide concentrations, 10% and 18% at a pH of 8.8, prepared using a linear gradient former and a

peristaltic pump. This allows a much greater range of protein weights and those of very similar size are separated better; large proteins enter the gel and start to separate immediately owing to the sieving nature of the gel while smaller proteins resolve at the smaller pore size towards the bottom of the gel; those of similar sizes therefore separate as two close sharp bands. Additionally the high pH ensures that most proteins have a net negative charge and will migrate towards the anode. The stacking gel contains large pore size polyacrylamide (3%) at pH 6.8 atop the resolving gel containing the loading lanes. This section allows the focusing of each protein into narrow bands and ensures that all the proteins enter the resolving gels at the same time.

2.2.2 Sample preparation for SDS-PAGE

Preparation of samples requires addition of 2x concentrate of Laemmeli sample buffer containing Tris base which acts as a buffer maintaining the correct pH. Glycerol makes the samples denser then the running buffer allowing them to settle into the bottom of the lanes, and addition of bromophenol blue allows visualisation of the electrophoresis progress. SDS breaks up the secondary and tertiary structure of the proteins by adding negative charge to the amino acids, as negative charges repel, therefore straighten out the protein thereby rendering the protein immediately functionless. The 2-mercaptoethanol was responsible to break the remaining disulphide bonds; residual quaternary structure was disrupted by boiling the sample for 5 minutes, transforming the samples from the globular to linear proteins ready to be used in SDS-PAGE **(Appendix I-4)**.

2.2.3 Methods for SDS-PAGE

Preparation of the specific percentage gel was dependent on the protein under investigation, by a gradient 10-18% or a single percentage 10% gel. Once the correct percentage was chosen it was placed in the gradient mixer and poured with the aid of a peristaltic pump **(Appendix I-5)**. Once at the correct level a small volume of methanol was pipetted atop the mixture ensuring a level and even surface and the resolving gel was left to polymerise for 1 hour. Following removal of the methanol, the 3% stacking gel was poured atop and a well forming comb inserted immediately, the mixture was left to polymerise for 20 minutes. Once the gel was secured in the electrophoresis module, the chamber was filled with running buffer **(Appendix I-6)** and the combs removed, allowing the loading of protein samples 10-30µg and the addition 10µl of a biotinylated protein ladder. This allows for greater accuracy when analysing the molecular weight of specific protein under investigation after running the gel at 120Volts for 2.5 hours.

2.2.4 Western Immunoblotting

Immunoblotting is a way of visualising the protein of interest using an antibody. For this technique the proteins have to be transferred out of the gel where they are inaccessible to the antibodies, to a polyvinylidene difluoride (PVDF) membrane. The membrane was pre-wetted with methanol for 1minute followed by a 10 minute wash with dH_2O and stored in transfer buffer until required. Following completion of SDS-PAGE the resolving gel was isolated from the casting plates, then sandwiched in the transfer cassette between sponges, blotting paper and the PVDF membrane kept saturated in running buffer while air pockets were removed prior to insertion into the

transfer tank, which was itself located in an ice box preventing overheating during the process consisting of 100Volts for 2.5 hours.

Following completion of the process of protein transfer after the allotted time, the PVDF membrane was removed and washed in $TBS_{T\ (0.1\%)}$ for two 5 minute intervals. The PVDF membranes were then placed in either 10% (w/v) BSA in $TBS_{T\ (0.1\%)}$ or 5% (w/v) milk for 30 minutes on a shaker leading to blocking of unoccupied binding sites on the membrane. The membrane was then washed for 5 minutes in $TBS_{T\ (0.1\%)}$ before been incubated with the primary antibody (in 2% w/v BSA in $TBS_{T\ (0.1\%)}$) at 4°C overnight. After this membranes were washed for two 5 minute intervals removing remaining primary antibody, then incubated with the appropriate secondary antibody (in $TBS_{T\ (0.1\%)}$) for 1 hour. Additional horse radish peroxidase (HRP)-conjugated anti-biotin antibody (1:10,000) was incubated to reveal the protein standard upon detection. After the incubation with the appropriate secondary antibody, membranes are washed 4-6 times in $TBS_{T\ (0.1\%)}$ removing excess remaining secondary antibody before detection with Enhanced Chemiluminescence (ECL) **(Appendix I-6)**. Membranes were incubated with ECL for 90 seconds while protected from light, then transferred to the exposure cassette and developed in a dark room, with the aid of Hyperfilm ECL and developer solutions, which were used to visualise the immunoblot. The film was then washed extensively in dH_2O before been placed in the fixer.

2.2.5 Stripping and reprobing of membranes

The purpose was to remove previous primary and secondary antibodies present upon the PDVF membrane to prevent any undue interference when re-probed using different antibodies.

The membrane was incubated at room temperature with the stripping buffer for the recommended duration of 20 minutes. The buffer was decanted away and the membrane was then bathed in $TBS_{T\ (0.1\%)}$ to remove residual traces of the corrosive buffer and once complete re-incubated with a new primary antibody.

2.3 Calcium Assay

2.3.1 Platelet preparation

Initial platelet preparation method was discussed in **2.1.1.** PRP was transferred to a new centrifuge tube with the addition of20µl/ml of 0.3M citric acid followed by 2µM of the fluorochrome Fluo-3 acetoxymethylester and the samples were incubated for 30 minutes at 37°C. After incubation indomethacin (10µM) was added to the PRP to prevent activation via thromboxane A_2 and samples were centrifuged at 800g for 12 minutes. The platelets were then resuspended at $1x10^8$ cells/ml in Ca^{2+}buffer, and apyrase (1U/ml) was added to prevent platelet activation via ADP **(Appendix I-9)**.

2.3.2 Measurement of cytosolic calcium

The fluorescent probe Fura-3 was used to investigate intracellular changes in cytosolicCa^{2+}in platelets. The platelet suspension and appropriate Ca^{2+} buffer were incubated at 37°C and each experiment carried out at $1x10^6$ cells/ml. Agonist induced increases in intracellular Ca^{2+} levels in Fluo-3 pre-loaded cells were measured at 530nm after excitation at 480nm and recorded using a fluorospectrometer (Photon Technology International). Graphs were constructed by measuring peak responses to agonist or antagonists and expressed as fluorescence intensity (counts per second).

2.4 SKOV-3 analysis

2.4.1 Cell culture

Human ovarian adenocarcinoma cells (SKOV3) were purchased from LGC Promochem (Middlesex, UK). The SKOV3 cells were cultured in a flask or a 10cm dish with DMEM-F12 medium (Invitrogen, UK) supplemented with 10% fetal calf serum, 100units/ml penicillin and 100µg/ml streptomycin, and maintained at 37°C under 95% air and 5% CO_2. Cells were seeded onto 48-well or 96-well culture plates or 6 cm dishes for experiments.

2.4.2 RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). The mRNA was reverse-transcribed to cDNA using M-MLV reserve transcriptase (RT) (Promega). The primer set for PCR was designed across an intron to avoid the genomic DNA contamination. Forward and reverse PCR primer sequences are given in **Appendix V**. Non-template or non-RT was set as negative control, and the TRPC mouse brain cDNA was used as positive control. Thermal cycling was 95°C (5 min), 40 cycles at 94°C (30 s), 53–60°C (1-3 min), 72°C (1 min), and 72°C (7 min). PCR products were detected on a 2 % agarose gel.

2.4.3 Antibodies

Rabbit polyclonal anti-TRPC antibodies were generated against the extracellular third loop (E3) region near the channel pore or targeting to the C-terminus. The specificity of E3-targeting antibodies including anti-TRPC1 (T1E3), anti-TRPC4 (T45E3), anti-TRPC5 (T5E3) and anti-TRPC6 (T367E3) were tested by ELISA, western blotting and functional assays demonstrated in (Xu & Beech, 2001; Xu et al., 2005b; Xu et al., 2006a; Xu et al., 2006b; Xu et al., 2008a).

2.4.4 Immunostaining

Cells were fixed with 4% paraformaldehyde and permeabilised by incubation in -20°C methanol for 1 minute and 0.1% Triton X-100 in PBS for 1 hour at room temperature. For un-permeabilised staining, the steps for methanol and Triton X-100 were omitted. Non-specific binding sites were blocked by incubation in 1% bovine serum albumin (BSA). Cells were then incubated in the appropriate TRPC primary antibodies (T1E3 at 1:500 dilutions, T45E3, T5E3 and T367E3 at 1:250 dilution) in PBS with 1% BSA at 4°C overnight. Cells were washed three times with PBS and then incubated in the secondary sheep anti-rabbit IgG conjugated with FITC (Sigma) at a dilution of 1:160 for 3 hours at room temperature. After three washes with PBS, cells were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). The stainings were photographed using Bio-Rad Radiance 2100 confocal microscope and acquisition software (Bio-Rad, USA).

2.4.5 Small interference RNA transfection

The siRNAs for TRPC channels were purchased from Sigma (UK) **(Appendix IV)**. TRPC siRNAs were transfected into SKOV3 cells using Lipofectamine 2000 (Invitrogen). For each 10 cm^2 plate surface area, the mixture of 10µl TRPC siRNA and 10µl Lipofectamine was incubated for 20 minutes at room temperature to allow the mixture of siRNA and Lipofectamine to form complexes. The siRNA and Lipofectamine complex was then added to the cells in a dish with 800µl of serum/antibiotics-free medium. Some transfections were performed onto cells in 96-well or 48-well cell culture plate. The final concentration of TRPC siRNAs was 100 nM. The wells or dishes without siRNA (no siRNA) or with scramble siRNA (Sigma)

or non specific pool siRNA were set as negative control in parallel. The Bcl-2 siRNA was used as positive control.

2.4.6 Cell proliferation assay

Cell proliferation was determined using WST-1 assay (Roche). WST-1 is a tetrazolium salt and can be cleaved by cellular enzymes, and thus the assay reflects the metabolic activity of the cultured cells. The overall cellular metabolic activity measured by optical absorption correlates well with the viable cell number in the culture dish/well as determined by cell counting.

2.5 Statistical analysis

All data are expressed as mean±SEM. The statistical significance was analysed using ANOVA and student t-test in the Origin 6.0 software (Northampton, MA, USA). Significance level was indicated by * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$).

CHAPTER 3 ROLES OF CALCIUM STORES AND SIGNALLING PROTEINS

3. Introduction

3.1 Store-operated calcium entry

Platelet activation is triggered by a significant number of agonists, including thromboxane A_2 (TxA_2), adenosine-5-diphosphate (ADP) and thrombin. These act on different receptors though all triggering an increase in intracellular Ca^{2+} concentration (Rink & Sage, 1990) and leading to activation of the Ca^{2+}/calmodulin dependent MLCK which phosphorylates the MLC. In platelets the elevation in cytosolic Ca^{2+} contributes to the various steps of cellular activation, such as the phosphorylation of the MLC, reorganisation of the cytoskeleton necessary for shape change and secretion (Hathaway & Adelstein, 1979; Kamm & Stull, 2001). Elevation in cytosolic Ca^{2+} can be derived from two main sources; the release from internalised stores and the influx of extracellular Ca^{2+} through the plasma membrane. Agonist stimulation leads to the activation of phospholipase C (PLC) isoforms, which hydrolyse phosphoinositide 4, 5 biphosphate (PIP_2) to inositol 1, 4, 5 trisphosphate (IP_3) and 1, 2 -diacyl glycerol (DAG). IP_3 binds to its receptors on the membrane of the intracellular Ca^{2+} stores and mediates the release of Ca^{2+} into the cytosol (Berridge *et al.*, 2003). This is inhibited by NO/cGMP which prevents the IP_3 mediated intracellular Ca^{2+} release (Schwarz *et al.*, 2001) a critical step in the signal transduction pathway that leads to full platelet activation (Jackson *et al.*, 2003). In platelets the major Ca^{2+} stores is the dense tubular system, upon depletion of the Ca^{2+}, it triggers a sustained influx of extracellular Ca^{2+} know as store operated Ca^{2+} entry (SOCE). The exact underlying signalling proteins in this process have only recently been elucidated, such as the stromal interacting molecule 1 (STIM1)

identified as a Ca^{2+} store sensor (Grosse *et al.*, 2007) with Orai 1 and members of the canonical transient receptor potential (TRPC) proposed to contribute to the SOC in platelets (Rosado *et al.*, 2002; Tolhurst *et al.*, 2008). The present work was directed to understanding the roles of SOC in platelets and in particular how this was potentially regulated by NO. The initial aspects of this work was to use immortalised ovarian cancer SKOV3 cells to establish the protocols for the assessment of the tools used to study the proteins involved in the SOC and apply these to platelets.

Ca^{2+} signalling is believed to play a central role in the signalling cascades in the tumorigenesis and neoplastic progression by controlling gene expression, progression through the cell cycle, and DNA synthesis (Berridge et al., 1998), as inhibitors of Ca^{2+}dependentsignalling suppress the proliferation of cancer cells in vitro and in solid tumours in vivo (Cole &Kohn, 1994; Holmuhamedov et al., 2002). Store-operated Ca^{2+} influx is one of the Ca^{2+} entry pathways and closely related to cell proliferation and apoptosis (Parekh & Penner, 1997). The importance of store-operated Ca^{2+} influx in the cancer development has been noticed for many years (Weiss et al., 2001; Peng et al., 2003; Kazerounian et al., 2005), however the role of TRP channels that act as the molecular constituents or subunits of SOCs or ROCs in cancer cell proliferation are largely unknown (Prevarskaya et al., 2007).

3.2 Results

3.2 Role of calcium stores and signalling proteins

3.2.1 Investigation into calcium signalling proteins

3.2.1.1 Confirmation of TRPC

The members of the TRPC family of cation permeable channels, have been proposed as G protein-coupled receptor-operated Ca^{2+} channels (ROC) or Ca^{2+} store-operated channels (SOC) (Xu & Beech, 2001; Clapham, 2003; Nilius et al., 2007). TRPCs are ubiquitously distributed in the body and play essential roles in human physiology, such as in the smooth muscle cell proliferation and migration (Bergdahl et al, 2005) and contraction (Bergdahl et al., 2003). Store-operated Ca^{2+} influx is one of the Ca^{2+} entry pathways and closely related to cell proliferation and apoptosis (Parekh & Penner, 1997), with the importance of store-operated Ca^{2+} influx in cancer development been noticed for many years (Weiss et al., 2001; Peng et al., 2003; Kazerounian et al., 2005). From the TRPC family, platelets have been shown to express TRPC6 and TRPC1, and are likely to express other TRPC and other SOC constituents (Authi, 2007). In the present study, the immortalised SKOV3 cells were used as a model for the TRPC expression prior to the application of the techniques across to investigate SOC in platelets.

To confirm the expression of TRPCs in SKOV3 cells, the messenger RNA was extracted and detected via reverse transcriptase polymerase chain reaction (RT-

PCR) shown in **Figure.3.1.a**. The β-actin was used as positive control, and the reverse transcript step omitting the reverse transcriptase (no RT) was set as negative control. TRPC1, TRPC3, TRPC4 and TRPC6 were positive in human ovarian adenocarcinoma SKOV3 cells. Two bands for TRPC1 were detected, which suggested the existence of splicing isoforms in the cells (Brinkman *et al.*, 2004). The protein expression of TRPC channels in **Figure.3.1.b.**was probed by western blotting.

The protein bands for TRPC1, TRPC3, TRPC4 and TRPC6 were detected using anti-TRPC1 (T1E3), anti-TRPC3 and anti-TRPC4 (T45E3) and anti-TRPC6 (T367E3) antibodies, respectively. The same size of TRPC6 protein band was detected by anti-TRPC6 antibody targeting the N-terminal shown in **Figure.3.1.b.ii.** SKOV3 cells were stained with anti-TRPC1 (T1E3), anti-TRPC3, anti-TRPC4 (T45E3) and anti-TRPC6 (T367E3) antibodies in **Figure.3.1.c**. TRPC1 was more evident in the cytoplasm of SKOV3 cells, but TRPC3, TRPC4 and TRPC6 were apparent in the plasma membrane. The cultured SKOV3 cells had an irregular and very flat cell shape and tightly attached onto the coverslips, so it was hard to obtain a Z-section with a typical imaging for plasma membrane staining.

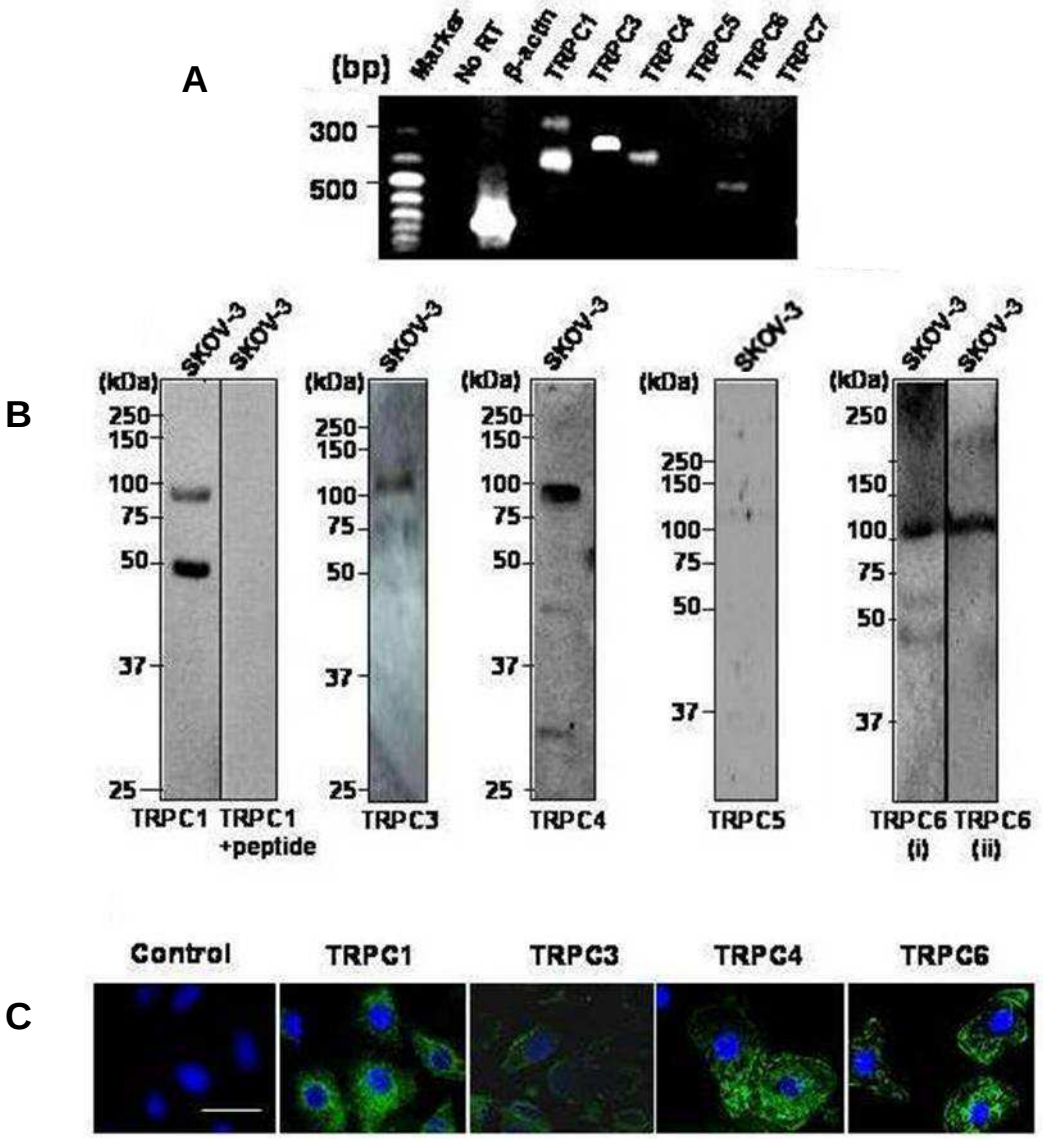

Figure.3.1 Expression of TRPC channels in SKOV3 cells

(A) Detection of TRPC mRNAs by RT-PCR using the primer set for TRPC1 (T1), TRPC3 (T3), TRPC4 (T4), TRPC5 (T5), TRPC6 (T6) and TRPC7 ((T7)**(Appendix V)**). **(B)** Western blotting detection of TRPC proteins using anti-TRPC1 (T1E3), anti-TRPC3, anti-TRPC4 (T45E3) and anti-TRPC6 **(i)** (T367E3) targeting the third extracellular loop. **(ii)** anti-TRPC6 targeting the N-terminal. No band was detected by anti-TRPC5 antibody (T5E3) in SKOV3. Two bands were detected by anti-TRPC1 (T1E3) and competed by antigenic peptide used at 10μM. **(C)** SKOV3 cells stained with anti-TRPC1 (T1E3), anti-TRPC3, anti-TRPC4 (T45E3) and anti-TRPC6 antibodies at a dilution of 1:500 and the secondary anti-rabbit antibody conjugated with FITC (green). No primary antibody was used as control. The nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI, blue). Scale bar is 25μm. The figures are representative of more than four independent experiments.

3.2.1.2 Confirmation of Stim and Orai

Studies have shown that TRPCs are not solely responsible for SOC activity; they operate as a component of a complex. Recently identified are two conserved proteins required for SOC entry. Stim functions as the sensor of endoplasmic reticulum (ER) luminal Ca^{2+} stores, which upon store depletion oligomerises and reorganises into puncta close to the plasma membrane leading to Orai activation, the pore forming subunit of the SOC channel (Liou et al., 2005; Ong et al., 2007).

Stim and Orai isoforms mRNA was detected in the SKOV3 cell line. The β-actin and GAPDH were used as positive controls in both cases. Stim1, Stim 2 and Orai 1, Orai 2 and Orai 3 were positive in human ovarian adenocarcinoma SKOV3 cells in **Figure.3.2**. The protein expression of Stim and Orai channels was detected using anti-Stim 1, anti-Stim 2 and anti-Orai 1, anti-Orai 2 and anti-Orai 3 antibodies, respectively. Stim 1 and Orai 3 were more evident in the plasma membrane of SKOV3 cells indicating its presence in pore/channel formation, but Stim 2, Orai 1 and Orai 2 were apparent in the cytoplasm signifying a function in the ER store (Ong et al., 2007).

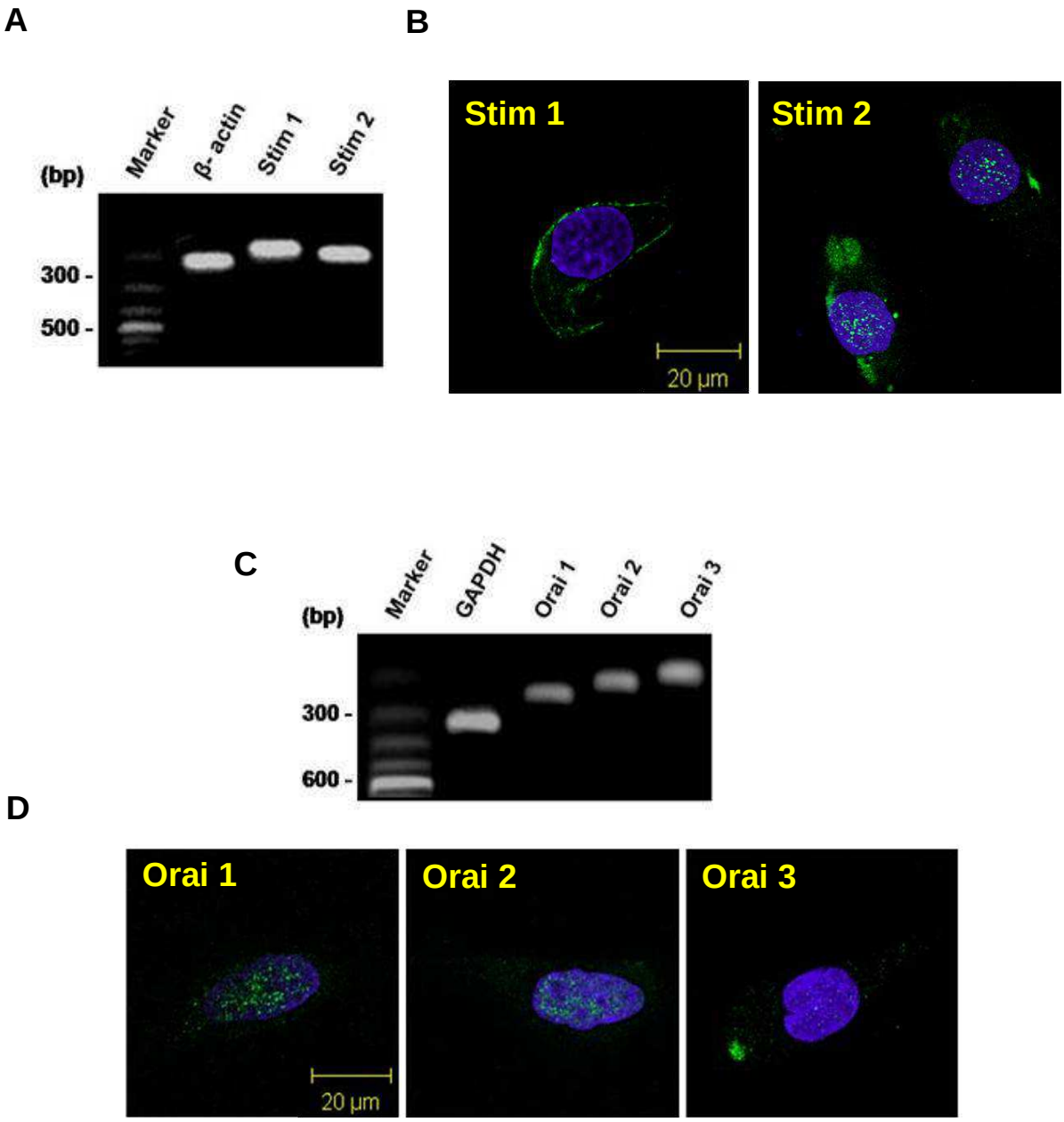

Figure.3.2 Expression of Orai and Stim channels in SKOV3 cells

Detection of Orai and Stim mRNAs by RT-PCR using the primer set for **(A)** Orai 1(O1), Orai 2 (O2), and Orai 3 (O3) **(C)** Stim 1 (S1) and Stim 2 (S2).**(B)** SKOV3 cells stained with anti-Stim 1, anti-Stim 2, antibodies at a dilution of 1:500 and the secondary anti-rabbit antibody conjugated with FITC (green). No primary antibody was used as control. The nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI, blue). Scale bar is 20µm. **(D)** SKOV3 cells stained with anti-Orai 1, anti-Orai 2 and anti-Orai 2 antibodies at a dilution of 1:500 and the secondary anti-rabbit antibody conjugated with FITC. No primary antibody was used as control. The nuclei were stained by DAPI. Scale bar is 20µm. The figures are representative of more than four independent experiments.

3.2.1.3 SKOV3 cell proliferation was inhibited by store-operated channel blockers

To investigate the role of SOC in SKOV3 cell growth, SOC blockers were used; 2-APB is a SOC blocker and a TRPC channel blocker (Flemming et al., 2003; Xu et al., 2008a). **Figure.3.3.a.** shows that 2-APB significantly inhibited the SKOV3 proliferation in a concentration-dependent manner. 2-APB has been shown to inhibit gap junctional channels, which might contribute to the anti-proliferative effect (Mesnil, 2002; Harks et al., 2003; Leithe et al., 2006; Vinken et al., 2006), therefore the SKOV3 cells were pretreated with carbenoxolone, a gap junctional blocker, to pharmacologically dissect out the contribution of store-operated channels. Carbenoxolone at 200μM and 400μM significantly inhibited the cell proliferation as shown in **Figure.3.3b** however, the anti-proliferative effect of 2-APB was still preserved in the presence of carbenoxolone in **Figure.3.3.c**, suggesting that the anti-proliferative effect of 2-APB could be explained by its inhibition on store-operated channel. To further enforce the active role of SOC in proliferation, a second SOC inhibitor was used, SKF-96365, a SOC channel blocker which also targets the Stim1/Orai1 pathway (Boulay et al., 1997; Okada et al., 1998; Liou et al., 2005). SKF-96365 significantly inhibited the cell proliferation of SKOV3 in a concentration-dependent manner in **Figure.3.3.d**, together with 2-APB suggesting the functional importance of SOC channels in the regulation of cell growth.

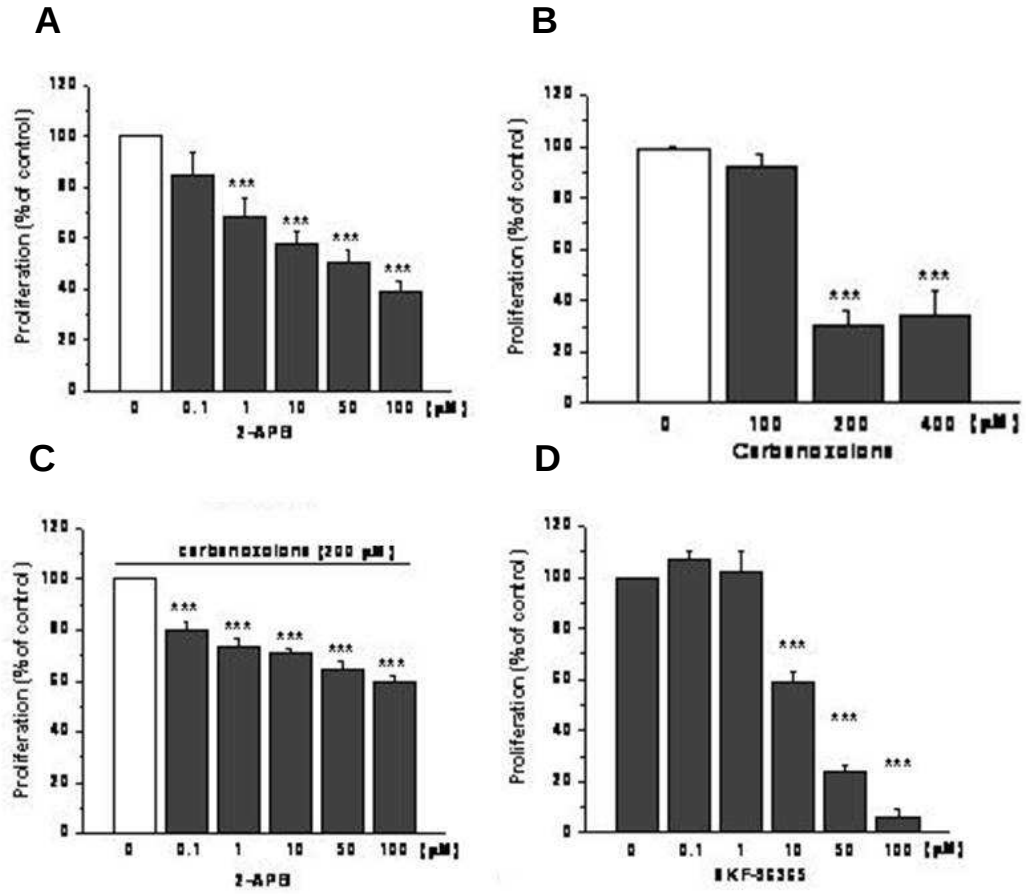

Figure.3.3 SKOV3 cell proliferation was inhibited by store-operated channel blockers

(A). SKOV3 were incubated with different concentrations of 2- APB for 24hours. The cell proliferation was monitored by WST-1 assay and the absorbance was measured at a wavelength of 450nm. **(B)**SKOV3 treated with carbenoxolone for 24hours. **(C)**. SKOV3 cells were incubated with carbenoxolone and 2-APB for 24hours. **(D)**. Effect of SKF-96365 on SKOV after 24hour incubation. Each experiment had 8 well repeats and the data was from three independent experiments. Data=mean±SEM. *** P<0.001 vs. untreated.

3.2.1.4 Role of TRPC channels in cell proliferation

Also applied were the TRPC siRNAs to demonstrate the active role of TRPCs in cancer cell proliferation. Shown in **Figure.3.4,** there was no difference among the groups transfected with scramble siRNA, pool siRNA and mock control transfection (no siRNA) on SKOV3 cell proliferation. However, the cell proliferation was significantly inhibited by the transfection with TRPC1, TRPC3, TRPC4 and TRPC6 siRNAs for 48 hours with no significant difference between their respective levels of inhibition. Bcl-2 siRNA, which targets the anti-apoptotic Bcl-2 gene, was used as a positive control, significantly inhibiting cell proliferation. These si-TRPC effects suggest that TRPC channels play a key role in proliferation of SKOV3, though there is controversy concerning the mode of activation and role in SOC and of particular TRPC channel subunits (Parekh et al., 2005).

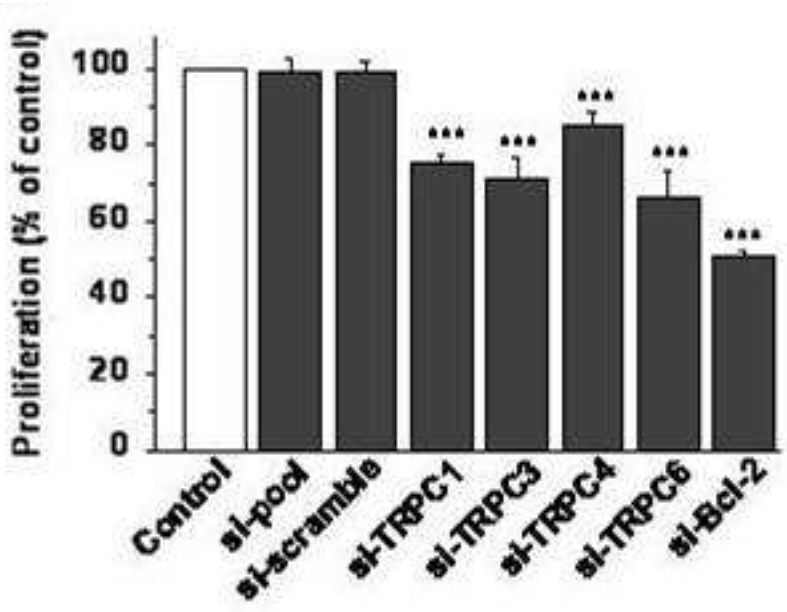

Figure.3.4 Role of TRPC channels in the cell proliferation

SKOV3 cells were transfected with TRPC siRNAs (100nM siRNA for each group) using Lipofectamine 2000. Cell proliferation was measured with WST-1 assay after 48hour transfection. The sham transfection (control), pool siRNA and scramble siRNA were negative control. Bcl-2 siRNA was used as positive control. n=16 for each group, *** $P<0.001$. Data is representative of three independent experiments. Data = mean±SEM.

3.2.2 Role of calcium pathway in platelets

Studies have confirmed an essential role for Ca^{2+} signalling during aggregate formation and thrombus growth in vitro (Nesbitt et al. 2003).One of the first stages upon the path to platelet aggregation is the reorganization of cytoskeletal protein such as actin and myosin, with the subsequent transition from the resting discoid cells to spheroidal cells and the production of pseudopodia (Siess, 1989). It has been established that a critical event in shape change is downstream of the G-protein coupled receptors, crucially at MLC phosphorylation (Daniel et al., 1984). The activation of MLC phosphorylation can occur through two routes; firstly, by a Ca^{2+}/calmodulin dependent pathway, mediated by MLCK, and secondly, by a Ca^{2+} independent pathway mediated by the GTPase RhoA and its effector Rho Kinase (ROCK) pathway (Paul et al., 1999).

To investigate the two pathways that can lead to platelet shape change, experimental conditions were performed in which either the cytosolic Ca^{2+} and/or the Rho-dependent pathway was inhibited. In the first instance, confirming the reported effects of NO on platelet intracellular Ca^{2+} levels, as recent studies indicate that the SOC component, TRPC1/3 can be regulated by NO in smooth muscle (Chen et al., 2009). **Figure.3.5** shows the possible effects of thrombin stimulation upon increases in cytosolic Ca^{2+}though the formation of IP_3 causing a release of Ca^{2+} from the intracellular stores via the IP_3R, and the effects of the inhibitors used to prevent this in a Ca^{2+} free buffer. Treatment of platelets with 1, 2-Bis (2-aminophenoxy) ethane-N, N, N, N-tetraacetic acid tetra (acetoxymethylester) ((BAPTA)(cell permeable Ca^{2+} chelator)) at 20µM was used to inhibit any cytosolic Ca^{2+} increases due to release from intracellular stores and resulted in a 89% inhibition of Ca^{2+} rise compared to

addition of thrombin (0.05U/ml) alone. Preincubation with S-Nitrosoglutathione ((GSNO) (PKG targeting the IP_3R and SOC proteins)) at 10µM similarly inhibited any rise by 86%, demonstrated in **Figure.3.5** while both experiments used ethylene glycol tetraacetic acid (EGTA) at 1mM, which prevents platelet aggregation by chelating extracellular divalent cations that are required for the stabilisation and function of the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ (Cicmil et al., 2000).

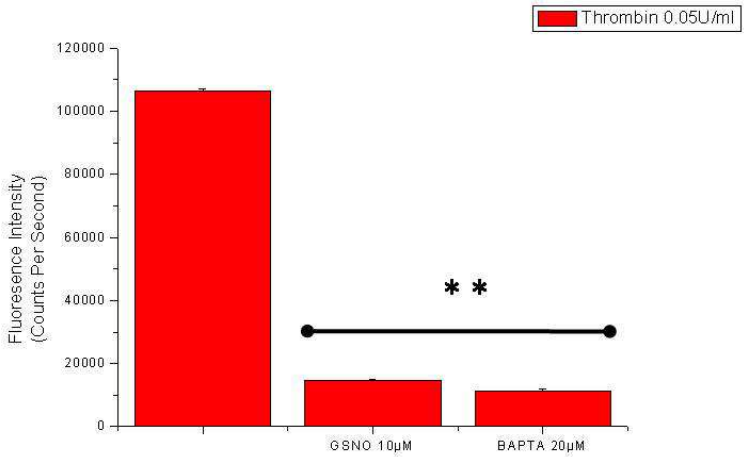

Figure.3.5. Changes in intracellular calcium in response to thrombin.

WP ($1x10^8$) were stimulated with thrombin (0.05U/ml) in the absence of extracellular Ca^{2+} and analysed at peak Ca^{2+} transit with preincubation with the Ca^{2+} chelator [BAPTA (20µM for 20mins)] or GSNO (10µM for 1min). This figure is representative of three independent experiments. Data=mean±SEM. **P<0.01 vs. agonist stimulation.

3.2.3 Influence of NO on calcium mediated MLC phosphorylation

An elevation of cytosolic free Ca^{2+} concentration is a major component of the signal transduction pathway to the activation of platelets following stimulation by thrombin (Davies *et al.*, 1989). This elevation of Ca^{2+} leads to the subsequent activation of MLCK and therefore the phosphorylation of MLC, a key marker in platelet activation and the reorganisation of the cytoskeleton necessary for shape change and secretion (Hathaway & Adelstein, 1979; Kamm & Stull, 2001). With the inference that the initial Ca^{2+} elevation was due to store-operated Ca^{2+} channels and that NO can negatively regulate this Ca^{2+} dependent pathway.

Platelet activation and subsequent Ca^{2+} elevation leads to the activation of the enzyme MLCK, involved in the phosphorylation of Ser^{19} upon MLC (Somlyo & Somlyo, 2003) and **Figure.3.6a** shows its concentration dependent inhibition via the cytosolic Ca^{2+} chelator BAPTA (1-20µM). The elevation of Ca^{2+} occurs by release from internal stores, the proposed candidates for this regulation is via the TRP channels. **Figure.3.6b** shows the addition of a SOC and TRPC blocker, 2-APB (80µM) inhibiting Ca^{2+} mobilisation upon addition of thrombin and **Figure.3.6c** similarly using SKF-96365 (25µM) a SOC inhibitor, demonstrated significant inhibition of Ca^{2+} mobilisation, both compared to the GSNO (10µM) a potent inhibitor of intracellular Ca^{2+} release and cation influx in human platelets.

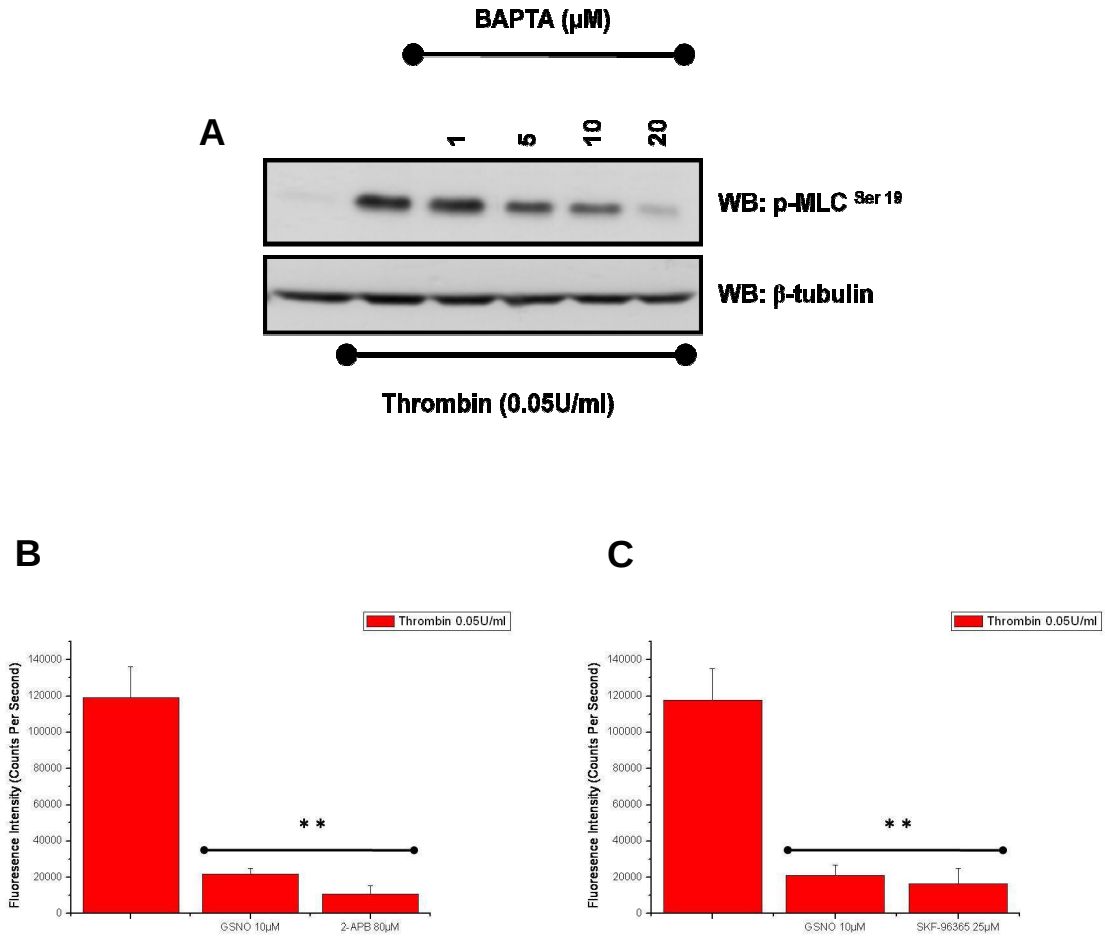

Figure.3.6 Intracellular effects of cytosolic calcium on MLC and its regulation in response to thrombin

(A) WP ($5x10^8$) were treated with BAPTA (1, 5, 10 and 20μM for 20mins)] and stimulated with thrombin (0.05U/ml) for 1 minute. Reactions were stopped by addition of and equal volume of 2xLaemmeli buffer. Proteins (30μg) were separated in a 10% gel and immunoblotted for anti-phospho-MLC^{Ser19}. This figure is representative of three independent experiments. **(B)** WP ($1x10^8$) were preincubated with the SOC inhibitor [2-APB (80μM for 3mins)] or GSNO (10μM for 1min) before stimulation with thrombin (0.05U/ml). The figure is representative of three independent experiments. **(C)** WP ($1x10^8$) were pre-incubated with the SOC inhibitor [SKF-96365 (25μM for 3mins)] or GSNO (10μM for 1min) before stimulation with thrombin (0.05U/ml). The figure is representative of three independent experiments. Data = mean±SEM. **P<0.01 vs. agonist stimulation.

3.3 Discussion

The store-operated Ca^{2+} channels (SOC) are the main mechanism for Ca^{2+} entry into non-excitable cells, and also regulating Ca^{2+} entry into cells; the Ca^{2+} homeostasis it maintains within cells controls a diversity of cellular processes such as morphological changes, exocytosis, enzyme control, gene regulation, cell growth and proliferation (Parekh et al., 2005). In platelets the initial response to thrombin induced stimuli is represented by shape change, which reportedly results in the reorganisation of cytoskeletal structure after subsequent phosphorylation of contractile proteins, in this case namely MLC (Johnson *et al.*, 2007). In this study it has been demonstrated that that this phenomenon can occur in both the presence and absence of cytosolic Ca^{2+} in response to stimulation with thrombin, confirming the findings of previous studies (Rink *et al.*, 1982; Negrescu *et al.*, 1995). In **Figure.3.6.a** BAPTA treatment significantly inhibits thrombin induced MLC phosphorylation, acting on the Ca^{2+} dependent pathway, confirmed by the use of fluorimetric studies on intracellular Ca^{2+} mobilisation and in agreement with (Trepakova *et al.*, 1999) that NO blocks intracellular Ca^{2+} mobilisation upon stimulation seen in **Figure.3.5.** The inhibition of the Ca^{2+} route alone is not sufficient to completely inhibit MLC phosphorylation as demonstrated with BAPTA, suggesting that the inhibition exerted by the inhibitors on the Ca^{2+} dependent pathway did not affect the Rho-Kinase dependent one.

One of the candidates proposed for SOC are the TRPC channels, which have been identified in many cell types; the CNS and in peripheral tissues such as bone, heart and prostate (Riccio, 2002), also smooth muscle (Xu et al., 2006) and platelets (Galan et al., 2009).

Demonstrated in this study, is that TRPC, Orai and Stim channels are highly expressed in both the mRNA and in protein form in the human ovarian cancer cell line SKOV3. Following the establishment of their expression, their possible role in SOC was investigated in **Figure 3.3**. This indicates that the possible effect of 2-APB is due to the inhibition on the SOC which is further supported by the use of another SOC blocker, SKF-96365. Since 2-APB is a broad-spectrum TRPC channel blocker and cannot distinguish the contribution of individual TRPC isoforms, the silencing of TRPC1, 3, 4, 6 with TRPC isoform-specific siRNA was untaken to confirm that they are an active component within the SOC and proliferation **(Figure.3.4)**.

To substantiate initial findings regarding the role of SOC in the model SKOV3 cell line, the contribution of the SOC in the regulation of agonist induced elevation of cytosolic Ca^{2+} in platelets was analysed via the use of fluorimetric study. In this, 2-APBwas used to block the SOC **(Figure.3.6.b)**, significantly inhibiting internal Ca^{2+} elevation upon agonist stimulation. To support the mechanism via which cytosolic Ca^{2+} elevation occurs in platelets, SKF-96365 another SOC and Orai1/Stim1 inhibitor (Liou et al., 2005) was used **(Figure.3.6.c)**, again confirming the model of Ca^{2+}efflux via SOC.

However due to the limited time available, the potential contribution of the subunits of SOC, such as TRPC, Stim and Orai proteins (Putney, 2007; Koslowski et al., 2008), studied in the SKOV3 model, could not be applied to the investigation of platelet activation.

However, recent TRPC1 extraction from human platelets has shown that it was phosphorylated by either PKA or PKG, and this phosphorylation decreased upon activation of platelets by thrombin (Authi, 2007). This suggests that the microenvironment around TRPC1 contains proteins that are substrates for PKA and PKG. Thereby in circulation when platelets are exposed to prostacyclin or nitric oxide leading to activation of PKA and/or PKG, respectively, their kinases would play an important regulatory role in maintaining low cytosolic Ca^{2+} levels via possible inhibitory phosphorylation of SOC proteins and the IP_3R regulating the IP_3 mediated intracellular Ca^{2+} release (Schwarz *et al.*, 2001), a critical step in the signal transduction pathway that leads to full platelet activation (Jackson *et al.*, 2003).

Collectively, the data presented in this chapter shows the inhibitory influence of NO upon cytosolic Ca^{2+}elevation; possibly through phosphorylation of key signalling proteins. The data also shows that the calcium dependent regulation of platelet activity is not the exclusive activatory pathway, with the presence of a calcium independent RhoA/ROCK dependent mechanism involved in the phosphorylation of the contractile protein, MLC.

CHAPTER 4 REGULATION OF PLATELET FUNCTION BY NITRIC OXIDE (NO)

4. Introduction

4.1 Regulation of platelet function by nitric oxide

Nitric oxide is a gaseous free radical messenger ($NO^{\cdot}$), which is synthesised from the amino acid L-arginine by various isoforms of NOS. NO is important in the endothelial dependent regulation of blood flow and pressure though also a key regulator of platelet function. Blood flow pushes platelets to the periphery of the vessel wall placing them next to the endothelium where they can respond rapidly to vascular damage. This blood flow also activates the release of the endothelial derived NO (Naseem, 2005).NO diffuses into platelets and via its close interaction with iron containing proteins binds to its intracellular receptor, the haem-containing enzyme called soluble guanylyl cyclase (sGC), activating it to 200 fold increase in producing the signalling molecular cyclic guanosine monophosphate (cGMP) (Schmidt et al., 1993), endothelial release of NO was linked to cGMP dependent platelet inhibition (Mellion et al., 1981), the cyclic nucleotide now can activate protein kinase G (PKG), the major mediator of NO (Feil et al., 2003). Defects in platelet cyclic nucleotide signalling may play a role in diseases such as ischemic heart disease, heart failure and diabetes, where platelet hyperactivity is due to reduced sensitivity of platelets to NO and thereby inhibition (Chirkov et al.,2007).

It has been shown that NO is able to block many aspects of platelet activation including early activatory signals such as the release of Ca^{2+} from the intracellular

stores, subsequent store operated Ca^{2+} entry, platelet adhesion, granule release, aggregation and apoptosis (Rukoyatkina et al.,2011; Schwarz, 2001; Nesbitt et al., 2003). Though as yet the exact signalling mechanisms involved in NO mediated inhibition are only partly understood. In the previous chapter the ability of NO to inhibit Ca^{2+} mobilisation was confirmed, although shown for the first time is an aspect of platelet signalling, that is, that phosphorylation state of MLC^{Ser19} can be regulated in a Ca^{2+}independent manner. In the present chapter experiments were designed to characterise the effects of NO on platelet function followed by a more detailed evaluation of the signalling mechanisms regulating MLC.

4.2 Results

4.2The regulation of platelet function by nitric oxide

4.2.1 Agonist induced platelet aggregation

The first experimental series was designed to investigate the actions of platelet agonists, via the use of thrombin and collagen typical agonists to ensure that the platelet isolation procedure produced a functionally responsive cell population. It was important to ascertain the most appropriate conditions for the use of the agonist. Thrombin and collagen were added to WP in aggregation tubes under continuous stirring ensuring an even suspension of platelets and their response to the addition of the agonists was measured using an aggregometer. These two agonists were chosen because they activate platelets by two distinct mechanisms.

Platelet preparations did not undergo spontaneous aggregation upon stirring. The addition of thrombin (0.005-0.1U/ml) to platelets induced a concentration-dependent increase in platelet aggregation. The initial effective aggregation was observed at 0.005U/ml to 8.3±4.7% and the maximum at the highest concentration used at 0.1U/ml is 71±2.1% **(Figure.4.1a)**. At concentrations of 0.05U/ml and above classical shape change could be observed.

Next the effects of the matrix protein collagen (0.1-2µg/ml), which promotes aggregation through tyrosine kinase linked receptors such as glycoprotein VI, was

examined. The initial dose responsible for activating aggregation was 0.1µg/ml 5.6±2.3%and maximum aggregation at 2µg/ml 70±2.5%**(Figure.4.1b)**. While higher concentration collagen caused maximal aggregation there was a significant lag phase compared to thrombin.

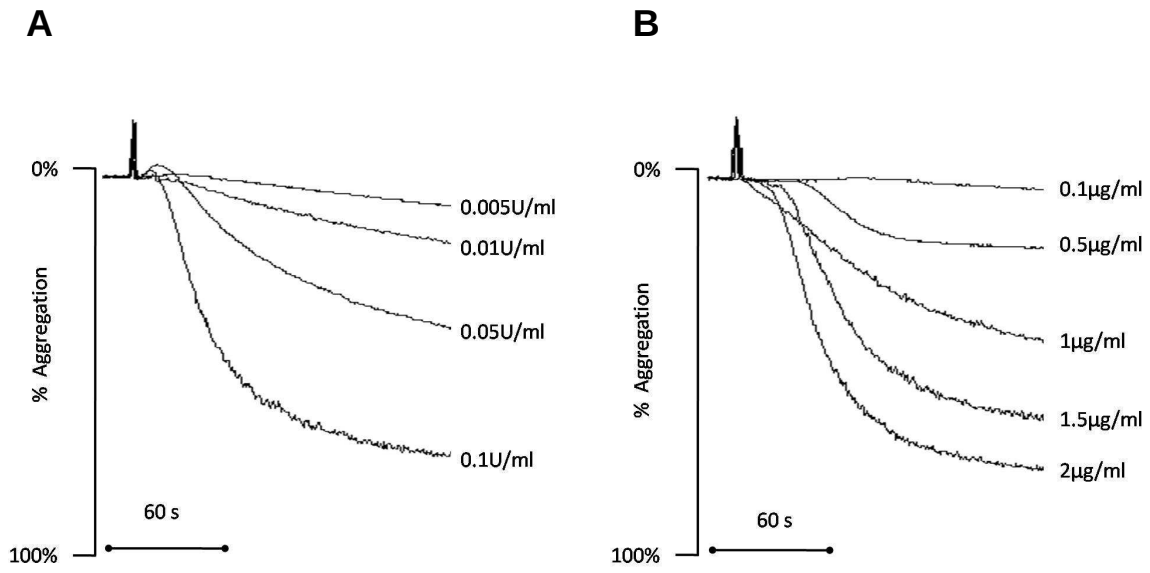

Figure.4.1 Agonist induced platelet aggregation

WP ($2.5x10^8$) was incubated at 37°C and aggregation measured as described in section **2.1.4.**Platelets were stimulated with varying concentrations of **(A)** thrombin and **(B)** collagen. Aggregation traces were recorded using a dual channel aggregometer and expressed as a % aggregation. Traces are representative of three independent experiments.

4.2.2 Influence of Nitric Oxide (NO) on platelet aggregation

4.2.2.1 Nitric Oxide inhibits platelet aggregation in a dose dependent manner

Having established concentrations of agonist required to induce aggregation the functional effects of the inhibitory pathway was tested. The NO-donor, S-nitrosoglutathione (GSNO), was used to stimulate the cGMP/PKG pathway. Platelets were incubated with GNSO (1-100μM) for 1 minute before addition of thrombin (0.05U/ml). The agonist alone caused 50±2.2% aggregation, which was then reduced to 49±9.2%, 27±3.2% ($P<0.05$), 13±1.9% ($P<0.05$) and 1.7±0.33% ($P<0.05$) in a concentration dependent manner in respect to 0.1, 1, 10, 100μM GSNO. Significant threshold inhibitory effects occurred by 1μM with 23±5.8% ($P<0.05$) inhibition, maximum inhibition was 100μM GNSO, resulting in 1.7±0.33% ($P<0.05$) aggregation **(Figure.4.2a)**. Importantly using 10μM and above completely abolished shape changes and therefore for further experiments GSNO was used at concentrations of 10μM and thrombin for 0.05U/ml.

Next to be examined was the affect of GSNO upon collagen agonist stimulated platelet aggregation. WP were again treated with GSNO (0.1-100μM) for 1 minute under continuous stirring then stimulated via the addition of collagen (1μg/ml). **Figure.4.2b** shows that collagen caused 60±0.33% aggregation, then upon addition of GSNO was reduced to 54±1.3% ($P<0.05$), 34±6.7% ($P<0.05$), 13±1.2% ($P<0.05$) and 2.3±0.88% ($P<0.05$) in a concentration dependent manner to GSNO at 0.1, 1, 10 100μM.Significantthreshold inhibitory effects occurred by 0.1μM with 5.3±1.7% ($P<0.05$) inhibition, maximum inhibition was 100μM GSNO, resulting in 2.3±0.88% ($P<0.05$) aggregation.

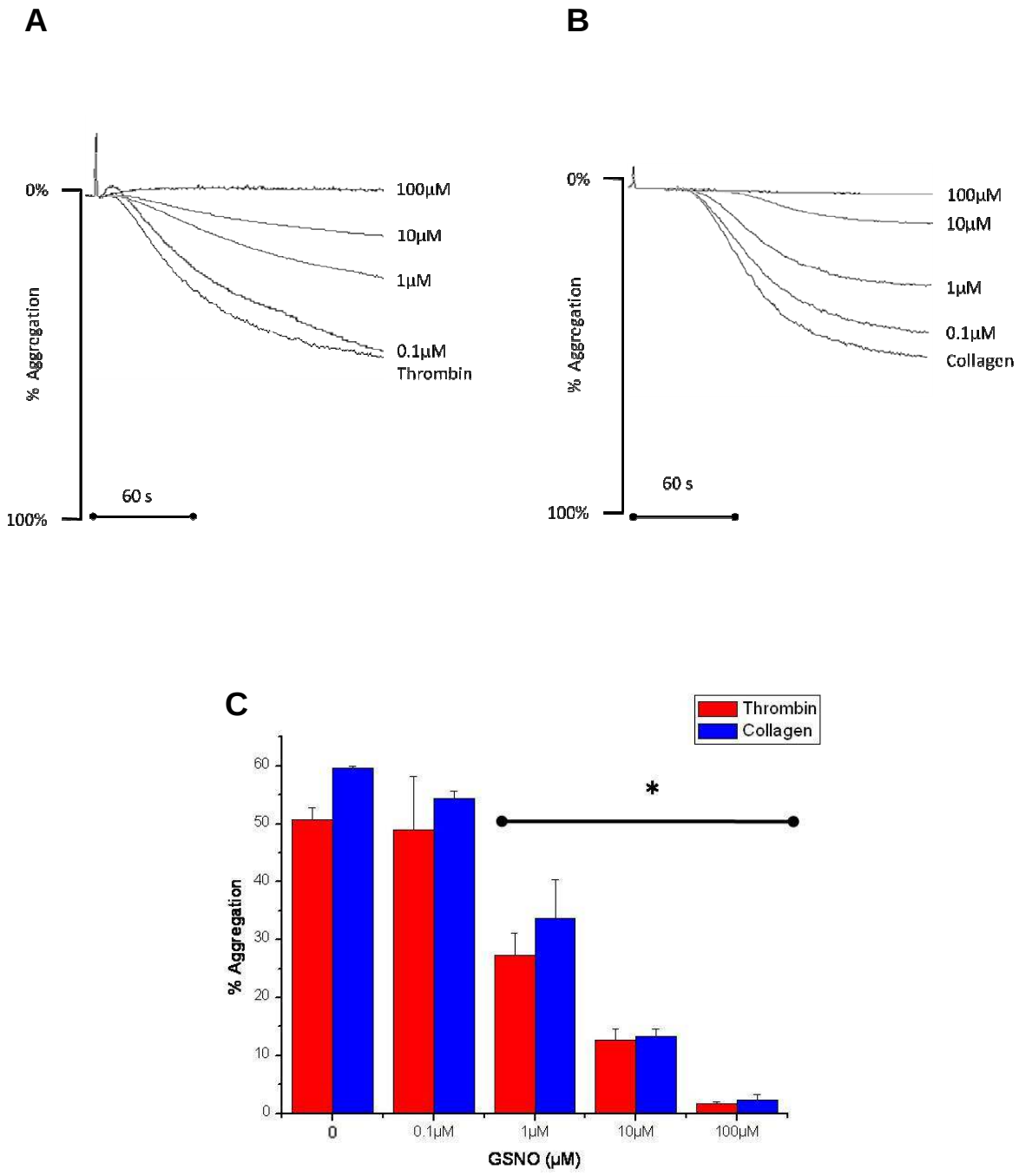

Figure.4.2 GSNO inhibits platelet aggregation in a dose dependent manner

WP ($2.5x10^8$) were stimulated with either **(A)** thrombin (0.05U/ml)**(B)** collagen (1µg/ml) or pretreated first with GSNO at the indicated concentrations prior to stimulation with the afore mentioned agonists. Responses were recorded for the indicated times and expressed as % aggregation. Traces are representative of three experiments. As is quantified in the graph **(C)**, P value compared to agonist alone. *P<0.05 vs. agonist alone sample.

4.2.2.2 Nitric Oxide inhibits platelet aggregation in a time dependent manner

The next aspect to be examined was the effectiveness of GSNO as a platelet inhibitor as a function of time. WP was incubated with an intermediate concentration of GSNO (10µM) from 30 seconds to 60 minutes before stimulation with the agonist of interest. **Figure.4.3a** shows that after 1 minute of incubation of GSNO, aggregation was inhibited by 84% upon addition of thrombin. The level of inhibition was reduced to 68% after 5 minutes and to 60% by 15 minutes. At 30 minutes 56% and for 60 minute the level of inhibition was negligible and comparable to addition of thrombin alone.

The effectiveness of GSNO as an inhibitor of collagen-mediated aggregation was tested using was collagen (1µg/ml) and 10µM of the inhibitor. **Figure.4.3b** shows that the maximum level of inhibition was reached at 1 minute with 67%, then to 26%, 11%, 8% and negligible for the times for 5, 15, 30 and 60 minutes respectively. The time courses allow us to demonstrate the changing platelet activity as a result of the degradation of GSNO over time and thereby its decline in the protective effects against agonist activation, though also the differing rates of inhibition regarding collagen and thrombin stimulation.

4.2.2.3 Nitric Oxide inhibits platelet aggregation via cGMP pathway

The key enzyme in the NO/PKG inhibitory pathway is soluble guanylyl cyclase (sGC). NO exerts its effects predominantly via activation of the sGC, which leads to the formation of cyclic guanosine mono-phosphate (cGMP) (Schaferet al., 2010). Therefore it was investigated if GSNO uses this sGC pathway to inhibit agonist-

induced aggregation. This was evaluated by the use of a selective sGC inhibitor (1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one) ODQ (Moroet al., 1996).

Preincubation of platelets with GSNO (10μM) for 1minute before the addition of thrombin (0.05U/ml) reduced aggregation from 58.3±3.4% to 14±4.6% ($P<0.05$). Incubation of platelet with ODQ (20μM) alone failed to affect aggregation induced by thrombin. However, when platelets were treated with ODQ before the addition of GSNO and thrombin, the NO-donor failed to inhibit aggregation. Under these conditions, thrombin induced 58±3.4% aggregation, which remained at 56±4.4% in the presence of ODQ and GSNO shown in **Figure.4.4**. Next platelets were stimulated with collagen after treatment with GSNO, the inhibitory effect is apparent; we see in **Figure.4.4** that GSNO causes a reduced aggregation from 57±6.01% to 19±4% ($P<0.05$). Incubation of platelets with only ODQ failed to affect aggregation induced by collagen, although when platelets were treated with ODQ before the addition of GSNO and collagen, again the NO-donor failed to inhibit aggregation. Under these conditions, collagen induced 57±6.01% aggregation, which in the presence of ODQ and GSNO 59±1.4%.

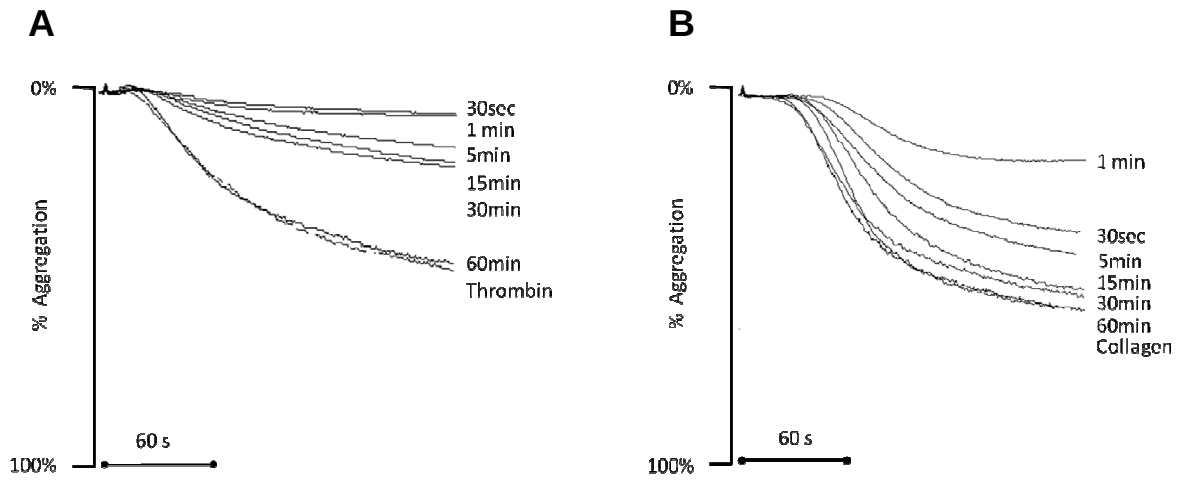

Figure.4.3 GSNO inhibits platelet aggregation in a time dependent manner.

WP ($2.5x10^8$) was incubated with GSNO (10µM) for up to 1 hour. At the indicated times the platelets were stimulated with **(A)** thrombin (0.05U/ml) **(B)** collagen (1µg/ml) for 3minutes. Aggregation traces were recorded on dual channel aggregometer and expressed as % aggregation. Traces represent two experiments.

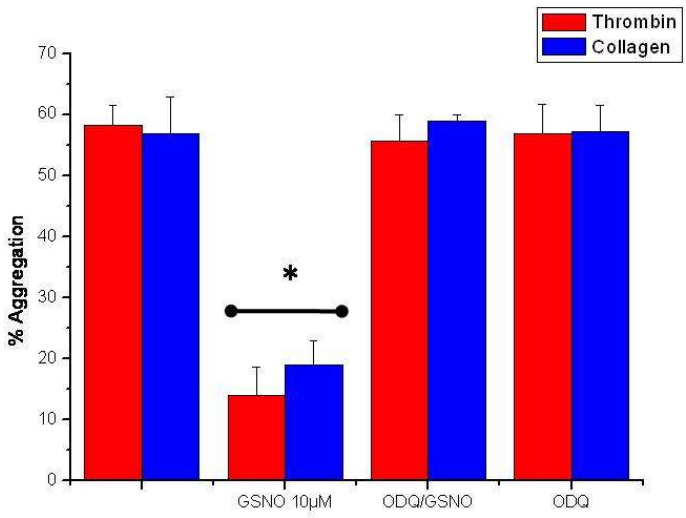

Figure.4.4 GSNO inhibits platelet aggregation via cGMP pathway

WP ($2.5x10^8$) was pre-incubated with sGC inhibitor [ODQ (20µM)] for 20 minutes. WP were then treated with GSNO (10µM) and stimulated with thrombin 0.05U/ml and collagen 1µg/ml. The graph is representative of three independent experiments. Data = mean±SEM. *P<0.05 vs. agonist stimulation.

4.2.3 Nitric Oxide induced phosphorylation of Vasodilator Stimulated Phosphoprotein (VASP)

4.2.3.1 NO induces a concentration and time dependent increase in VASP at serine 239

Stimulation with NO leads to the activation of cGMP dependent protein kinase G (PKG) and subsequent phosphorylation of various proteins, one of these is VASP, a major substrate for cyclic nucleotide dependent kinases, to be located in focal adhesions, stress fibres, cell–cell contacts, and highly dynamic membrane regions (Reinhard et al., 2001). In platelets, it is present in particularly high concentrations (Eigenthaler et al., 1992). Phosphorylation of this protein can be taken as an indicator of PKG activity. Three phosphorylation sites have been identified in VASP (Ser^{157}, Ser^{239} and Thr^{278}), although Ser^{239} is preferentially phosphorylation by PKG (Smolenski et al., 1998). Furthermore VASP phosphorylation correlates with vasodilator induced inhibition of platelet activation and aggregation (Horstrup et al., 1994). Therefore taking VASP as a marker, the previous conditions were analysed for correlation with inhibition of aggregation via the western blotting technique.

In unstimulated platelets no phosphorylation of VASP was observed. Treatment of WP with incremental higher doses of GSNO (0.1-100µM) for 1 minute incubation led to similar increase in phosphorylation of $VASP^{Ser239}$ **(Figure.4.5a)**, from the 1µM to a maximum at 100µM (highest concentration tested). This phosphorylation of $VASP^{Ser239}$ correlated directly with the inhibition of aggregation shown in **Figure.4.2** upon addition of the agonist. Incubation of platelets with the sGC inhibitor ODQ prior to GSNO resulted in the inhibition of $VASP^{Ser239}$ phosphorylation, confirming that the inhibitory pathway and its subsequent marker of effect are both mediated by the

cGMP/PKG pathway. Having established a dose dependent increase in VASP^{Ser239} phosphorylation, an intermediate concentration of GSNO (10µM) was selected and the time dependent phosphorylation of VASP^{Ser239} analysed in **Figure.4.5b**. Initial phosphorylation of the residue was detected within 30 seconds and was maintained for 15 minutes hereafter the inhibitory phosphorylation triggered by GSNO declines and by 60 minutes is no longer present.

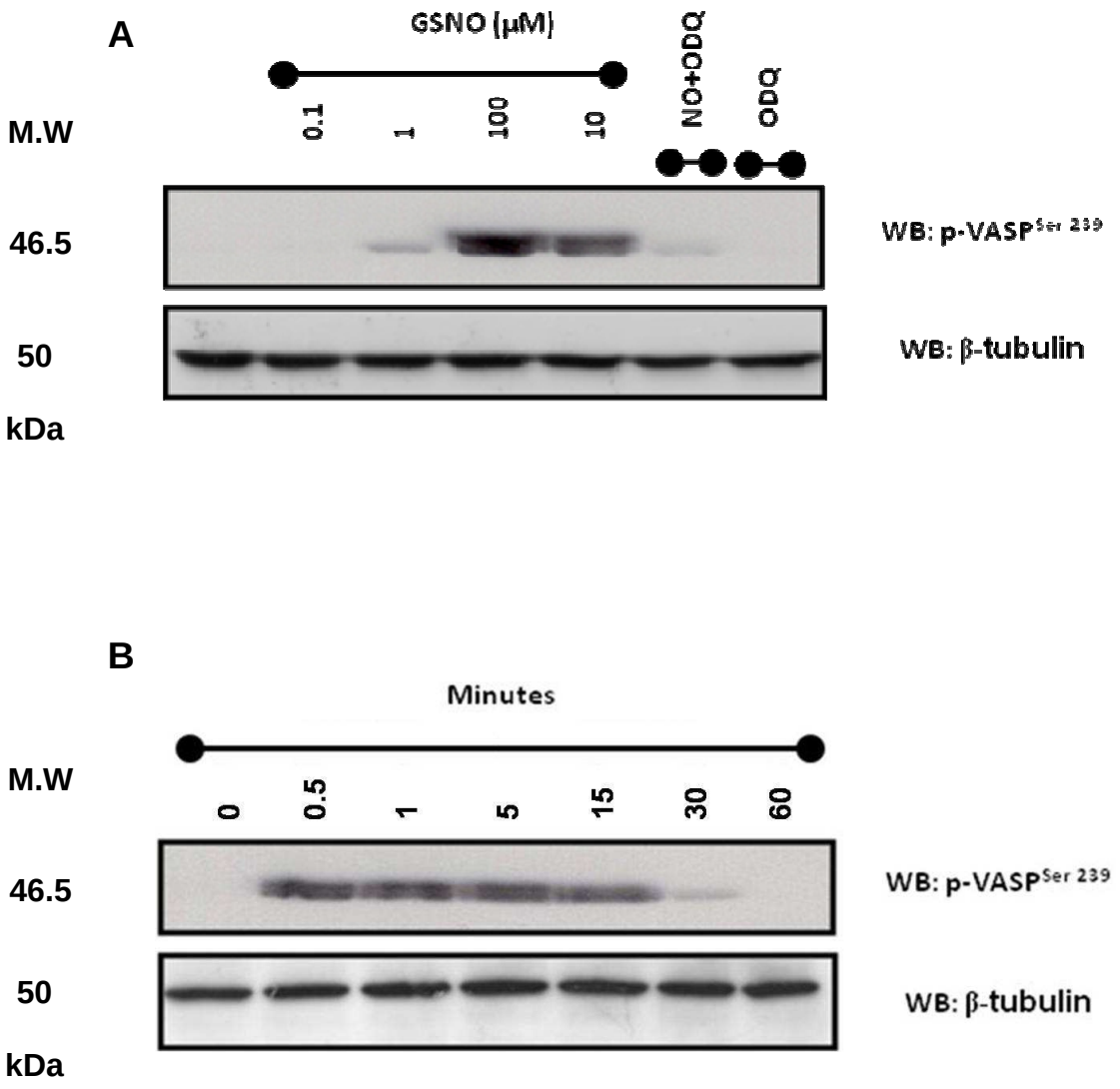

Figure.4.5 GSNO induces a concentration and time dependent increase in VASP at serine 239

(A) WP ($5x10^8$) was incubated with varying doses of GSNO for 1 minute in the presence or absence of the sGC inhibitor [ODQ (20μM].Each sample was treated at 37°C with stirring (1000rpm). Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (30μg) were separated in 10% gradient gels and immunoblotted for anti-phospho-$VASP^{Ser239}$.
(B) WP ($5x10^8$) was incubated with GSNO (10μM) for differing times. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (30μg) were separated in 10% gradient gels and immunoblotted for anti-phospho-$VASP^{Ser239}$. Blots are representative of three independent experiments.

4.2.4 Inhibition of PAR 1 and 4 induced platelet aggregation via Nitric Oxide

4.2.4.1 Inhibition of Protease Activated Receptors (PARs) 1 and 4 via a dose and time dependent manner

The multifunctional serine protease thrombin is generated at the sites of vascular injury during the coagulation cascade and activates various types of cells in the vasculature like platelets and endothelial cells (Macfarlane et al., 2001; Hirano, 2007), and plays a key role in haemostasis and thrombosis. Unlike all other ligands that are involved in platelet activation that act by binding in a reversible manner to their receptors, thrombin activates its PAR receptors by cleaving an N-terminal part at a consensus site (Vu et al., 1991), and is rapidly inactivated by uncoupling from its signalling pathway, then internalised and degraded in lysosomes (Trejo et al., 1998). The activation of human platelets is predominately mediated through PAR-1, a high affinity receptor for activation at low concentrations of thrombin and also PAR-4 induced platelet responses which are less pronounced and is it a low affinity receptor, signalling at primarily high thrombin concentrations (Andersen et al., 1999). To address the signal transduction surrounding thrombin induced aggregation and how this was affected by cGMP signalling, the action of the PAR receptors was examined upon exposure to GSNO.

The activation of PARs is achieved by the proteolytic unmasking of a tethered peptide ligand that resides in the receptor's N-terminal exodomain containing the recognition sequence. The synthetic peptides mimic this sequence and function as agonists that activate PARs independent of receptor cleavage (Kataoka et al., 2003). In the first instance appropriate concentration of the PAR agonists were determined

utilising previous work (Bilodeau et al., 2007; Kataoka et al., 2003). PAR-1 agonist with a sequence of H-Ser-Phe-Leu-Leu-Arg-Asn-NH2/SFLLRN-NH_2at 6µM giving 58% aggregation and PAR-4 agonist with a sequence of H-Ala-Tyr-Pro-Gly-Lys-Phe-NH2/AYPGKF-NH^2at 60µM for 51% aggregation **(Figure.4.6a-b)**.

Upon preincuabtion of platelets with GSNO (1µM) for 1 minute before the addition of PAR-1 (6µM), this reduced aggregation seen in **Figure.4.6a** from 58±7.5% to14±7.5% ($P<0.05$) to 4.3±2.2% ($P<0.01$) and then 2.1±2.4% ($P<0.01$) for 10µM and 100µM GSNO respectively. Incubation of platelet with ODQ (20mM) alone failed to affect aggregation induced by PAR-1. However, when platelets were treated with ODQ before the addition of GSNO (10µM) and the PAR-1 agonist, the NO-donor failed to inhibit aggregation. Under these conditions, PAR-1 induced 58±7.5% aggregation, which remained at 54±19% in the presence of ODQ and GSNO shown in**Figure.4.6c**.

Similarly, **Figure.4.7b** with the PAR-4 (60µM) incubation with GSNO (1µM) for 1 minute reduced aggregation from61±10.5% to 14±10%to 2.5±2.5% ($P<0.05$) and then to 3±1% ($P<0.05$) for 10µM and 100µM GSNO respectively. Incubation of platelet with ODQ (20mM) alone failed to affect aggregation induced by PAR-4. However, when platelets were treated with ODQ before the addition of GSNO (10µM) and PAR-4 agonist, the NO-donor failed to inhibit aggregation. Under these conditions, PAR-4 induced 61±10.5% aggregation, which remained at 54±6% in the presence of ODQ and GSNO shown in **Figure.4.6d.**

Next to be examined was if the effectiveness of GSNO at inhibiting PAR-1 induced platelet aggregation was dependent on the time of incubation, in order establish the time of peak inhibition following its addition. WP was incubated with an intermediary concentration of GSNO at 10µM for allotted time intervals before stimulation with the agonist of interest. **Figure.4.7a**shows that after 30seconds of incubation of GSNO (10µM) before PAR-1 (6µM) aggregation was reduced from 52.7±6.4% to 0.3±0.3% ($P<0.01$). The maximum level of inhibition of aggregation was reached at 30seconds, then remained at 0.6±0.5% ($P<0.01$) and 1±1.4% ($P<0.01$) aggregation for the times 1 and 5minutes until the duration of 15 minutes incubation with GSNO, 3.7±3.2% ($P<0.01$) aggregation and for 30minutes 15.7±7.8% ($P<0.05$), 60minutes 48.5±6.5%.

Figure.4.7b demonstrates that following 30secondsof GSNO incubation, PAR-4 agonist induced aggregation is reduced from 68.3±2.4% to 24.7±9.2% ($P<0.05$), and subsequently to 6.7±4.1% ($P<0.01$), 5.3±2.9% ($P<0.01$), 0.5±0.5% ($P<0.01$) and 18.7±12.2% ($P<0.05$) for 1, 5, 15 and 30 minutes respectively, and for 60 minutes the level of aggregation had recovered to 39±14.6% suggesting distinct signalling differences between PAR1 and 4 upon addition of GSNO and its differing rates of inhibition over time.

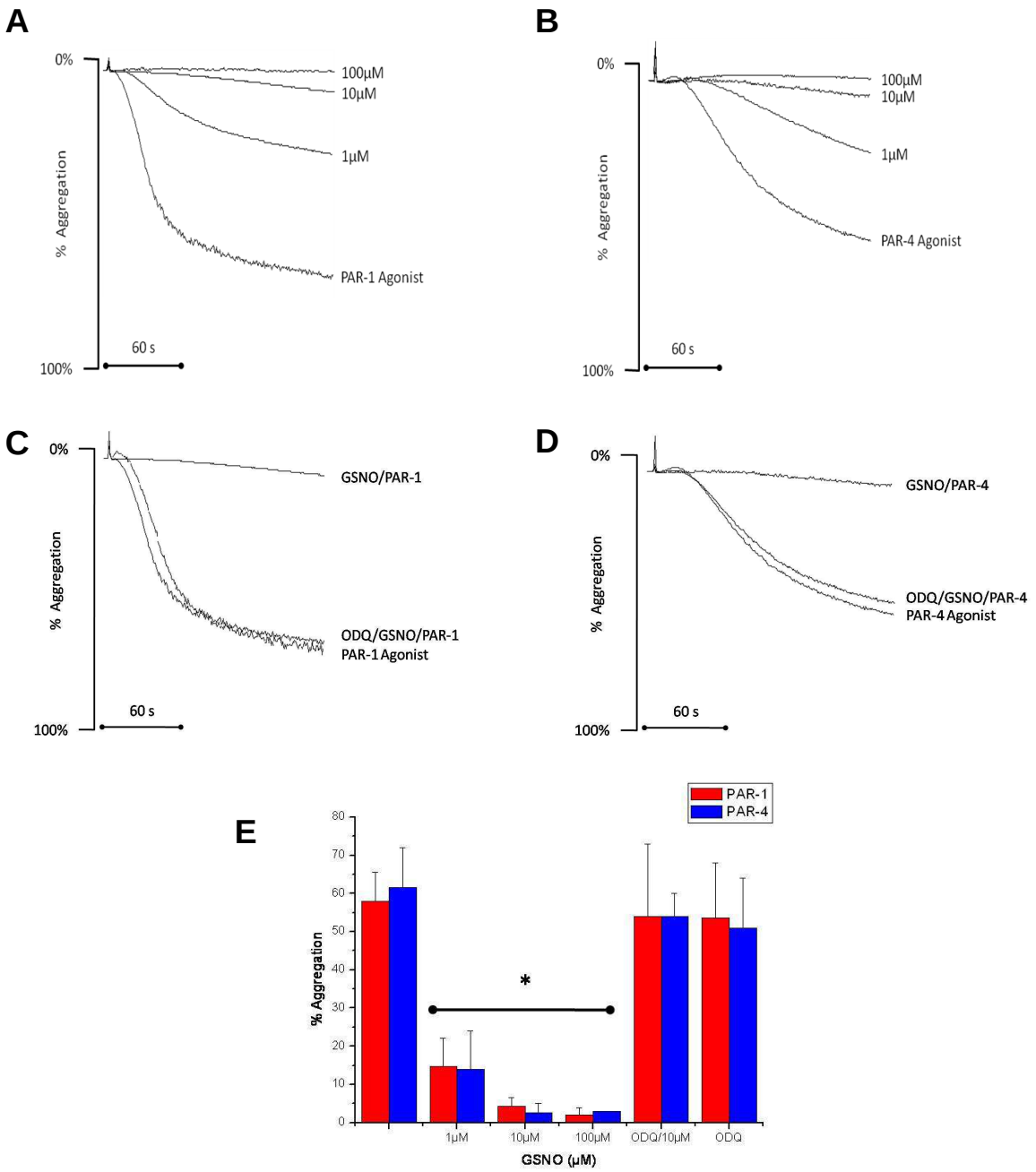

Figure.4.6 Inhibition of Protease Activated Receptors agonists 1 and 4 via GSNO

WP ($2.5x10^8$) were stimulated with either **(A)** PAR-1 **(B)** PAR-4 agonists or pretreated first with GSNO at the indicated concentrations prior to stimulation with the aforementioned agonists. Responses were recorded for the indicated times and expressed as % aggregation. Traces are representative of three experiments. WP ($2.5x10^8$) were preincubated with sGC inhibitor [ODQ (20µM)] for 20 minutes. WP were then treated with GSNO (10µM) and stimulated with **(C)** PAR-1 **(D)** PAR-4. Responses were recorded and expressed as % aggregation. Traces are representative of three experiments. The graph **(E)** is representative of the above experiments. Data = mean±SEM. *P<0.05 vs. agonist stimulation.

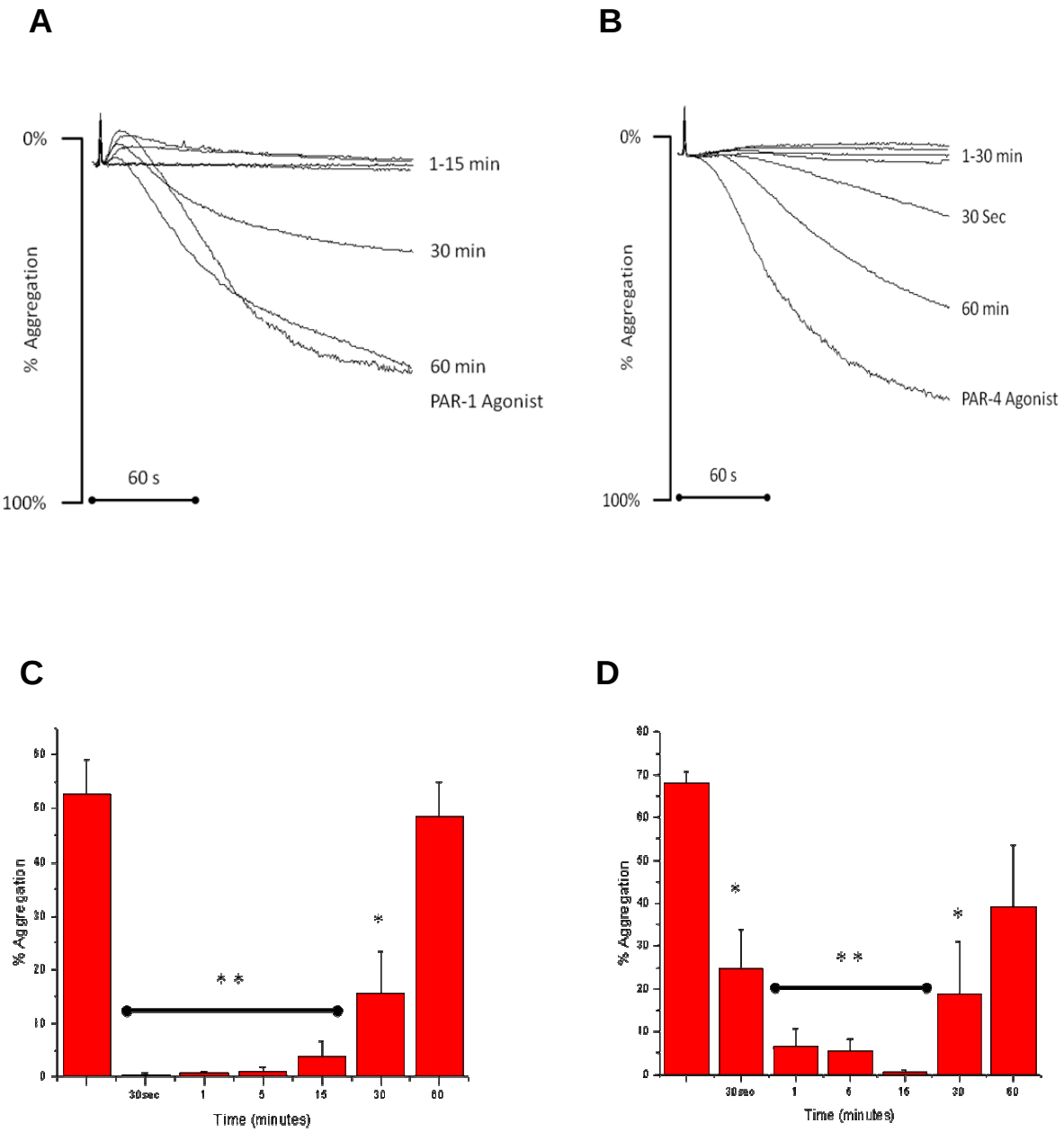

Figure.4.7 GSNO inhibits PAR 1-4 induced platelet aggregation in a time dependent manner

WP ($2.5x10^8$) was incubated with GSNO (10µM) for up to 1 hour. At the indicated times the platelets were stimulated with **(A)** PAR-1 at 6µM and **(B)** PAR-4 at60µM for 3 minutes. Aggregation traces were recorded on dual channel aggregometer and expressed as % aggregation. Traces represent three experiments. The graphs **(C)** PAR-1 **(D)** PAR-4 are representative of three independent experiments. Data = mean±SEM. *P<0.05 **P<0.01 vs. agonist stimulation.

4.3 Discussion

NO acts as an important paracrine signalling molecule in the cardiovascular system (Radomski et al., 1987). By the diffusion across the plasma membrane of platelets it is able to alter intracellular enzyme activity, with its main receptor being NO sensitive guanylyl cyclase receptor leading to the production of cyclic guanosine monophosphate (cGMP) from the nucleotide guanosine triphosphate (GTP) substrate. Cyclic GMP is present in almost all cells (Hardman et al., 1969), and is a secondary messenger which regulates many signalling events; activation of protein kinases, ion channels and phosphodiesterases (PDEs). Increased levels of cGMP increases the activity of the cGMP-dependent protein kinase (PKG), this being the NO dependent mechanism of platelet regulation (Schwarz, 2001). Cyclic GMP is a strong negative regulator able to interfere with platelet adhesion, activation, aggregation, secretion and shape change. Though there are also cGMP independent effects that are mediated by reactive nitrogen species produced as a result of the interaction of NO with oxygen (O_2), the superoxide radical ($O_2^{\cdot -}$) or metals which can lead to tyrosine nitration and nitrosylation of numerous proteins, including transcription factors and signalling molecules (Friebe et al., 2003). The present study is focussed on the mechanisms by which NO and cGMP inhibited platelet function.

The NO donor chosen an S-Nitrosothiol, to investigate this process is present *in vivo* as major redox form of NO in the plasma (Stamler et al., 1992) with the most abundant intracellular RSNO the low molecular weight; S-nitrosoglutathione (GSNO) (Stamler et al., 1992), this has the ability to inhibit platelet function, and as NO, stimulate guanylyl cyclase thereby producing intracellular levels of cGMP and attenuating platelet function primarily via this mechanism. With release of NO from

GSNO occurring via enzymatic GSNO reductase activity, by catalytic redox effects of transition metals (Cu^{+}, Cu^{2+}, Fe^{2+}) or protein disulphide isomerase(Hogg, 2002;Xiao et al., 2011).

To begin to address the mechanisms by which NO and cGMP inhibited platelet function, the relationship by which NO induces the inhibition of agonist stimulation mediated by agonists such as collagen and thrombin was examined.

Consistent with previous studies using a range of GSNO concentrations, from threshold inhibitory responses at 0.1µM to where maximum inhibition was achieved at 100µM, we confirmed that GSNO at the 10µM concentration significantly inhibits platelet aggregation by thrombin (0.05U/ml) and collagen (1µg/ml)(Roberts et al., 2009). Demonstrated here is the platelet response upon addition of NO to inhibit agonist induced platelet aggregation in a concentration and time-dependent manner via the use of aggregometry.

Many of the cellular effects of thrombin are initiated by the protease activated receptors (PARs), which are GPCR whose activation occurs via proteolytic generation of a tethered ligand. PARs were selectively activated by synthetic peptides (Vu et al., 1991; Faruqi et al., 2000) and subsequently intracellularly activate G_q and G_{12}pathways (Offermanns, 2006). The results reflect that individual PAR agonist can activate platelet aggregation in the same dose and time dependent manner as thrombin. It is also known that thrombin has a higher affinity for PAR1 than PAR4 and that more PAR1 are expressed on platelet surface compared with PAR4 (Offermanns, 2006). Due to the high PAR1 affinity, lower concentrations of

thrombin and its mimic are required for activating PAR1 (Covic et al., 2000).As the cleavage of human PAR-4 required a higher concentration of thrombin than does cleavage of PAR-1, it is likely thatPAR-1 is the predominant signalling receptor at low thrombin concentrations (Shapiro et al., 2000). Demonstrated here is the fact that PAR1 and PAR4 are sensitive to cGMP dependent actions which is consistent with the current signalling models for these receptors and indeed a common pathway of activation and signal transduction of that established by thrombin, its inhibition by GSNO and reversal by ODQ (Holinstat et al., 2007; Murugappa et al., 2006).

In a wider context, the results demonstrate here the efficiency of the anti-aggregatory effects of NO linked to the activation of GC, and that ceasing the addition of the NO donor and subsequent decline in cGMP production over time, allow the pro-aggregatory agonist, thrombin, to reverse the otherwise anti-aggregatory pathways. The reversibility of NO mediated platelet inhibition has been shown to correlate with a cGMP decrease, thereby allowing platelet pro-aggregatory signals to become dominant and also the relaxation of smooth muscle (Smolenski, 2012; Rivero-Vilches et al., 2003; Nakamura et al., 2007; Kitazawa et al., 2009).

Although the inhibitory effect of NO on agonist induced aggregation, adhesion and Ca^{2+} mobilisation is certain, whether the inhibitory function was of a cGMP-dependent or independent was uncertain. Data on cGMP-independent signalling uses mainly the NO-GC inhibitor ODQ, relying on oxidation of the central iron of the enzymes prosthetic haem from Fe^{2+} to Fe^{3+} preventing NO binding (Schrammel et al., 1996).The data here suggests that the action of the NO donor GSNO inhibits human platelet activation in main part via a sGC dependent mechanism. Though

high concentrations of the NO donor SNAP (100µM) can partially overcome this (Moro et al., 1996; Schrammel et al., 1996).In NO-CG deficient mice, at the high NO concentrations suggested for cGMP-independent effects, NO donors, such as DEA-NO (100µM-4mM), SNP (0.8mM) including GSNO (100µM-4mM) failed to inhibit knock out platelet aggregation by agonists such as thrombin (Dangel et al., 2010). Furthermore the inhibitory actions of GSNO and ODQ are determined by the platelet preparation used, although ODQ effectively blocks the inhibitory actions of NO donors in washed platelet preparation, the effect in platelet rich plasma is reduced or abolished (Crane et al., 2005; Sogo et al., 2000), due to ODQ binding to plasma proteins and thus a reduction in the concentration to inhibit GC. The platelet rich plasma also contains free nitrosothiols at concentrations of 1-3µM (Stamler et al., 1992) and micromolar concentrations of nitrosated proteins that serve to transmit NO bioactivity and regulate protein function via mechanism corresponding to phosphorylation (Naseem et al., 1996).

To uncover the effect of GSNO upon vasodilator-stimulated phosphoprotein (VASP) in platelets, as one of the major regulatory substrates for activated PKG is VASP, we sought to determine the effect of GSNO upon Ser^{239} phosphorylation in a dose and time dependent manner. Revealing that the phosphorylation of VASP on Ser^{239} results in a mobility shift which was detected as a double band, with the upper 50KDa band corresponding specifically with phosphorylated Ser^{239} VASP and the lower 46KDa band corresponding to dephosphorylated Ser^{239}VASP, serving as an indicator of VASP phosphorylation state (Horstrup et al., 1994). Our data demonstrate that GSNO stimulation induces rapid phosphorylation and subsequent dephosphorylation over time due to the limited half-life of the NO-donor of Ser^{239}on

VASP in human platelets. This rapid phosphorylation has been shown to be cGMP/PKG-dependent, and dephosphorylation is dependent on the down-regulation of PKG, suggesting that VASP and its phosphorylation on Ser^{239} are important elements in the mechanism of human platelet aggregation (Lindsay et al., 2007; Brindle et al., 1996).

The aggregation traces detail human platelets with exposures to NO prior to agonist stimulation provide a good match to the concentration and time dependent response for VASP phosphorylation. Up to duration of 1 minute after the addition of NO the inhibitory action continues to increase in agonist induced aggregation, though the negative regulation of platelet activity gradually declines after this time due to the decline in the NO signal along with the actions of phosphodiesterases (PDEs) terminating cGMP signalling (Schwarz et al., 2001). PDE5 specifically degrades cGMP, providing negative feedback on cGMP levels by being activated by cGMP and additionally phosphorylated by PKG, activating PDE5 catalytic activity and long term desensitisation of an NO induced cGMP response (Mullershausen et al., 2003). The tight regulation of the cGMP level is thereby required to maintain the sensitivity of the response to the incoming signal and rapid adjustment to changes in that signal (Francis et al.,2010).

This phosphorylation of VASP corresponds to the vasodilator induced inhibition of platelet activation and aggregation upon residue Ser^{239}, affecting actin-filament mediated cytoskeletal reorganisation required for platelet aggregation and spreading (Harbeck et al., 2000). Previous studies showed that a lack of the PKG target VASP results in megakaryocyte hyperplasia and enhanced agonist induced action of P-

selectin expression and fibrinogen binding (Hauser et al., 1999) as well as enhanced collagen induced activation and impaired inhibition via cGMP dependent inhibition of platelet aggregation (Aszodi et al., 1999)

In conclusion, these results demonstrate that GSNO exerts its inhibitory effects via a sGC dependent mechanism in turn generating the cyclic nucleotide cGMP and subsequent activation of PKG leading to protective effect against agonist induced aggregation. Subsequent phosphorylation of VASP and the IP_3 receptor by PKG results in the reduced rearrangement of the cytoskeletal proteins and reduced Ca^{2+} release from internal stores; in the absence of sGC GSNO has no effects (Friebe et al., 2003).

CHAPTER 5 REGULATION OF MYOSIN LIGHT CHAIN PHOSPHORYLATION BY NITRIC OXIDE

5. Introduction

5.1 Regulation of myosin light chain phosphorylation by nitric oxide

Platelets mediate haemostasis via amplifying an initial agonist stimulus and aggregating at the site of injury. Activated platelets change shape, secrete α-granules and dense granules, and release positive feedback mediators (Holmsen, 1994). When platelets are initially stimulated, the first event is a rearrangement of the cytoskeletal proteins (actin and myosin), and the inert disc-shaped platelets change into spheres with filopodia (Wurzinger, 1990). Activation of phospholipase A_2 releases arachidonic acid from membrane phospholipids, which is converted into thromboxane A_2. Serotonin and ADP, released from dense granules, and thromboxane A_2 function as positive feedback mediators, which recruit more platelets into a primary haemostatic plug (Shattil et al., 1997).

During platelet shape change, a prerequisite for secretion and platelet aggregation, changes are driven by the rearrangement of the actin-myosin cytoskeleton and the contraction of actin filaments, with myosin, a hexamer ATPase motor protein, playing a central role in the regulation of actin filaments contraction (Hartwig, 1992). Phosphorylation of the myosin light chain (MLC) on $Serine^{19}$ (MLC^{Ser19}) regulates the ATPase activity of myosin which upon ATP hydrolysis generates a mechanical force the causes actin filaments contraction (Johnson et al., 2007). MLC phosphorylation is regulated in a Ca^{2+}/Calmodulin-dependent manner by myosin light chain kinase (MLCK) and in a Ca^{2+} independent manner by RhoA/Rho-Kinase (ROCK) (Bauer et

al., 1999). Activation of the later pathway leads to the inhibition of myosin light chain phosphatase (MLCP) allowing MLC phosphorylation to take place (Kimura et al., 1996). NO/cGMP inhibits platelet shape change, though the mechanism remains unclear. In smooth muscle cells the NO/cGMP pathway decreases the phosphorylation of the MLC and increases phosphorylation of MLCP resulting in vasorelaxation (Wooldridge et al., 2004). In the previous chapter the data indicated that NO completely abolished shape change. Therefore it was possible that NO may influence the biochemical mechanisms regulating this morphological process. In this chapter the mechanism by which NO modulated platelet shape change is examined in detail.

5.2Results

5.2 Regulation of Myosin Light Chain phosphorylation by NO

5.2.1 Platelet shape change

Shape change is mediated through two separate pathways, Ca^{2+} dependent and independent. To examine the potential role of NO on these two pathways, inhibitors were used to isolate the pathways in the presence and absence of NO. Platelets were stimulated with thrombin, in the presence of apyrase (1U/ml), indomethacin (10µl/ml) and EGTA (1mM) to prevent secondary signalling though underwent shape change but not aggregation. Shape change through the pathway that was independent of an increase in cytosolic Ca^{2+}, that is in the presence of BAPTA, was delayed though the maximal response was not reduced. The role of the RhoA/ROCK pathway was investigated by preincubation of platelets with Y-27632 (10µM) shown to specifically inhibit the kinase activity of p160ROCK purified from platelets (Uehata et al., 1997). Under these conditions shape change was weakly inhibited upon addition of thrombin. However, pre-treatment with both the Y-27632 and BAPTA, completely inhibited shape change, suggesting that thrombin stimulates both pathways and that Ca^{2+} and Rho-kinase pathways play independent roles in the initiation of this morphological response. In contrast, GSNO alone abolishes shape change completely.

The inhibitors were then used in the presence of NO and thrombin induced shape change was investigated. **Figure.5.1**shows that when platelets were preincubated with either BAPTA in combination with GSNO, leading to complete inhibition of shape change. Similarly, the combination of Y-27632 with GSNO also ablated shape change. Together these data indicate that NO has the capacity to target the Ca^{2+} dependent and ROCK dependent pathways independently of each other.

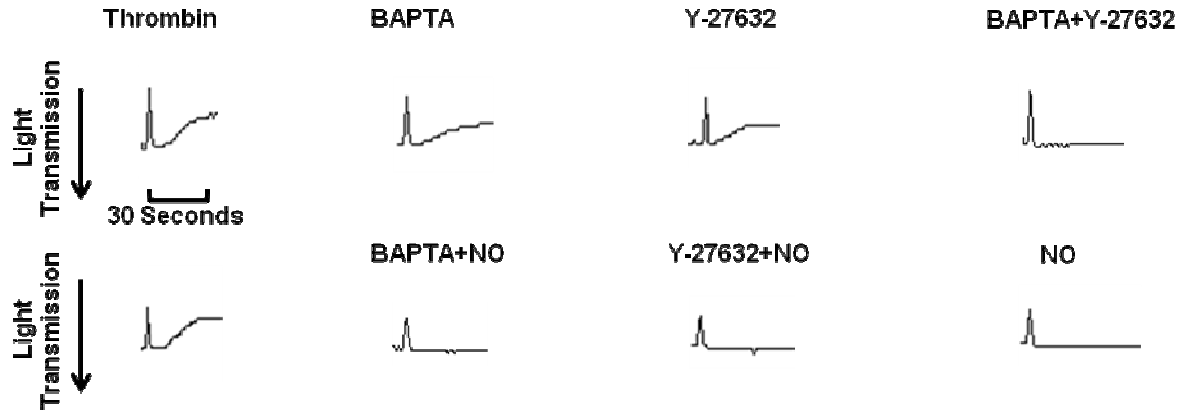

Figure.5.1 Platelet shape change in response to thrombin

WP ($2.5x10^8$) stimulated with thrombin (0.01-0.05U/ml) or pre-incubated first with the Ca^{2+} chelator [BAPTA (20μM for 20mins)], Rho-kinase inhibitor [Y-27632 (10μM for 20mins)], GSNO (10μM for 1min) before stimulation of Apyrase (1U/ml) and Indomethacin (10μl/ml) treated platelets. The figure is representative of three independent experiments.

5.2.2 Thrombin induces a dose and time dependent MLC phosphorylation in platelets

Actin and myosin interactions have a critical role in platelets (Daniel, 1984) and their interactions are regulated via the MLC and its phosphorylation by agonist stimulation upon the residues Ser^{19} and Thr^{18} (Itoh et al., 1992). In this study we focused upon the MLC^{Ser19} phosphorylation site, as it has been shown to be phosphorylated downstream of both the Ca^{2+} dependent G_q activation and Ca^{2+} independent $G_{12/13}$ pathways, while the residue Thr^{18} phosphorylation occurs subsequent to former residue and is principally phosphorylated via the Ca^{2+} independent $G_{12/13}$ pathway (Getz et al.,2010). After addition of thrombin platelets undergo shape change as demonstrated in **Figure.5.1** and can subsequently aggregate, during which it is reported that phosphorylation of MLC^{Ser19}takes place (Buaer et al 1999).

As part of the study it was important to establish the effect of NO signalling on MLC phosphorylation. However, prior to this, the conditions for MLC phosphorylation in response to platelet activation had to be determined. No phosphorylation of MLC was observed under basal conditions. The phosphorylation of MLC^{Ser19} occurs in a dose dependent manner upon addition of increasing concentrations of thrombin (**Figure.5.2)**. Initial phosphorylation was observed at concentrations as low 0.025U/ml thrombin, with more robust phosphorylation observed at 0.05U/ml and 0.1U/ml. Selecting an intermediate dose of thrombin (0.05U/ml), the phosphorylation state of MLC was examined over a period. This revealed a time dependent phosphorylation which was observed as early as 15secondspeaking at 1minute after stimulation thereafter returning to basal conditions.

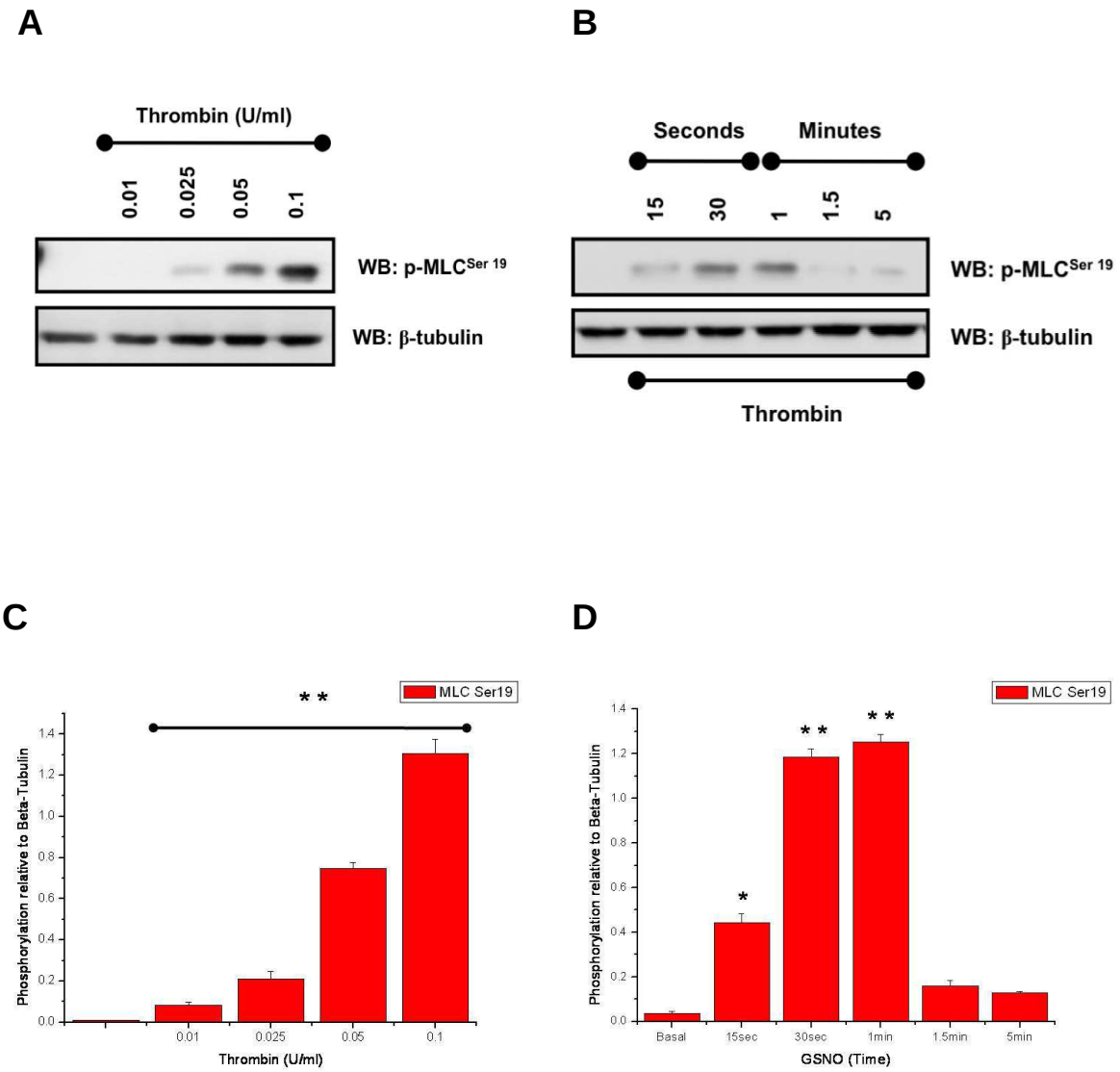

Figure.5.2 Effect of thrombin on MLC phosphorylation

(A) WP (5x10 ^{8}) stimulated with varying doses of thrombin for 1minute. Each sample was treated at 37°C with stirring (1000rpm). Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (30µg) were separated in 10% gradient gels and immunoblotted for phospho-MLC^{Ser19}. Blot is representative of three individual experiments, and **(C)** densitometric analysis of these immunoblots. **P<0.01 vs. basal sample. **(B)** WP (5x10^{8}) were incubated with 0.05U/ml of thrombin for up to 5 minutes. Each sample was treated at 37°C with stirring (1000rpm). At the indicated times the reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (30µg) were separated in 10% gradient gels and immunoblotted for anti-phospho-MLC^{Ser19}.Blot is representative of three individual experiments, and **(D)** densitometric analysis of these immunoblots. *P<0.05, **P<0.01 vs. basal sample.

5.2.3 Thrombin induces MLC phosphorylation via biphasic mechanism

It has been previously shown in **Figure.5.1** that thrombin induced platelet shape change is mediated by both ROCK and Ca^{2+}dependent pathways and that thrombin in **Figure.5.2** affects the phosphorylation state of MLC (Bauer et al., 1999). Having confirmed that stimulation of platelets with thrombin induced MLC phosphorylation on $Ser^{19}$the pathways regulating this phosphorylation event were examined. Firstly, to determine whether the agonist-mediated phosphorylation of the residue is Ca^{2+} dependent, platelets were incubated with BAPTA (20μM) prior to stimulation with thrombin. Thrombin induced MLC phosphorylation was significantly reduced, indicating that phosphorylation is mediated, at least in part, in Ca^{2+} dependent manner. To delineate the Ca^{2+}independent signalling pathway upstream of MLC phosphorylation platelets were incubated with Y-27632, a ROCK inhibitor (Uehata et al., 1997). Similarly to BAPTA, thrombin-induced MLC phosphorylation was partially, but significantly reduced under conditions of ROCK inhibition. Importantly, incubation of platelets with both inhibitors prior to stimulation with thrombin completely abolished MLC phosphorylation. This data suggests that MLC phosphorylation in response to thrombin is dually regulated byCa^{2+} and ROCK dependent mechanisms. The dual regulation of MLC phosphorylation by ROCK and Ca^{2+} in platelets is in agreement with other studies using different agonists, such as ADP, thromboxane analogue U46619 and convulxin (Wilde et al., 2000; Bauer et al., 1999).

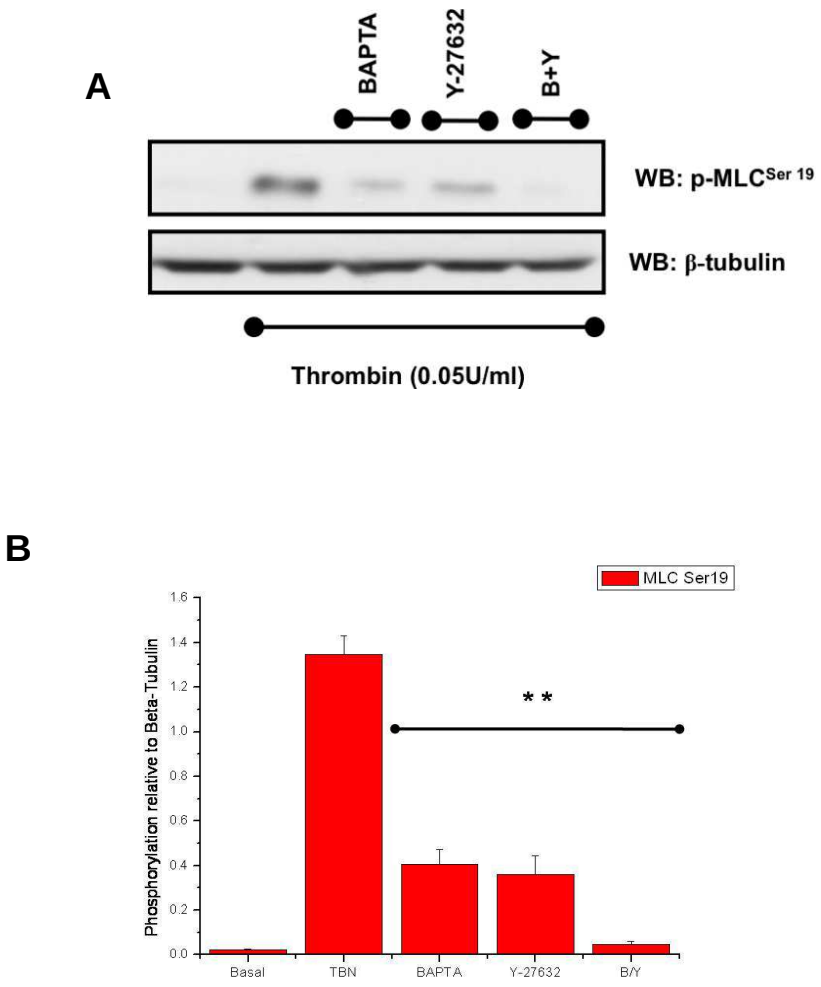

Figure.5.3 Effect of ROCK inhibition by Y-27632 and chelation of cytosolic calcium by BAPTA on thrombin induced MLC phosphorylation

(A)WP ($5x10^8$) stimulated with thrombin (0.05U/ml) or pre-incubated first with the Ca^{2+} chelator [BAPTA (20μM for 20mins)], Rho-kinase inhibitor [Y-27632 (10μM for 20mins] before stimulation. After 1 minute incubation with agonist the reactions were stopped with addition of an equal volume of 2xLaemmli buffer. Proteins (30μg) were separated in 10% gradient gels and immunoblotted for phospho-MLC^{Ser19}. Blot is representative of three individual experiments and **(B)** densitometric analysis of these immunoblots. **P<0.01 vs. TBN (Thrombin) sample.

5.2.4 Reversible phosphorylation via the action of NO

The aim of the next series of experiments was to determine the effect of GSNO on thrombin induced phosphorylation of MLC^{Ser19}. **Figure.5.4a** shows the agonist stimulation of platelets with thrombin after preincubation for 1 minute with increasing concentrations of GSNO (1-50µM) the phosphorylation of MLC^{Ser19}. Phosphorylation of the $MLC^{Ser19}$was increased significantly upon addition of thrombin. Preincubation of platelets with GSNO (0.1-50µM) inhibited phosphorylation of the MLC triggered by the agonist and maintained the basal phosphorylation state; this occurred at all concentrations tested. Within the same samples the phosphorylation of VASP was also examined. Minor phosphorylation of VASP was observed at 1µM; with maximal phosphorylation observed at 10µM.The mechanism of action of GSNO was investigated via the use of a soluble guanylyl cyclase (sGC) inhibitor ODQ. In the presence of this inhibitor the action of GSNO is blocked, that is, thrombin was still able to induce phosphorylation of MLC. The confirmation that ODQ is blocking cGMP mediated signalling is evidenced by the lack of VASP phosphorylation under these conditions, this data indicative of a cGMP dependent mechanism.

The first series of experiments highlighted the ability of NO to prevent thrombin stimulated phosphorylation of MLC. In the next series of experiments, the ability of NO to potentially dephosphorylate MLC that had been phosphorylated in response to thrombin was investigated. To achieve these platelets were treated with Thrombin for 1min followed by the addition of GSNO for 1min. **Figure.5.4b** shows that stimulation of thrombin for 1minute led to the phosphorylation of MLC^{Ser19}. However, the addition of GSNO after thrombin reversed the phosphorylation of MLC; at all concentrations of GSNO tested. The paired examination of VASP phosphorylation demonstrated

that cGMP signalling was activated. Though it was noticeable that GSNO induced dephosphorylation of MLC was maximal at lower concentrations than required to induce maximal phosphorylation of $VASP^{Ser239}$. The potential dephosphorylation of MLC was cGMP dependent, since when sGC activity was blocked by ODQ, the level of MLC phosphorylation return that that found with thrombin alone.

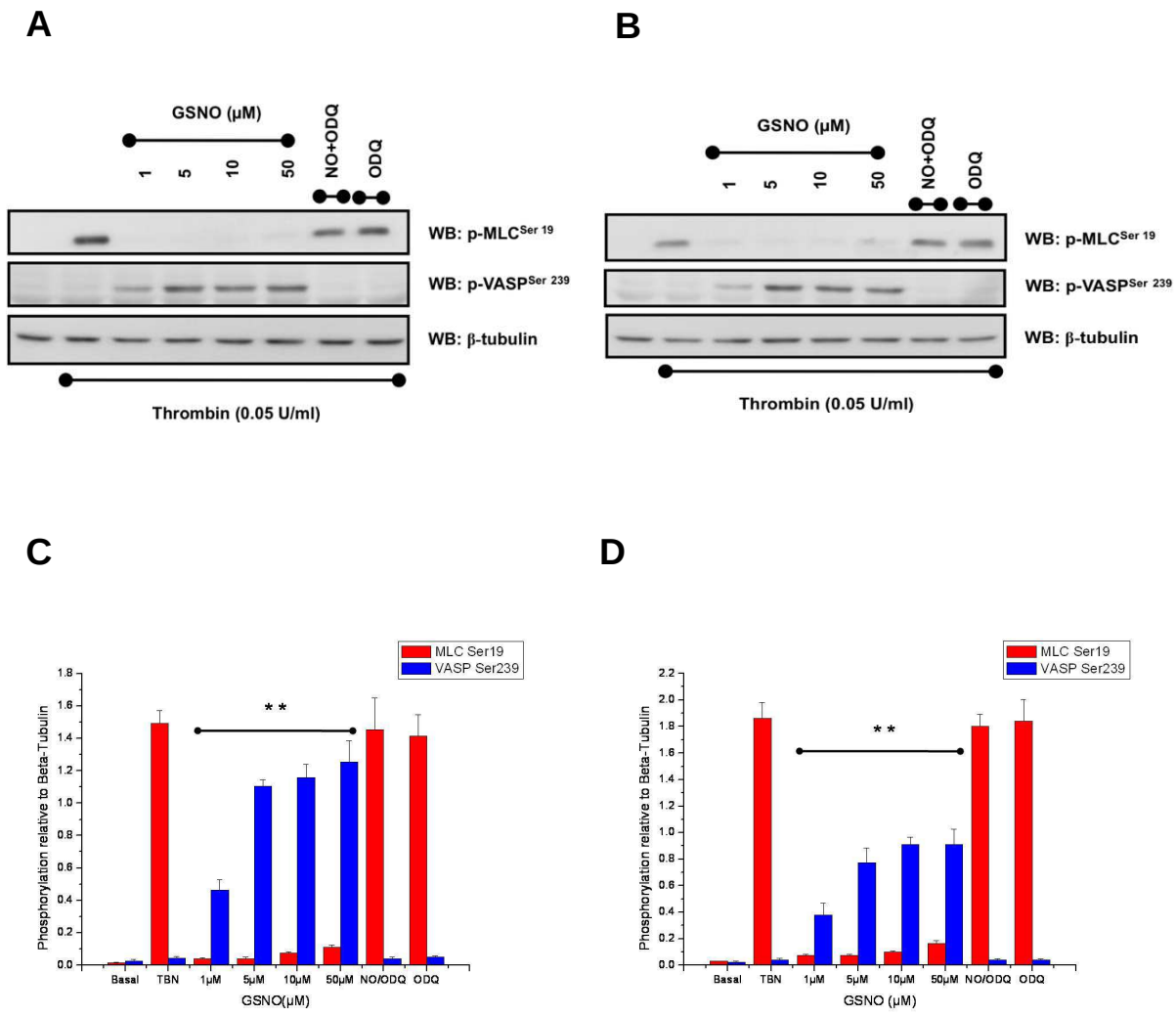

Figure.5.4 Effect on NO mediated inhibition of MLC phosphorylation

(A)WP ($5x10^8$) pre-incubated with increasing concentrations of GSNO for 1minute prior to stimulation with thrombin (0.05U/ml) for 1minute or pre-incubated first with the sGC inhibitor [ODQ (10μM for 20mins]. After 1 minute incubation with thrombin the reactions were stopped with addition of an equal volume of 2xLaemmli buffer. Proteins (30μg) were separated in 10% gradient gels and immunoblotted for anti-phospho-MLC^{Ser19} and anti-phospho $VASP^{Ser239}$. Blot is representative of three individual experiments and **(C)** densitometric analyses of these immunoblots. **P<0.01 vs. TBN (Thrombin) sample.**(B)** WP ($5x10^8$) stimulated with thrombin (0.05U/ml) for 1minute then increasing concentrations of GSNO incubated for 1 minute post stimulation, or pre-incubated first with the guanylyl cyclase inhibitor [ODQ (10μM for 20mins]. After 1 minute incubation with GSNO the reactions were stopped with addition of an equal volume of 2xLaemmli buffer. Proteins (30μg) were separated in 10% gradient gels and immunoblotted for anti-phospho-MLC^{Ser19} and anti-phospho $VASP^{Ser239}$. Blot is representative of three individual experiments and **(D)** densitometric analysis of these immunoblots. **P<0.01 vs. TBN (Thrombin) sample.

5.2.5 Dissection of the pathways regulating NO mediated regulation of MLC phosphorylation

Following on from the actions of NO was the requirement to explore the pathways that are regulated by NO, and the subsequent effects upon the phosphorylation state of MLC^{Ser19}. Having confirmed in section **5.2.3**that MLC phosphorylation required two independent pathways; we examined the effect of NO on theCa^{2+} dependent/MLCK route through the use of the Ca^{2+} chelator BAPTA and the Ca^{2+} independent/ROCK route via the inhibitor Y-27632. First, the individual effects of the inhibitors were examined, and then in combination with NO to gauge if the alternate pathway was also abolished. **Figure.5.5a** shows that following stimulation by thrombin there was a large increase in phosphorylated MLC^{Ser19}. Incubation with BAPTA, which prevents the rise in cytosolic Ca^{2+} concentration, resulted in a dramatically reduced phosphorylation compared with agonist alone stimulated platelets, although phosphorylation was not abolished suggesting a role for ROCK activity in Ca^{2+} independent MLC phosphorylation. Consistent with this **Figure.5.5b**showsY-27632 (10μM) pre-treatment also exhibits significant inhibition of thrombin-induced phosphorylation. Again the phosphorylation was not abolished by Y-27632 indicating that phosphorylation is not entirely ROCK dependent.

In contrast to the pathway specific inhibitors, GSNO completely abolished MLC phosphorylation, suggesting that it targeted both pathways. Importantly the combination of either BAPTA or Y27632 with GSNO (10μM) abolished phosphorylation remaining when using the inhibitor alone. The protective effect of NO against phospho$MLC^{Ser19}$was prevented by incubation of platelets with the inhibitor ODQ, confirming that its protective effect is via cGMP signalling and this

principally occurs through dual inhibition of the cytosolic Ca^{2+} increases and RhoA/Rho-kinase activity in platelets thus reducing MLC phosphorylation, platelet shape change and ultimately aggregation.

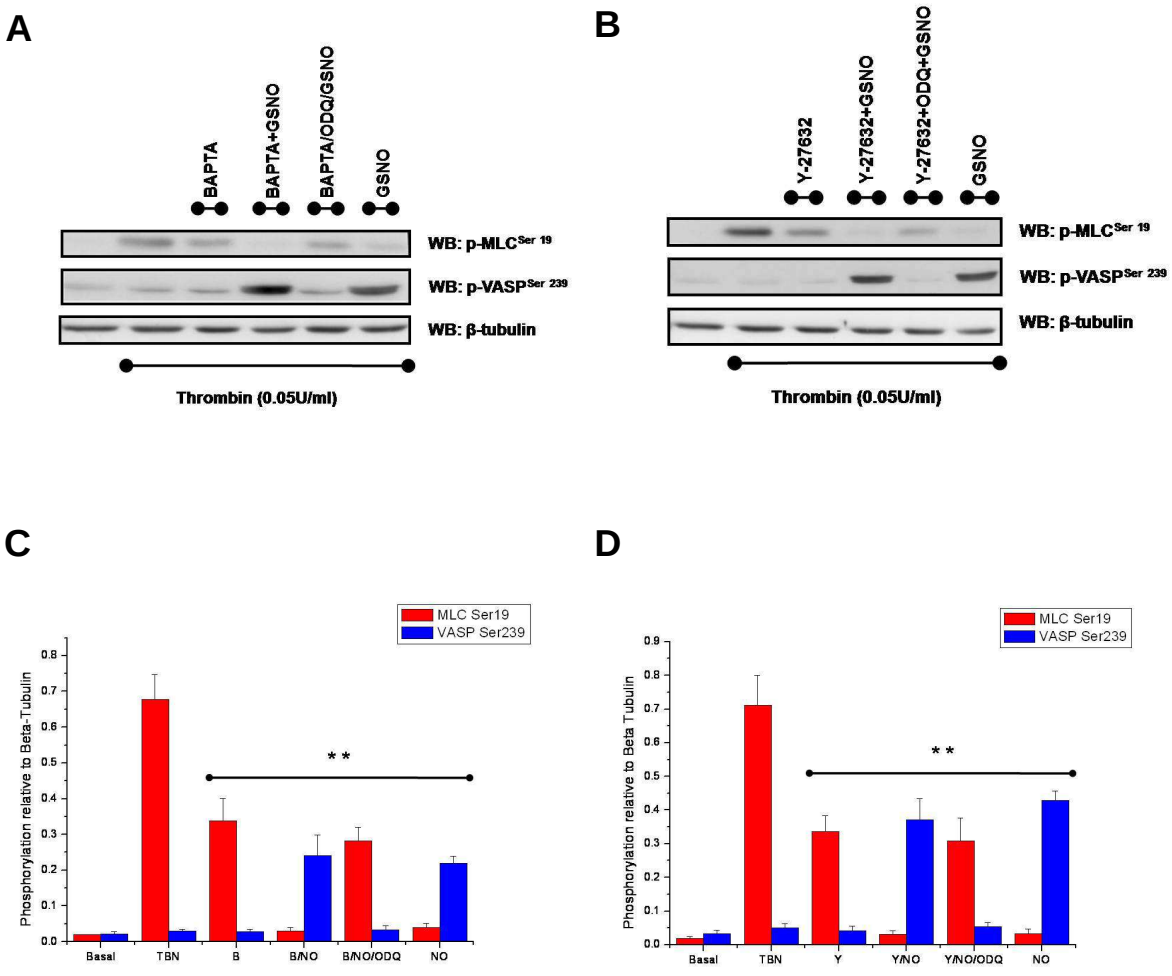

Figure.5.5 Influence of NO on MLC phosphorylation

WP ($5x10^8$) stimulated with thrombin (0.05U/ml) for 1minute. **(A)** Pre-incubated first with the Ca^{2+} chelator [BAPTA (20μM for 20mins)], the guanylyl cyclase inhibitor [ODQ (10μM for 20mins] or GSNO for (10μM for 1minute) before stimulation. **(C)** Densitometric analysis of these immunoblots. **P<0.01 vs. TBN (Thrombin) sample. **(B)** After 1 minute thrombin stimulation addition of GSNO for (10μM for 1minute)and or preincuabtion with the Rho-kinase inhibitor [Y-27632 (10μM for 20mins] and guanylyl cyclase inhibitor [ODQ (10μM for 20mins]. Reactions were stopped by addition of2xLaemmli buffer. Proteins (30μg) were separated in 10% gradient gels and immunoblotted for phospho-MLC^{Ser19} and anti-phospho $VASP^{Ser239}$. Blot is representative of three individual experiments and **(D)** densitometric analysis of these immunoblots. **P<0.01 vs. TBN (Thrombin) sample.

5.2.6 NO modulation of the RhoA-mediated pathway

Platelet stimulation via thrombin is mediated through G-protein coupled receptors which activate G_q, $G_{12/13}$ and G_i (Offermanns et al., 1994). $G_{12/13}$ is the primary signalling pathway to RhoA activation (Getz et al., 2010). This enables the activation of Rho guanine nucleotide exchange factors, leading to the switching from the inactive GDP form to RhoA-GTP bound active state that are interconvertable by GDP-GTP exchange and GTPase reaction. Stimulation of ROCK results in MLC^{Ser19} phosphorylation, increasing actomyosin contractility and regulation of microtubule coils during shape change and dense granule secretion (Offermanns, 2006). Data presented in section **5.2.5** suggested that GSNO can inhibit the ROCK pathway that leads to phosphorylation of MLC, however, it was still unclear how NO modulated the pathway. The classic model of Rho protein regulation involves a cycle between active GTP-bound and inactive GDP-bound conformations (Etienne-Manneville et al., 2002). Various agonists induced signals, such as G protein-coupled receptor agonists, activate guanine nucleotide exchange factors and increase the level of GTP-RhoA. Activated GTP-RhoA is readily prenylated at its carboxy terminus and then translocated to the cell membrane, where it interacts with effector proteins like ROCK to generate downstream signalling. Ser^{188} phosphorylation of RhoA is thought to uncouple this cycle and to terminate RhoA signalling by allowing the guanine dissociation inhibitors to bind and sequester GTP RhoA in the cytosol (Lang et al., 1996; Rolli-Derkinderen et al., 2005). Previous studies demonstrate that PKG phosphorylates RhoA at Ser^{188} in vitro and in cultured cells (Ellerbroek et al., 2003; Sauzeau et al., 2000; Sawada et al., 2001). **Figure.5.6** demonstrates in platelets that increasing doses of GSNO (1-50µM) lead to a concentration dependent increase in phosphorylation of RhoA at Ser^{188}. The level of phosphorylation at Ser^{188} on RhoA

closely parallels that of VASP phosphorylation at Ser^{239}, the site selectively phosphorylated by PKG (Smolenski et al., 1998). The results indicate that PKG phosphorylates RhoA at Ser^{188} in human platelets, with phosphorylation initiated at 1µM and maximal at 50µM, making this consistent with the profile for the inhibition of RhoA. The ability of GSNO to drive the phosphorylation of RhoA is blocked by ODQ. Thus, activation of the NO/PKG pathway leads to inhibition of RhoA dependent functions, potentially through the inhibitory phosphorylation.

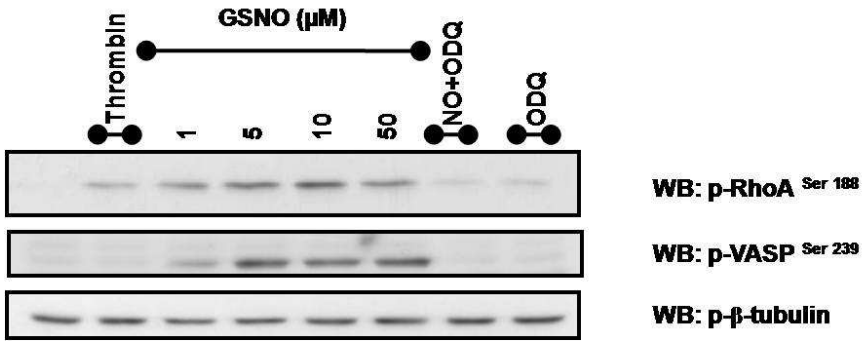

Figure.5.6 NO mediated inhibition of Rho-mediated inhibitory pathway

WP ($5x10^8$) was stimulated with Thrombin (0.05U/ml) for 1 minute or incubated first with the guanylyl cyclase inhibitor [ODQ (10μM for 20mins] or GSNO for (1, 5, 10 and 50μM for 1 minute) before the reactions were stopped with addition of an equal volume of 2xLaemmli buffer. Proteins (30μg) were separated in 10% gradient gels and immunoblotted for phospho-RhoA^{Ser188} and phospho-VASP^{Ser239}. Blot is representative of two individual experiments.

5.3 Discussion

Platelets respond to various stimuli by rapid changes in shape followed by aggregation and subsequent secretion of granule contents. Platelet shape change is the earliest functional response following activation with physiological agonists, which is accompanied by the rearrangement of the cytoskeleton (Daniel et al., 1984). Cytoskeletal rearrangements such as filament assembly, surface membrane folding and centralization of secretory granules are thought to be mediated by the phosphorylation of Myosin IIA (Siess, 1989). The phosphorylation of the MLC results in an increased development of actin-activated ATPase activity and reflects the contractile activity of actomyosin (Getz et al., 2010).Previous studies have demonstrated that platelet shape change can occur both in the presence and in the absence of an increase of cytosolic Ca^{2+} concentration in response to thrombin, ionomycin and the thrombin receptor activating peptide (Negrescu et al., 1995). In contrast to thrombin, the thromboxaneA_2analogue U46619 was not able to induce a rise of intracellular Ca^{2+} concentration in G_q deficient platelets and the addition of the ROCK inhibitor Y-27632inhibited the U46619-induced MLC phosphorylation in G_q-deficient mouse platelets. These observations led the authors to conclude that a Rho/Rho kinase pathway regulating MLC phosphorylation operated in platelets and that ROCK mediates the Ca^{2+}independentpathway. These data suggested that a dual regulation of platelet activation and aggregation is present; thereby extension also requires a reversible inhibition of these pathways to allow rapid platelet responses to changing internal environment. The intracellular mechanism remains undetermined, though shown here is the inhibitory capability of NO to potentially target both pathways.

The dual regulation of MLC phosphorylation and shape change by ROCK and Ca^{2+} elevation inhuman platelets is demonstrated here via thrombin through PAR receptors which couple to both heterotrimeric G proteins G_q and $G_{12/13}$. The activation of G_q and ultimately PLCβ, resulting in the mobilisation of intracellular Ca^{2+} and MLCK activity resulting in platelet shape change was prevented via the addition of BAPTA, as shown in the fluorimetric data in **Figure.3.6**, thus leading to delayed shape change and reduced MLC phosphorylation. Consistent with a role of NO in modulating intracellular Ca^{2+} concentrations, we demonstrate that thrombin induced Ca^{2+} mobilisation can be inhibited by GSNO. However, as seen in **Figure.5.1** and **5.3**inhibition of the Ca^{2+} mobilisation alone is not sufficient to completely inhibit shape change and MLC phosphorylation. In the presence of BAPTA approximately 50% of thrombin induced phospho MLC is lost, clearly indicating an additional pathway. The inhibition of ROCKbyY-27632 only slightly affected shape change and again did not completely inhibit MLC phosphorylation induced by thrombin; a complete inhibition of shape change was only achieved when both pathways were simultaneously inhibited by Y-27632 and BAPTA. This confirmed a biphasic stimulation by thrombin involved in shape change and that there is dual regulation of MLC phosphorylation, one via the Ca^{2+} dependent MLCK and the other by the Ca^{2+} independent ROCK.

Circulating platelets are exposed to endothelium-derived NO and also low levels of thrombin(Shimokawa et al., 1995) and alterations in NO concentrations might influence this balance, as evidenced by increased susceptibility to thrombosis in patients with impaired NO production (Freedman et al., 1996). Data in this thesis demonstrated that there is the dual inhibitory effect of NO upon both of the pathways

required for initiation of shape change, MLC phosphorylation and subsequent aggregation. The cGMP pathway negatively regulates cytosolic Ca^{2+} elevation including all types of Ca^{2+} oscillations that have been observed under flow conditions (Mazzucato et al., 2002). Some of these effects are thought to be mediated by direct phosphorylation of IP_3-R (Tertyshnikova et al., 1998) or by the phosphorylation of IRAG on intracellular stores (Hofmann, 2005; Schwarz et al., 2001). Though Ca^{2+} levels are further regulated by the transient receptor potential channel 6 (TRPC6) in platelets (Hassock et al., 2002), which is involved in store-operated Ca^{2+} entry, and forms a complex with type II IP_3R in platelets (Redondo et al., 2008).

The data presented in this section of the work show for the first time the ability of NO via PKG, to block the Ca^{2+} independent Rho-pathway. This is a critical observation in understanding the mechanisms by which NO regulates platelet function. Since Ca^{2+} mobilisation is so central to platelet activity it has often been assumed that NO mediates its major effects by blocking Ca^{2+}. However, our data demonstrates that regulation of platelets by NO requires other targets. The mechanism by which NO/PKG inhibits RhoA activation is unclear, but could involve the inhibitory phosphorylation of $RhoA^{Ser188}$, inactivating RhoA. This phosphorylation event in other cells is thought to prevent the translocation of the active GTP-bound RhoA to the membrane and interactions with the kinase present there necessary for activation of the ROCK and all subsequent downstream affects (Totsukawa et al., 2000). ROCK activation is required indirectly for the phosphorylation of MLC, since it will cause the inhibitory phosphorylation of MYPT1 substrate, which in turn allows MLC kinase to phosphorylate MLC unhindered. Thus, it is possible that PKG regulation of shape change is due to its ability to block RhoA/Rock inhibition of MLCP, thus ultimately

allowing for reversible dephosphorylation of the MLC phosphorylation state by the MLCP.

Shown here is that NO prevents and reverses the thrombin induced platelet shape change and MLC phosphorylation via a pathway dependent on cGMP/PKG activation, confirmed by the action of the sGC inhibitory ODQ, which consequently led to abolition of the protective NO dependent pathway upon thrombin stimulation. The effects of NO on platelets have been previously demonstrated to be rapidly reversible and at concentrations produced by stimulated endothelial cells (Kanai et al., 1995). Therefore circulating platelets should thus be able to respond to fluctuations in concentrations of NO with a rapid and reversible shape change. More importantly we identify RhoA as a novel target for PKG signalling and potentially a new mechanism of regulation of platelets by the cyclic nucleotide regulated pathway.

CHAPTER 6 THE REGULATION OF MYOSIN LIGHT CHAIN PHOSPHATASE (MLCP)

6. Introduction

6.1 The regulation of myosin light chain phosphatase

Myosin Light Chain Phosphatase (MLCP) is responsible for dephosphorylation of the phosphorylated MLC and was identified as the major serine/threonine phosphatase component in the platelet-cytoskeleton fraction (Kiss et al., 2002). MLCP from human platelets is composed of three subunits, a 38kDacatalytic subunit of type 1 protein phosphatase PP1c, 130-kDamyosin phosphatase target subunit 1 MYPT1 and a 20kDa subunit of unknown function (Nakai et al., 1997), as is also the case for MLCP present in smooth muscle, cerebral arteries (Kitazawa et al., 2009; Neppl et al., 2009) and endothelial cells (Hartel et al., 2007). MYPT1 targets PP1c to its main substrate myosin and thus is involved in the formation of the active MLCP complex. Inhibition of MLCP can occur though phosphorylation of inhibitory sites: MYPT1; $Thr^{696/853}$ result in inhibition of PP1c activity. Both sites mentioned are major phosphorylation sites for Rho-Kinase (Feng et al., 1999). The activation of the MLCP can occur through phosphorylation of Ser^{695} in response to PKG activity, which also prevents the phosphorylation of the inhibitory site Thr^{696} (Wooldridge et al., 2004) via ROCK, thereby blocking inhibition. Having already explored the relationship between NO/cGMP signalling and regulation of MLC, this relationship between these two was further explored by examining MLCP activity and its role in MLC phosphorylation.

6.2 Results

6.2 The regulation of myosin light chain phosphatase

6.2.1 Rho-Kinase dependent inhibition of MLCP

Having examined the action of thrombin and GSNO induced responses, the mechanisms that lead to assembly and activity of the phosphatase complex were now probed through the controlling phosphorylation at residues Thr^{696} and Thr^{853} upon the MYPT1 subunit.

In **Figure.6.1** treatment of WP with thrombin (0.05U/ml) resulted in robust phosphorylation of both MLCP inhibitory residues $Thr^{696/853}$ and at MLC^{Ser19}. However, pre-incubation with the ROCK inhibitor, Y-27632 (10µM) prevented the inhibitory phosphorylation and decreased phospho MLC^{Ser19}residues, confirming that all lie downstream of ROCK signalling. To explore the effect of Ca^{2+} elevation, ML-7 (5µM) was used to inhibit the Ca^{2+} sensitive MLCK. In the presence of ML-7, thrombin phosphorylation of MLC^{Ser19} was reduced, while the inhibitory phosphorylation of MLCP was maintained. These data confirm that MLC phosphorylation is under the control of MLCK and potentially ROCK dependent regulation of MCLP.

6.2.2 Agonist triggered phosphorylation of MYPT1 residues

Upon agonist stimulation intracellular Ca^{2+} concentration is elevated and GDP-Rho converted to GTP-Rho leading to its activation. In parallel activation of RhoA/ROCK phosphorylates the MYPT1 subunit of the MLCP at $Thr^{696/853}$ (Kitazawa et al., 2009). Utilising thrombin (0.05U/ml) the contribution of MYPT1 phosphorylation was explored focusing upon the inhibitory threonine residues. **Figure.5.2** has previously shown that phosphorylation of the MLC is time dependent, now we determine if the inhibitory phosphorylation of MYPT1 residues has a similar behaviour. Treatment of the WP with thrombin (0.05U/ml) resulted in a time dependent phosphorylation of the inhibitory sites on the MYPT1, although there were differences in the kinetics. Thr^{696} was maximally phosphorylated at 5seconds post thrombin and maintained for 30seconds before undergoing full dephosphorylation by 60 seconds. The Thr^{853} is phosphorylated at 5seconds, but is maximal at 30seconds before declining to basal by 60seconds, possibly indicating preferential targeting by ROCK activity for Thr^{696}.

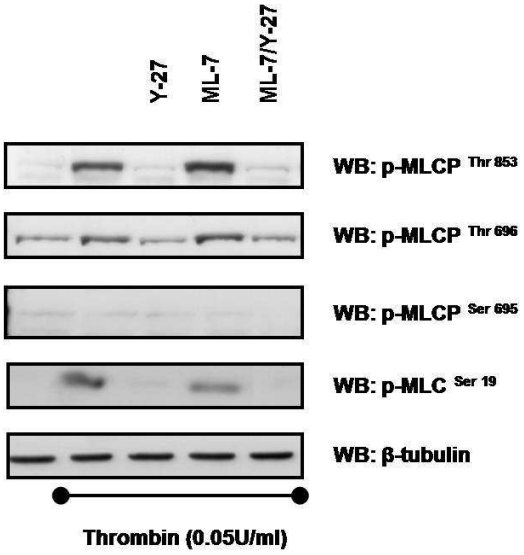

Figure.6.1 Thrombin initiated MLCP inhibitory phosphorylation via Rho-Kinase

WP ($5x10^8$) stimulated with thrombin (0.05U/ml) for 30seconds or pre-incubated first with Rho-kinase inhibitor [Y-27632 (10μM for 20mins], and or the MLCK inhibitor [ML-7 (5μM for 20mins)]. Reactions were stopped by addition of 2xLaemmli buffer. Proteins (40μg) were separated in 10% gradient gels and immunoblotted for anti-phospho $MLCP^{Thr853,}$ anti-phospho-$MLCP^{Thr696}$, anti-phospho-$MLCP^{Ser695}$, anti-phospho $VASP^{Ser157}$ and phospho-MLC^{Ser19}. Blot is representative of two individual experiments.

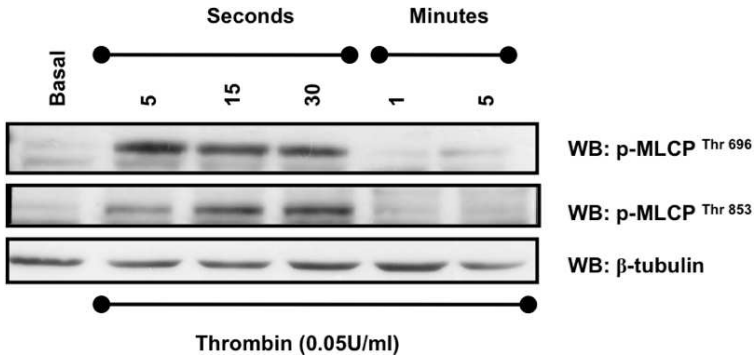

Figure.6.2 Thrombin triggered phosphorylation of inhibitory residues

WP ($5x10^8$) was incubated with thrombin (0.05U/ml) for differing times. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (40μg) were separated in 10% gradient gels and immunoblotted for anti-phospho-$MLCP^{Thr696}$ and anti-phospho-$MLCP^{Thr853}$. Blots are representative of two independent experiments.

6.2.3 Influence of NO on platelet MLCP phosphorylation

A downstream target of RhoA/ROCK and NO/PKG pathways, MLCP activity is represented though phosphorylation and dephosphorylation of the MYPT1 (Kitazawa et al., 2009; Hartel et al., 2007). The NO/PKG pathway results in the reactivation and disinhibition of MLCP (Wu et al., 1998) via two possible routes, one, phosphorylation at Ser^{188} on RhoA inhibits its activity and subsequently ROCK mediated phosphorylation of MYPT1 at inhibitory site Thr^{853}, or two, PKG mediated phosphorylation of MYPT1 at Ser^{695}, which may interfere with phosphorylation of the adjacent Thr^{696}, thus potentially removing the inhibition of MLCP thereby reactivating the MLCP (Nakamura et al., 2007). The ability of NO/cGMP signalling to modulate MLCP activity was investigated. Treatment of WP with 10µM GSNO **(Figure.6.3)**, a concentration that blocks MLC phosphorylation, elicited a time dependent increase in phosphorylation. This increase in MYPT1 phosphorylation at Ser^{695} is consistent with previous studies of (Nakamura et al., 2007) and (Wooldridge et al., 2004) following treatment elevating cGMP levels in smooth muscle. Concomitant increases in VASP phosphorylation at Ser^{239}in response to GSNO treatment suggest that PKG phosphorylates both MYPT1 and VASP at the sites examined in human platelets, with initial phosphorylation of the Ser^{695} residue occurring after 15 seconds with maximum being reached after 60 seconds incubation. These strongly suggest that PKG is the kinase responsible for phosphorylating these serine sites when active and possibly suggesting that the $MYPT1^{Ser695}$ can be used as a marker of a functional endothelial NO/cGMP signalling (Neppl et al., 2009).

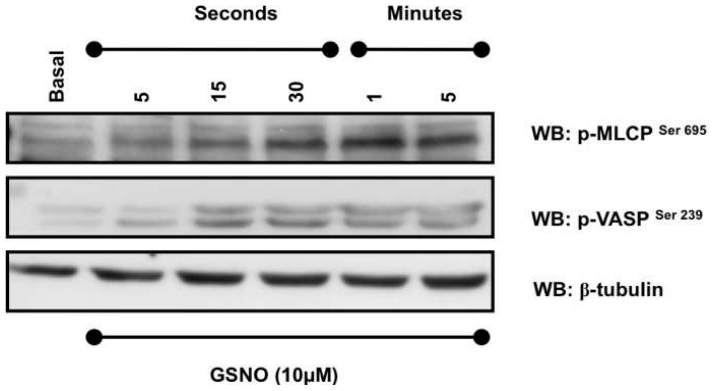

Figure.6.3 NO triggered activatory phosphorylation of MLCP

WP ($5x10^8$) was incubated with GSNO (10μM) for differing times. Reactions were stopped with an equal volume of 2xLaemmli buffer. Proteins (40μg) were separated in 10% gradient gels and immunoblotted for anti-phospho-MLCP^{Ser239} and anti-phospho-VASP^{Ser239}. Blots are representative of two independent experiments.

6.2.4 Reversible phosphorylation of MLCP upon influence of NO

In sections **6.2.2** and **6.2.3** the action of thrombin and NO upon their respective phosphorylation residues on MYPT1 was observed, and the consequent action on the phosphorylation of MLC^{Ser19}. The data suggested that the phosphorylation of MLC^{Ser19} can be reversed by the action of the NO/PKG pathway specifically by the activity of the MLCP. The ability of NO/cGMP signalling to modulate the phosphorylation state of MLC^{Ser19} and the key controlling residues of MLCP activity was investigated. In **Figure.6.4a** WP were pre-incubated with thrombin before addition of GSNO (10µM) under increasing durations demonstrating the inhibitory action of the endothelium. Thrombin pre-incubation for 25 seconds before addition of GSNO demonstrated phosphorylation at the inhibitory residues of MYPT1 at $Thr^{696/853}$ and also MLC^{Ser19}, though all diminished markedly as addition of GSNO for 5 seconds. The dephosphorylation of the inhibitory residues continued in a NO time-dependent manner, as did the dephosphorylation of MLC^{Ser19}.The phosphorylation of the MLCP activatory residue, Ser695 become apparent only upon NO alone incubation, indicating the strong inhibitory nature of thrombin possibly induced by phosphorylation of $MLCP^{Thr696}$ residue. In **Figure.6.4b** GSNO was first to be incubated with WP, this accurately replicates an *in vivo* system as platelets will be exposed to nitric oxide in blood vessels prior to encountering a site of potential injury. Incubation of WP with GSNO (10µM) for increasing time points strongly inhibited thrombin (0.05U/ml) induced phosphorylation. Pre-treatment with GSNO for 5 seconds before addition of thrombin inhibited, inhibitory phosphorylation of the residues $Thr^{853/696}$ and maintained activity of MLCP by Ser^{695} phosphorylation. This is evident through the lack of phosphorylation at MLC^{Ser19}, and the action and activity of PKG in this role confirmed via phosphorylation of $VASP^{Ser239}$.

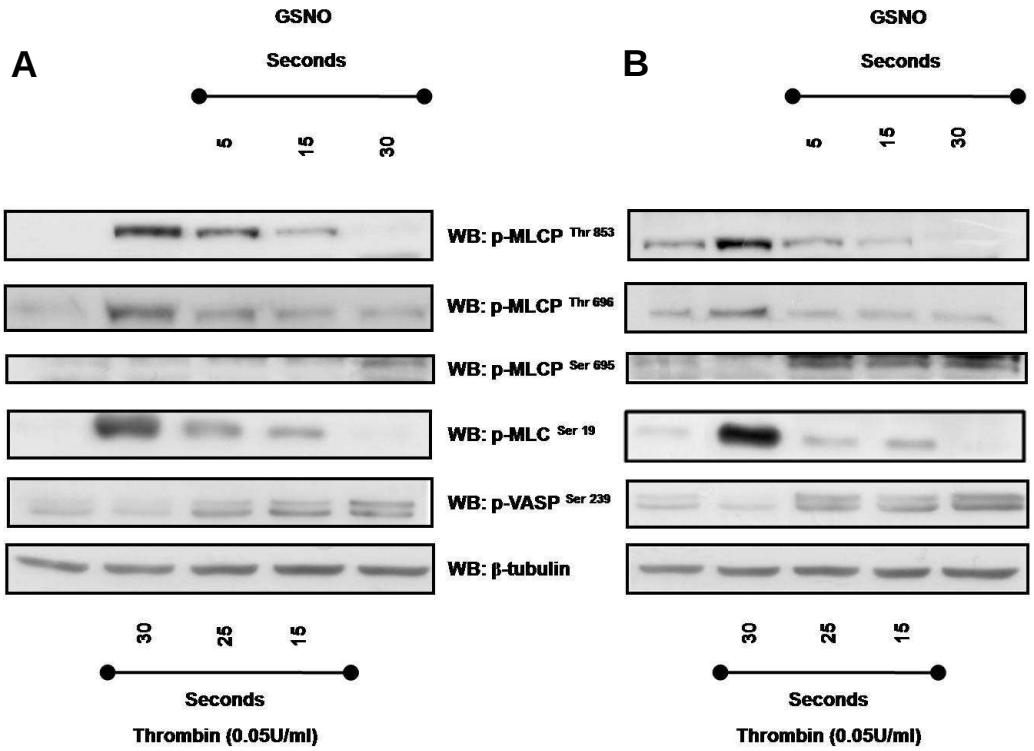

Figure 6.4 Reversible phosphorylation of MLCP upon influence of NO

(A)WP ($5x10^8$) stimulated with thrombin (0.05U/ml) for 30seconds or pre-incubated thrombin first then addition of GSNO (10µM) for increasing time periods after stimulation. After 30 seconds incubation with the thrombin GSNO mix the reactions were stopped with addition of an equal volume of 2xLaemmli buffer. Proteins (40µg) were separated in 10% gradient gels and immunoblotted for anti-phospho $MLCP^{Thr853,}$ anti-phospho-$MLCP^{Thr696}$, anti-phospho-$MLCP^{Ser695,}$ anti-phospho-MLC^{Ser19} and anti-phospho $VASP^{Ser239}$. Blot is representative of two individual experiments. **(B)** WP ($5x10^8$) stimulated with thrombin (0.05U/ml) for 30 seconds or pre-incubated first with GSNO (10µM) for increasing time periods before addition of thrombin. After 30seconds incubation with GSNO and thrombin mix the reactions were stopped with addition of an equal volume of 2xLaemmli buffer. Proteins (40µg) were separated in 10% gradient gels and immunoblotted anti-phospho $MLCP^{Thr853,}$ anti-phospho-$MLCP^{Thr696}$, anti-phospho-$MLCP^{Ser695,}$ anti-phospho-MLC^{Ser19} and anti-phospho $VASP^{Ser239}$. Blot is representative of two individual experiments.

6.2.5 Action of NO mediating MLC phosphorylation via MLCP

To further investigate the mechanisms by which NO regulates MLC phosphorylation, its effects on the inhibitory phosphorylation of MLCP induced by thrombin were studied. Stimulation with thrombin (0.05U/ml) induced phosphorylation of the inhibitory residues $Thr^{696/853}$ and subsequently at MLC^{Ser19}. Thrombin induced inhibitory MYPT1 phosphorylation was completely blocked by Y-27632, a direct inhibitor of ROCK, confirming that both phosphorylation sites are targeted by ROCK, though the remaining MLC phosphorylation occurring via the cytosolicCa^{2+} elevation as indicated in **Figure.6.5**. The addition of GSNO (10μM) prior to thrombin addition eliminated not only the Ca^{2+} dependent pathway, but also the inhibition of ROCK dependent phosphorylation of the inhibitory residues upon MYPT1, preventing phosphorylation of MLC at Ser^{19}. The combination of Y-27632 with NO mirrored the affects of either agent alone upon the MLCP indicating the same role.

To examine whether MLCP was active under basal conditions, the serine phosphatase inhibitor calyculin A (100nM) was used. Incubation of platelets with calyculin A prior to addition of thrombin led to a strong phosphorylation of MLC, indicating that MLCP is constitutively active in order to prevent phosphorylation of MLC^{Ser19} or possibly suggesting an active kinase (Deng et al., 2001). Subsequently, it was examined whether NO acts though the regulation of the MLCP or MLCK. Inhibition of the phosphatase via prevention of the assembly of the MLCP subunits with calyculin A resulted in the inhibitory affects of NO been lost and phosphorylation of inhibitory residues, on $MLCP^{Ser695}$, and ultimately MLC^{Ser19} phosphorylation, thus indicating that NO acts via the activity of MLCP and not MLCK, confirmed by addition of ML-7 (5μM) failing to cause effect in combination with NO.

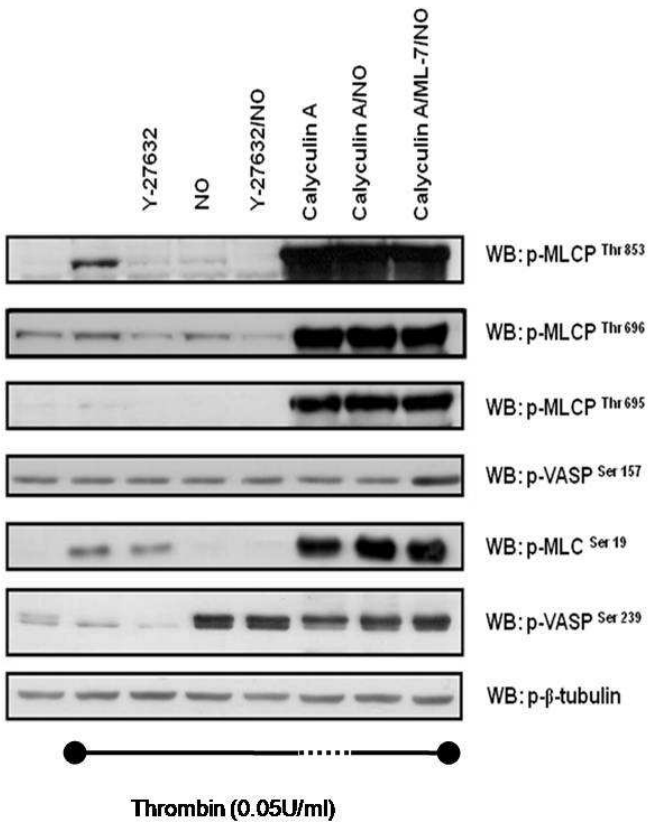

Figure.6.5 NO prevents MLC phosphorylation via the MLCP

WP ($5x10^8$) stimulated with thrombin (0.05U/ml) for 30seconds or pre-incubated first with NO [GSNO (10µM for 1 min)], the Rho-kinase inhibitor [Y-27632 (10µM for 20mins)], the MLCK inhibitor [ML-7 (5µM for 20mins)], and or the MLCP inhibitor [Calyculin A (100nM for 20mins)] except for the dashed line in the thrombin underline indicating no addition. Reactions were stopped by addition of2xLaemmli buffer. Proteins (40µg) were separated in 10% gradient gels and immunoblotted for anti-phospho $MLCP^{Thr853,}$ anti-phospho-$MLCP^{Thr696}$, anti-phospho-$MLCP^{Ser695}$, anti-phospho $VASP^{Ser239}$, and phospho-MLC^{Ser19}. Blot is representative of two individual experiments.

6.3 Discussion

In the present chapter the effects of the NO signalling pathway on the regulation of MLCP were examined as a mechanism by which the pathway influences the phosphorylation of MLC. It is established in both smooth muscle cells and platelets that G-protein coupled signalling pathways lead to the phosphorylation of MLCP on $Thr^{696/853}$. Phosphorylation of MLCP by ROCK, although other kinases have been implicated, on these residues is associated with inhibition of the phosphatase activity. Our data suggests a key role of ROCK in the phosphorylation of these residues in response to thrombin. The phosphorylation of both of these inhibitory residues was blocked by the ROCK inhibitor Y-27632. In contrast, phosphoMLCP-$Thr^{696/853}$ in response to thrombin was unaffected by the MLC kinase inhibitor ML7. This is consistent with previous reports suggesting MYPT1 phosphorylation of the Thr^{853}, which has been shown to inhibit MLCP activity by reducing the affinity of MYPT1/MLCP towards myosin (Velasco et al., 2002), and the Thr^{696} site apposing the adjacent activatory Ser^{695} site which yields an inactive form of MLCP (Muranyi et al., 2005) are primarily regulated via ROCK (Feng et al., 1999) confirmed by the incubation of the specific ROCK inhibitor Y-27632. Our data also clear demonstrates that NO signalling regulates the ability of the thrombin to induce this inhibitory phosphorylation, which is consistent with previous reports in smooth muscle (Nakamura et al., 2007; Wu et al., 1996; Sawada et al., 2001; Kitazawa et al., 2009).Moreover this seems to happen in two ways. Firstly, the addition of the NO prior to the addition of thrombin can prevent the ability of thrombin to induce inhibitory phosphorylation of MLCP. Secondly, addition of NO after thrombin also caused a rapid reduction in the phosphorylation of phospho $MLCP^{Thr696/853}$. These two sets of observations suggest that there may be multiple mechanisms at work.

The inhibition of RhoA that was observed in **Figure.5.6** could probably be the ability of GSNO to prevent phosphorylation in response to thrombin, that is, blocking RhoA prevents activation of ROCK and therefore phosphorylation of MLCP. However, other mechanisms may also be present. In smooth muscle cells MLCP has been shown to be a direct substrate for PKG. The phosphorylation of MYPT1^{Ser695} by a PKG dependent mechanism (Wooldridge et al., 2004) has been described. Phosphorylation of this site is associated with increasing activity of MLCP. The pre-phosphorylation at Ser^{695} blocked the adjacent inhibitory phosphorylation of Thr^{696} by thrombin, preventing MLCP inhibition and also reducing the total phosphorylated Thr^{696} after agonist addition, reducing the inhibition of MLCP activity. The study also demonstrated that the prior phosphorylation of Ser^{695} affected the rate at which the inhibitory sites and adjacent site Thr^{696} was phosphorylated, consistent with previous reports that Ser^{695} phosphorylation can interfere with the subsequent Thr^{696}phosphorylation (Wu et al., 1996). However, this phosphorylation of MLCP at Ser^{696}was thought to be primarily driven by PKG1α, an isoform not present in platelets. Data from this study clearly demonstrate that NO can cause the phosphorylation of this site, which was mirrored by the phosphorylation of VASP, indicating a cGMP dependent kinase that phosphorylated the site. Therefore it is possible that NO acts via two mechanisms to prevent the phosphorylation of MLC, (I) inhibition of RhoA, which prevents the activation of ROCK, and (ii) the direct phosphorylation of MLCP, which may prevent subsequent inhibitory phosphorylation. Furthermore the findings suggest that MYPT1^{Ser695} is an additional marker for NO/cGMP signalling in platelets, which has been verified in cerebral vessels and the pulmonary artery, where L-NAME treatment completely abolished Ser^{695} phosphorylation (Neppl et al., 2009).

Under conditions where the activity of the MLCP is inhibited by the incubation of calyculin A, the protective action of NO was lost on Ser^{695}, and ultimately MLC^{Ser19} phosphorylation. Previous reports indicate that this triggers a spatial redistribution of MYPT1 subunits, thereby being unable to de-phosphorylate the MLC (Lontay et al., 2005). This indicates that NO acts via the activity of MLCP and not MLCK, that were confirmed by addition of ML-7 failing to cause effect in combination with NO, revealing that MLCP activity but not MLCK activity was affected by NO/PKG pathway, consistent with reports from smooth muscle cells (Nakamura et al., 2007; Lee et al., 1997).

The ability of NO to cause the dephosphorylation of MLCP at $Thr^{696/853}$ occurs through an unknown mechanism. There were two potential explanations that could account for these effects of GSNO, (I) there could be a phosphatase that regulates the phosphorylation of MLCP, which in turn could be activated by NO, or (ii) NO directly modifies MLCP to such a degree that it is more susceptible to phosphatase activity. It has previously been shown in vascular smooth muscle (Kitazawa et al., 2009) that the regulation of MLCP activity by NO is associated with a reduction in the phosphorylation of the specific inhibitory protein CPI-17 at Thr^{38}, thereby causing MLCP disinhibition. In endothelial cells cAMP/PKA plays a similar role (Aslam et al., 2010). However the role of NO to influence CPI-17 phosphorylation and activity and the regulation of MLCP in platelets is unknown, therefore these possibilities require further investigation.

Shown in this chapter for the first time is the influence of NO/PKG on RhoA signalling in relation to downstream phosphorylation of MLCP in platelets and the ability of NO

to induce phosphorylation of RhoA resulting in reduced inhibitory phosphorylation of MLCP and unveiled the novel ability of NO to induce disinhibitory phosphorylation of MYPT1.

CHAPTER 7 GENERAL DISCUSSION

7. General Discussion

Platelets play a vital role in haemostasis, stopping haemorrhaging following vascular injury though their ability to rapidly adhere to the damaged endothelium and sub endothelia matrix proteins and to aggregate (Ruggeri, 2002). The processes of platelet adhesion and aggregation are promoted via differing factors. VWF, thrombin and ADP activate multiple intracellular signal transduction mechanisms which lead to shape change. This drives the formation of filopodia and lamellipodia, secretion of granule contents, aggregate formation and ultimately thrombus formation (Johnson et al., 2007). Acto-myosin driven contraction is initiated by the phosphorylation of regulatory MLC by a network of kinases, including the Ca^{2+}/calmodulin-dependent MLCK and Ca^{2+}-independent ROCK (Somlyo et al., 2003). The present study initially wished to examine how NO regulated different aspects of Ca^{2+} mobilisation in platelets. However, during the course of the investigation we discovered a novel and potentially important mechanism of platelet regulation by NO that did not involve its known effects on intracellular Ca^{2+} levels.

Platelet activation is tightly regulated under physiological conditions by antagonists such as the endothelium derived inhibitor, NO, which the platelets encounter as they circulate at the periphery of the blood vessel. NO inhibits platelet activation, adhesion and aggregation *in vitro* and *in vivo* (Naseem et al., 2008). The NO donor chosen here, an S-Nitrosothiol, to investigate this process is present *in vivo* as major redox form of NO in the plasma (Stamler et al., 1992) with the most abundant intracellular RSNO being the low molecular weight S-nitrosoglutathione (GSNO) (Stamler et al., 1992). Release of the NO activates the haem containing enzyme sGC, elevating levels of cGMP and causing activation of PKG, which now

phosphorylates target proteins leading to the inhibition of platelet activation (Schwarz et al., 2001). There are number of PKG substrates that have been identified including inositol trisphosphate (IP_3) receptors (Cavallini et al., 1996), the focal adhesion protein vasodilator-stimulated phosphoprotein (VASP) (Halbrugge et al., 1990), IRAG, RapIb (Danielewski et al., 2005) and the TxA_2 receptor (Wang et al., 1998). However, what remains to be established is how the phosphorylation of these targets is linked to the inhibition of specific platelet functions. Moreover, many of these targets were identified using non-physiological activation of the cGMP system. Therefore it is important to determine more physiological targets to fully understand how NO regulates platelets.

Platelets respond to various agonistic stimuli by rapid changes in shape followed by aggregation and subsequent secretion of granule contents. Platelet shape change is the earliest functional response following activation with physiological agonists, which is accompanied by the rearrangement of the cytoskeleton (Daniel et al., 1984). This investigation utilised thrombin to induce the phosphorylation of contractile proteins, in this case namely MLC (Johnson et al., 2007) resulting in the reorganisation of cytoskeletal structure and in shape change. The phosphorylation of the MLC results in an increased development of actin-activated ATPase activity and reflects the contractile activity of actomyosin (Getz et al., 2010). Thrombin's intracellular signalling is mediated and confirmed through the use of PAR analogues of the PARs coupling to G_q and $G_{12/13}$ families of GPCR, which ultimately activate signalling cascades leading to MLCK and ROCK activation (Macfarlane et al., 2001). Both of these pathways contribute to the regulation of the phosphorylation state of MLC. Ca^{2+}/calmodulin acts through the MLCK mediated phosphorylation at

MLC^{Ser19}residue; ROCK contributes by both deactivating the MLCP and potentially directly phosphorylating the Ser^{19} residue (Somlyo, 2003; Yazaki et al., 2005).

7.1.1 First key finding

The major observation in this study was the ability of NO to inhibit the phosphorylation of MLC.

The experimental approaches used the NO donor GSNO, but found that the inhibitory effect of GSNO was solely mediated via NO-GC and cGMP, as NO mediated effects were abolished under conditions of sGC inhibition, confirming it as the critical node in the NO signalling cascade in platelets (Riba et al., 2008). This was important since GSNO has been shown to have cGMP-independent effects on platelets (Irwin et al., 2010). These findings of a cGMP dependent mechanism that reduces MLC phosphorylation is consistent with previous findings suggesting NO role in smooth muscle and endothelial cells in inducing relaxation via this mechanism (Rivero-Vilches et al., 2003; Nakamura et al., 2007).

7.1.2 Second key finding

This study has confirmed ROCKs dual role in platelets and shown that this mechanism does not therefore increase phosphorylation directly but potentially decreases the dephosphorylation upon the MLC residue.

Phosphorylation of MLC triggers activation of the contractile machinery, regulated by the Ca^{2+} dependent MLCK and Ca^{2+} independent Rho-Kinase (ROCK) (Goeckeler et

al., 1995; Garcia et al., 1995). In the context of the present study ROCK is of special interest. It was postulated that ROCK may have a dual role; this had previously been shown in smooth muscle firstly via the direct phosphorylation of the MLC (Amano et al., 1996), and secondly through the phosphorylation on MYPT1$^{Thr696/853}$, resulting in the inhibition of MLCP catalytic activity, and its affinity to bind to myosin (Fukata et al., 2001; Velasco et al., 2002), thereby increasing in MLC phosphorylation.

The crucial role of ROCK in mediating sustained MLC phosphorylation and in smooth muscle contraction has focused attention on the ability of NO to inactivate RhoA and its effector ROCK (Etter et al., 2001; Sauzeau et al., 2000). Previous studies on smooth muscle and NK cells have suggested the negative regulation of Rho-dependent signalling via PKA phosphorylating activated, membrane bound RhoA at the site Ser^{188}, and accelerating its inactivation and its dissociation from its membrane bound substrates (Murthy et al., 2003).The data in this study shows for the first time that NO/PKG signalling pathways induce phosphorylation of RhoA on Ser^{188}. This study demonstrates that NO/PKG phosphorylates RhoA, which inhibits its signalling probably by retaining RhoA in the cytosol or by terminating an already active RhoA signalling pathway in platelets (Murthy et al., 2003).

7.1.3 Third key finding

This study shows for the first time that NO/PKG can phosphorylate MYPT1 at Ser^{695} in platelets.

Though as PKG cannot dephosphorylate MLC^{Ser19} directly, it was anticipated that it would act indirectly via altering activity of the MLCP or inhibition of the MLC kinase

(Bauer et al., 1999). The inactivation of RhoA^{Ser188} via addition of GSNO was thought likely to inhibit the activation of ROCK, which in turn may account for the observations that inhibitory phosphorylation of MYPT1 at the residues Thr^{853} and Thr^{696} in human platelets is reduced. These sites have also been shown to be governed by ROCK in endothelial cells (Ito et al., 2004).

Although regulation of the MLCP via addition of NO has been demonstrated in endothelial, smooth muscle and porcine aorta (Hartel et al., 2007; Nakamura et al., 2007; Eto et al., 1995), the exact regulation of MLCP by PKG in human platelets has not. As mentioned previously, this is the first study which shows that NO/PKG can phosphorylate MYPT1 at Ser^{695} in platelets. This is associated with reduced phosphorylation of the inhibitory residues Thr$^{696/853}$ by ROCK, possibly by steric hindrance (Kitazawa et al., 2009), leading to MLC dephosphorylation. NO potentially reduces the ability of the agonist to inhibit phosphatase activity and when reversed, the pre-phosphorylation of Thr^{696} can be overturned. Phosphorylation of the residue Ser^{695} on MYPT1 demonstrates the mutual exclusion of the phosphorylation of the inhibitory residues by ROCK based on the fact of being adjacent to the inhibitory site and PKG upstream deactivation (Nakamura et al., 2007).

7.1.4 Fourth key finding

The prior phosphorylation of Ser^{695} affected the rate at which the inhibitory sites and adjacent site Thr^{696} was phosphorylated. This was shown to be consistent with previous reports that Ser^{695} phosphorylation can interfere with the subsequent Thr^{696} phosphorylation in smooth muscle (Wooldridge et al., 2004; Wu et al., 1996).

GSNO significantly reduced MLC^{Ser19} phosphorylation and diminished the ability of thrombin to activate the RhoA pathway thereby preventing inhibitory phosphorylation of MYPT1 residues. Though the pre-phosphorylation at the inhibitory sites upon MLCP affects that rate at which activatory phosphorylation at Ser^{695} accrues.

These findings suggest that MYPT1$^{Ser\ 695}$ is an additional marker for of NO/cGMP signalling which has been alternatively verified in cerebral vessels and endothelial denudation of pulmonary artery, where L-NAME treatment completely abolished Ser^{695} phosphorylation (Neppl et al., 2009).

The data produced here provides new insights into the mechanism by which GSNO induced PKG activation inhibits Ca^{2+} elevation required for MLCK activity, blocks the two Rho dependent pathways; phosphorylation of MLC and inhibitory phosphorylation of MYPT1. While phosphorylating the Ser^{695} site resulting in an increase in MLCP activity leading to the dephosphorylation of the MLC, thus inhibiting cytoskeletal rearrangement and platelet shape change, though critically in the study we have identified two novel substrates for PKG signalling in platelets.

8. Further Work

This further work to be carried out will reinforce the preliminary data that has already been accumulated; to advance our understanding of the mechanisms which NO regulates MLCP activity in platelets.

- **The modulation of RhoA/ROCK mediated inhibition of MLCP by NO/PKG**

  Aim: To examine if NO activates MLCP through inhibition of RhoA/ROCK.

  NO inhibits shape change mediated by RhoA/ROCK. Since RhoA/ROCK mediate their effects though inhibitory phosphorylation of MYPT1, the data here suggests a link between NO inhibition of shape change and the phosphorylation of MLCP. The influences of NO/PKG on RhoA signalling in relation to downstream phosphorylation of MLCP will be further investigated.

  - The influence of NO on RhoA/ROCK mediated inhibition of MLCP.

    - The activity of RhoA in agonist and NO treated platelets will be established using a GST-pull down assay.

    - To determine if inhibition of RhoA influences the activity of MYPT1 performed using Sensolyte fluorimetric phosphatase assay with immunoprecipitated MLCP.

- The influence of NO on RhoA/ROCK/MLCP interactions and subcellular localisation.

  Activation of smooth muscle RhoA requires translocation from the cytosol to the membrane (Gong *et al.,* 1997) where it interacts with ROCK and potentially MLCP. It will be established the effect of NO on this process in platelets.

  - Determine if NO can influence either the translocation of RhoA to the membrane or accelerate its translocation back to the cytosol utilising differing fractions that will be immunoblotted for RhoA in its native or phosphorylated form over several time points.

  - Examine if these proteins form a multi-protein complex and if NO inhibits this association to prevent inhibition of MYPT1.

- Does PGI_2/PKA modulate RhoA/ROCK mediated inhibition of MLCP?

  RhoA is phosphorylated, inhibited and relocalised from the membrane to cytosolic compartments by PKA in numerous cell types (Murthy *et al.,* 2003).

  - Although study focus on NO, it is fundamentally important to examine if PGI_2 influences the activity of MYPT1.

- **The role of phosphorylation in the regulation of platelet MYPT1 activity by NO/PKG**

  Aim: To determine how NO/PKG signalling influences phosphorylation of MYPT1, downstream activity and subcellular localisation of MLCP.

  This will characterise the effects of NO on the multiple phosphorylation sites of MYPT1 and how these post-translational modifications influence MLCP activity, structure of the haloenzyme and cellular localisation.

  - Characterisation of phosphorylation of MYPT1 Ser^{695} by NO signalling: to confirm that the phosphorylation of MYPT1 on Ser^{695} increases MLCP activity.

  - The influence of NO on inhibitory phosphorylation of MYPT1.

    Examine the interplay between the activatory and inhibitory phosphorylation sites, and the influence of NO on the phosphorylation sites and MLCP activity.

    - To determine the influence of MYPT1 phosphorylation on enzyme activity.

  - Is MYPT1 phosphorylation associated with changes in cellular location?

Stimulation of smooth muscle leads to the translocation of MLCP from the cytosol to the membrane by ROCK dependent mechanisms (Shin *et al.,* 2002: Lontay *et al.,* 2005), leading to potential inhibition of the enzyme. The cellular location and potential translocation of platelet MLCP under NO treatment requires clarification.

- If translocation is required for inhibitory phosphorylation of MLCP or the reverse.

- If the different phosphorylation states reflect differences in cellular location.

- How does NO influence the structure of the MLCP holoenzyme.

  Under resting conditions platelet MLCP exists as a 130kDa heterotrimer (Suzuki *et al.,* 1999), although it is unclear if this is maintained under activatory conditions.

  - Is the MLCP maintained as a heterotrimer or dissociate into different cellular fractions according to its phosphorylation state?

➢ **NO regulation of MLCP activity through modulating CPI-17**

Aim: To examine the ability of NO and PGI_2 to modulate CPI-17 mediated inhibition of MLCP.

CPI-17 is an important endogenous regulator of MLCP (Ito *et al.,* 2004). However the ability of both cGMP/PKG and cAMP/PKA to influence CPI-17 regulation of MLCP in platelets is unknown and so the influence of NO and PGI_2 on platelet CPI-17 shall be characterised.

- Does NO influence the phosphorylation and activity of CPI-17?
    - Does ROCK phosphorylate CPI-17 or multiple pathways?
    - Influence of NO on CPI-17, if NO prevents and/or induces dephosphorylation of CPI-17 and its effects on MLCP activity.
    - If CPI-17 affects the interaction between PP1c and the MLCP
- Does PGI_2/PKA influence the phosphorylation and activity of CPI-17?

Appendix I

1-Isolation of human platelets via pH method

Buffers

- Acid Citrate Dextrose (ACD) pH 6.5

COMPOUND	MOLARITY (mM)
Glucose	113.8
Tri-Sodium citrate	29.9
Sodium Chloride	72.6
Citric Acid	2.9

- Wash Buffer pH 6.5

COMPOUND	MOLARITY (M)
Citric Acid	0.036
EDTA	0.010
Glucose	0.005
Potassium Chloride	0.005
Sodium Chloride	0.09

- Tyrodes Buffer pH 7.4

COMPOUND	MOLARITY (mM)
Sodium Chloride	150
HEPES sodium salt	5
NaH_2PO_{4anh}	0.55
$NaHCO_{3anh}$	7
Potassium Chloride	2.7
Magnesium Chloride	0.5
Glucose	5.6

Equipment

- Falcon Tubes (15 and 50ml)……Sarstedt

- Centrifuge

- Butterfly-21 Venisystems……Abbot Labs

2-Calculation of platelet count

Buffer

- Ammonium Oxalate; (1%w/v) in dH_2O

Equipment

- Neubauer cell counter

- Inverted light microscope

3-Determionation of protein content

Buffer (Ratio-1:1)

- Tyrodes Buffer

COMPOUND	**MOLARITY (mM)**
Sodium Chloride	150
HEPES sodium salt	5
NaH_2PO_{4anh}	0.55
$NaHCO_{3anh}$	7
Potassium Chloride	2.7
Magnesium Chloride	0.5
Glucose	5.6

- Lysis Buffer

COMPUND	MOLARITY (mM)
Sodium Chloride	150
Tris-base	10
EGTA	10
EDTA	1

Assay Kit

- DC Protein Assay……..BioRad

Equipment

- 96-well cell culture plate…….Sarstedt

- Multiplate reader with 750nm filter

4-Measurment of platelet aggregation

Buffers

- Tyrodes Buffer

COMPOUND	MOLARITY (mM)
Sodium Chloride	150
HEPES sodium salt	5
NaH_2PO_{4anh}	0.55
$NaHCO_{3anh}$	7
Potassium Chloride	2.7
Magnesium Chloride	0.5
Glucose	5.6

Equipment

- Aggregation module-duel channel……Payton

4-Sample preparation for Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Buffer

- Laemmeli buffer (2x) – pH6.8

COMPOUND	MOLARITY
SDS	4%w/v
2-Mercaptoethanol	10%v/v
Glycerol	20%v/v
Tris-Base	50mM
Bromophenol blue	Trace

Equipment

- Aggregation module-duel channel……Payton
- Aggregation cuvettes

5-Methods for SDS-PAGE

Buffers

- Buffer 1 pH 8.8

COMPOUND	MOLARITY
Tris-Base	0.5M
SDS	0.4%w/v

- Buffer 2 pH 6.8

COMPOUND	MOLARITY
Tris-Base	1.5M
SDS	0.4w/v

Equipment

- Miniprotean 3 cell……BioRad
- Gradient mixer……BioRad
- Peristaltic pump
- Butterfly-21 Venisystems……..Abbot Labs
- Plastic tubing
- Biotin protein ladder……Cell signalling

Gradient gel (10-18%) composition for 1.5mm casting plates

COMPOUND	10% Running Gel	18% Running Gel	3% Stacking Gel
dH_2O	1.418ml	0.708ml	4.87ml
Buffer 1	0.886ml	0.886ml	
Buffer 2			1.87ml
30% Acrylamide	1.182ml	1.961ml	0.75ml
TEMED	2µl	2µl	10µl
Ammonium Persulphate 10%	18µl	18µl	75µl

10% gel for 2x 1.5mm casting plates

COMPOUND	10% Running Gel	3% Stacking Gel
dH_2O	6.48ml	4.87ml
Buffer 1	4ml	
Buffer 2		1.87ml
30% Acrylamide	5.3ml	0.75ml
TEMED	5.3µl	10µl
Ammonium Persulphate 10%	65µl	75µl

6-Western Immunoblotting

Buffers

- Transfer Buffer

COMPOUND	MOLARITY
Tris-Base	25mM
Glycine	0.2M
Methanol	20% v/v

- Tris Buffered Saline Tween ($TBS_{T0.1\%}$) pH 7.4

COMPOUND	MOLARITY
Sodium Chloride	150mM
Tris-Base	20mM
Tween-20	0.1% v/v

- ECL-1

COMPOUND	MOLARITY
Luminol	250mM
P-Coumoric	90mM
Tris-Base(pH 8.5)	100mM

- ECL-2

COMPOUND	MOLARITY
H_2O_2 (30%)	0.2% v/v
Tris-Base (pH 8.5)	100mM

- ECL 1 & ECL 2 combined in 1:1 ratio before use

- Developer solution; prepared in 1:5 ratio with dH_2O

- Fixer solution; prepared in 1:5 ratio with dH_2O

Equipment

- Hybond-P PVDF membrane……...GE Health Care
- Mini Trans-Blot electro transfer cell……BioRad
- Exposure cassette……Sigma
- Hyper film……GE Health Care
- Microplate shaker

9-Platelet preparation for calcium assay

Buffer

- Calcium buffer pH 7.4 With the addition of $CaCl_2$ (1mM) or EGTA (1mM) as required

COMPOUND	MOLARITY
Sodium Chloride	145mM
Potassium Chloride	5mM
Magnesium Chloride	1mM
HEPES	10mM
Glucose	10mM

Equipment

- Fluorospectrometer…….PhotonTechnology International
- Fura-3……..Molecular Probes

Appendix II

Inhibitors/Activators

Compound	Concentration	Target
2-APB	0.1-100μM	SOC
SKF-96365	0.1-100μM	SOC
Carbenoxolone	100-400μM	GAP Junctional Channels
Gd^{3+}	0.1-100μM	TRPC4/5
GSNO	0.1-100μM	sGC
BAPTA	20μM	Intracellular Ca^{2+}
EGTA	1mM	Extracellular Ca^{2+}
SFLLRN-NH_2	6μM	PAR-1
AYPGKF-NH_2	60μM	PAR-4
ODQ	20μM	sGC
Y-27632	10μM	RhoA
Thrombin	0.01-0.1U/ml	PAR-1/4
Calyculin A	100nM	Ser/Thr Phosphatases
ML-7	5μM	MLCK
Indomethacin	10μM	TxA_2
Apyrase	1μ/ml	ADP

Appendix III

Antibody List

Antibody	Company
Anti-rabbit IgG: HRP	Amersham
Anti-Biotin protein ladder	Cell Signalling
Anti-mouse IgG: HRP	Amersham
Anti-phospho VASP (Ser^{239})	Cell Signalling
Anti-β tubulin	Upstate
Anti-phospho RhoA (Ser^{188})	Santa Cruz
Anti-phospho MLC (Ser^{19})	Santa Cruz
Anti-phospho MLCP (Thr^{853})	Cell signalling
Anti-phospho MLCP (Thr^{696})	Santa Cruz
Anti-phospho MLCP (Ser^{695})	Santa Cruz
Anti-TRPC1 (T1E3)	Leeds University
Anti-TRPC4 (T45E3)	Leeds University
Anit-TRPC5 (T5E3)	Sigma
Anti-TRPC6 (T367E3)	Leeds University
Anit-TRPC6 (N-Termini)	Alomone
Anti-Stim1	Sigma
Anti-Stim2	Sigma
Anti-Orai1	Sigma
Anti-Orai2	Sigma
Anti-Orai3	Sigma
Goat Anti-Rabbit-IgG: FITC	Sigma

Appendix IV

SiRNA List

siRNA	Company
si-pool	Sigma
si-scramble	Sigma
si-TRPC1	Sigma
si-TRPC3	Sigma
si-TRPC4	Sigma
Si-TRPC6	Sigma
si-Bcl_2	Sigma

Appendix V

Primer sequences for RT-PCR

Gene Name		Primers (5' to3')
β-actin	F	TTGTAACCAACTGGGACGATATG
	R	GATCTTGATCTTCATGGTGCTGG
GAPDH	F	CCATCCACAGTCTTCTGG
	R	CCCTCCAAAATCAAGTGG
TRPC1	F	TGGTATGAAGGGTTGGAAGAC

(T1)	R	GGTATCATTGCTTTGCTGTTC
TRPC3	F	TGACTTCCGTTGTGCTCAAATATG
(T3)	R	CCTTCTGAAGTCTTCTCCTTCTGC
TRPC4	F	TCTGCAGATATCTCTGGGAAGGATGC
(T4)	R	AAGCTTTGTTCGAGCAAATTTCCATTC
TRPC5	F	TGAGAACGAGAACCTGGAG
(T5)	R	TACTCGGCCTTGAACTCATTC
TRPC6	F	TACGATGGTCATTGTTTTGC
(T6)	R	TCTGGGCCTGCAGTACATATC
TRPC7	F	ATCTTCGTGGCCTCCTTCAC
(T7)	R	AACGCTGGGTTGTATTTGGC
STIM1 (S1)	F	TGTGGAGCTGCCTCAGTATG
	R	AAGAGAGGAGGCCCAAAGAG
STIM2 (S2)	F	CAGCCATCTGCACAGAGAAG
	R	AGGTTCGTGCACTGCTATCC
ORAI1	F	AGGTGATGAGCCTCAACGAG
(O1)	R	CTGATCATGAGCGCAAACAG
ORAI2	F	CATAAGGGCATGGATTACCG
(O2)	R	CGGGTACTGGTACTGCGTCT
ORAI3	F	GGCTACCTGGACCTCATGG
(O3)	R	GGTGGGTACTCGTGGTCACT

Note: F forward, R: reverse.

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