H24/3462

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON.....29 July 2003.....
.....[redacted].....
Sec. Research Graduate School Committee

Addendum.

p 6, para 3, line 1: Change " canuliculi" for " canaliculi"

p 35, third line: Change "desialylated" for "desialated"

p 58, para 2 last sentence: Change "(Shapiro, 1976)" for "Shapiro (1976)"

p 61, first sentence: Change "RBCs" for "RBC's"

p 104, para 2, line 9: Change "sustained aggregate" for "sustained aggregates"

The reference of Beigi, 1999 on pg 124, para 1, line 11 was omitted from the bibliography. The reference is:

> Beigi, R., Kobatake, E., Aizawa, E., Dubyak, G.R. (1999) Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. *Am J Physiol*, **276,** C276-278.

Figure legend of Figure 3.1: Change "Effect" for "The effect"

Figure legend of Figure 3.3: Change "Effect" for "The effect"

Figure legend of Figure 3.4: Change "Effect" for "The effect"

Figure legend of Figure 3.5: Change "Effect" for "The effect"

Figure legend of Figure 3.6: Change "Effect" for "The effect"

Figure legend of Figure 3.8: Change "Effect" for "The effect"

Figure legend of Figure 3.9: Change "Effect" for "The effect"

Figure legend of Figure 4.3: Change "Effect" for "The effect"

Figure legend of Figure 4.4: Change "Effect" for "The effect"

Figure legend of Figure 4.5: Change "Effect" for "The effect"

Figure legend of Figure 4.6: Change "Effect" for "The effect"

Figure legend of Figure 4.9: Change "Effect" for "The effect"

Figure legend of Figure 6.2: Change "Effect" for "The effect"

Figure legend of Figure 6.3: Change "Effect" for "The effect"

Figure legend of Figure 6.4: Change "Effect" for "The effect"

Figure legend of Figure 6.8: Change "Effect" for "The effect"

CALCIUM SIGNALLING REGULATING PLATELET ADHESION AND THROMBUS GROWTH.

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

Simon Giuliano

Department of Medicine

Monash Medical School

Box Hill Hospital, Box Hill

Victoria 3128

AUSTRALIA

February, 2002

Table of contents

Statement of originality

This thesis does not contain any material which has been previously accepted for the award of any other degree in any university. The data presented in this thesis is exclusively the work of the author and contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Simon Giuliano.

Acknowledgments.

Firstly I would like to thank Prof. Hatem Salem and Dr. Shaun Jackson for allowing me the privilege to undertake my PhD studies at Box Hill Hospital. In particular, I thank Shaun for his never-ending encouragement and supervision. I am forever grateful for your support, and for the many words of wisdom you have shared with me over the years. Our long discussions have taught me many valuable lessons even beyond the scope of science. It has been a great learning experience.

A big thank you to Warwick Nesbitt for all your help towards my PhD studies, your advice and guidence have helped me to be a better scientist. Thankyou even more so, for your friendship and your unique sense of humour which has made life in the lab so much more bearable. Thank you also to Su Kulkarni, for being such a great friend for the last seven years. God bless, and may all your ambitions come true.

Many thanks especially to my family who have been such a great source of strength and inspiration for me. Thankyou for your unconditional love and support throughout my studies. None of this would have been possible without you guys, I will always be grateful.

Above all else I would like to thank my wife Michelle for being so patient and always believing in me. Thankyou for being so understanding, for sharing all of my joys and helping me through the many tough times.

Summary.

The arrest of bleeding is critically dependent on the ability of blood platelets to adhere and to form a haemostatic plug at sites of vascular injury. The interaction of circulating platelets with injured blood vessels is mediated through the binding of specific surface receptors with immobilised adhesive proteins in the vascular subendothelium. It is generally accepted that the initial recruitment of platelets to the vessel wall is mediated by the binding of the GP Ib/V/IX receptor with immobilised vWf on the subendothelium. This interaction not only mediates platelet adhesion, but also results in signalling processes leading to the activation of the major platelet integrin, $\alpha_{IIb}\beta_3$, and subsequently to firm adhesion, spreading and aggregation. There is now strong evidence to suggest that this multi-step adhesion process also underlies the formation of occlusive thrombi that precipitate stroke and myocardial infarction. While the importance of GP Ib/V/IX and $\alpha_{IIb}\beta_3$ in platelet function is undisputed, the precise signalling mechanisms regulating the conversion from surface translocation to stationary adhesion, and subsequent aggregation, remain unclear. Recent studies have demonstrated that binding of vWf to GP Ib/V/IX results in the elevation of cytosolic calcium and in the activation of a key calcium dependent signalling protein, protein kinase C (PKC). Both calcium and PKC have been shown to play important roles in integrin $\alpha_{IIb}\beta_3$ activation and soluble agonist secretion, however, the exact signalling relationship between calcium and PKC in co-ordinating these processes remains controversial. Another key unresolved issue that is fundamental to haemostasis and thrombosis is the mechanism regulating the extent of thrombus formation at sites of vascular injury. It is unclear if platelet adhesion receptors alone are sufficient to regulate platelet aggregation, or whether this process requires co-stimulation through other platelet agonist receptors.

The first section of this thesis examines the requirement for cytosolic calcium in integrin $\alpha_{IIb}\beta_3$ activation, in response to stimulation by adhesive substrates and soluble agonists. These studies demonstrated that cytosolic calcium is absolutely essential for platelet $\alpha_{IIb}\beta_3$ activation in response to multiple physiological platelet agonists. Pre-treatment of platelets with the membrane permeable calcium chelator dimethyl-Bapta-AM (DM-BAPTA,AM) totally abolished the ability of platelets to bind PAC-1 and aggregate in response to thrombin, soluble type I collagen and ADP, and also inhibits the ability of platelets to spread on vWf and adhere irreversibly to vWf and collagen under high shear conditions. Detailed analysis of cytosolic calcium changes in both suspension and adhesion based assays demonstrated that platelet integrin $\alpha_{IIb}\beta_3$ activation is only observed in association with elevated cytosolic calcium levels, and that treatment with sufficiently high concentrations (100μM) of DM-BAPTA,AM abolishes cytosolic calcium transients induced by weak and strong platelet agonists.

The second section of this thesis investigates the role of PKC in regulating $\alpha_{IIb}\beta_3$ activation and calcium flux on immobilised vWf. These studies demonstrated a complex signalling relationship between calcium, PKC and $\alpha_{IIb}\beta_3$. The requirement for PKC during vWf-mediated $\alpha_{IIb}\beta_3$ activation was highlighted by the fact that PKC inhibitors could reduce $\alpha_{IIb}\beta_3$ activation on vWf. However, contrary to previous studies suggesting a major role for GP Ib/V/IX in PKC activation, the present studies demonstrated that PKC is primarily activated through $\alpha_{IIb}\beta_3$ signalling. Moreover, these studies demonstrated that PKC is involved in potentiating platelet calcium flux by promoting integrin $\alpha_{IIb}\beta_3$-dependent calcium mobilisation and establishing a positive feedback loop leading to stationary adhesion formation and sustained cytosolic calcium oscillations. Furthermore, these studies demonstrated that while PKC is a key early signalling event during $\alpha_{IIb}\beta_3$

activation, when the cytosolic calcium levels exceeds a certain threshold, integrin $\alpha_{IIb}\beta_3$ activation could proceed in the absence of PKC activity.

The studies in the third section of this thesis examine the role of calcium in regulating platelet thrombus formation under flow conditions. These studies demonstrate that the ability of platelets to form stable aggregates under high shear depends on the propagation of calcium through a population of coherent platelets. This process, termed inter-platelet calcium communication (ICC), was found to determine the rate and extent of thrombus growth. A key finding of these investigations was that the propagation of ICC is mediated largely by secretion of endogenous ADP. These studies define a novel synergistic signalling mechanism operating through integrin $\alpha_{IIb}\beta_3$, and the purinergic ADP receptor, $P2Y_{12}$, promoting thrombus development.

In the last section of this thesis, the role of ADP in primary platelet adhesion to vWf was investigated. These studies demonstrated that platelet activation and stationary adhesion formation on immobilised vWf could occur directly through adhesion receptor engagement, independent of ADP co-stimulation. However, the efficiency of this process was shown to be very low, with only a small proportion of the vWf adherent population under flow conditions forming stationary adhesion contacts. These studies demonstrated that co-stimulation of vWf-adherent platelets with ADP resulted in a dramatic increase in the ability of platelets to form irreversible adhesion contacts on vWf, supporting further a synergistic relationship between integrin $\alpha_{IIb}\beta_3$ and ADP receptors during platelet activation. Moreover, the results of these studies suggest a multi-step signalling process regulating $\alpha_{IIb}\beta_3$ activation, whereby signalling through the purinergic G_q-linked $P2Y_1$ receptor mediates transient and reversible integrin activation, while concomitant signalling through the G_i-linked $P2Y_{12}$ receptor stabilises the $\alpha_{IIb}\beta_3$ receptor and promotes irreversible stationary adhesion.

Abbreviations.

ADP	adenosine diphosphate
AR-C69931MX	N(6)-(2-methyl-thioethyl)-2-(3,3,3-trifluoropropylthio)-beta,gamma-dichloromethylene-ATP
A3P5PS	adenosine 3'-phosphate, 5'-phosphosulfate
°C	degrees Celsius
Ca^{2+}	Calcium ion
$CaCl_2$	calcium chloride
DAG	diacylglycerol
DIC	differential interference contrast
$DiOC_6$	3,3'-dihexyloxacarbocyanine iodide
DM-BAPTA,AM	5,5'-dimethyl-BAPTA, acetomethyl ester
DMSO	dimethyl sulphoxide (Me_2SO_4)
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
FACS	fluorescence activated cell sorter
FITC	fluroscein isothiocyanate
GP	glycoprotein
HEPES	4-(2-hydroxymethyl)-1-piperazineethanesulphonic acid
IgG	immunoglobulin
IP_3	inositol 1, 4, 5-trisphosphate
IP_4	inositol 1,3,4,5-tetrakisphosphate
kDa	kilo dalton
μM	micromolar

mAb	monoclonal antibody
Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
NP-EGTA	o-nitrophenyl EGTA
PH	pleckstrin homology
PI 3-K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PPP	platelet poor plasma
PRP	platelet rich plasma
PS	phosphatidylserine
PWB	platelet washing buffer
RGD	arginine-glycine-aspartic acid
rpm	revolutions per minute
S.E.M	standard error of the mean
s^{-1}	inverse seconds
SCCS	surface connecting canalicular system
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SERCA	sarco-endoplasmic reticulum calcium ATPase
SH2	src homology 2
SH3	src homology 3
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2(hydroxymethyl 1)-1,3-propandiol

v/v	volume in volume
vWD	von Willebrand's disease
vWf	von Willebrand factor
x g	gravities

CHAPTER 1:

INTRODUCTION

Chapter 1.

Introduction.

1.1 Historical Overview of Platelet Research.

The platelet is a specialised blood cell that plays a major role in the control of bleeding at sites of vascular injury and in the repair of damaged blood vessels. Platelets are the smallest of the known blood cells found in the human circulatory system. Platelets are generally capable of most cellular metabolic processes, but because they are derived from the fragmented cytoplasm of larger parent cells called megakaryocytes, (found in the bone marrow and the lungs), they do not possess a nucleus and therefore virtually lack protein synthesising ability (George, 1985; Heynes Adu, 1985; Holmsen, 1986). In their resting state, most circulating platelets are discoid in shape and have an average size of approximately 3µm in diameter and a mean thickness of around 1µm (Tangelder et al., 1989). In humans, the number of circulating platelets ranges between 150 and 400 x 10^9 per litre, and whilst the platelet count between individuals varies greatly, the number of circulating platelets within one individual is generally regulated within narrow limits (Heynes Adu, 1985).

Platelets were first identified by Donne in 1842, however at the time they were assumed to be artefacts of the preparation of blood specimens rather than a normal constituent of healthy blood, and consequently attracted very little scientific interest (Donne, 1842). It was not until the late 1800's that a physiological role for platelets was defined and they were described as the first blood elements to accrue at sites of vascular injury. A study published in 1882 by Giulio Bizzozero, (who is today accredited with the discovery of platelets), described for the first time the presence of platelets in blood and their role in thrombosis and coagulation. In his paper, Bizzozero published the first

observations that following incision of a vessel wall, bleeding was not arrested by coagulation of extravasated blood, but by the formation of a white thrombus at the site of the lesion (Bizzozero, 1882; de Gaetano, 2001; Donne, 1842). In the two decades following Bizzozero's publication, other investigators also reported a role for platelets in thrombus formation and coagulation at sites of vascular injury (Eberth, 1886; Hayem, 1882). In 1893, Krauss (1883) and later Hayem (1900), discovered that the number of platelets in the blood of children suffering from purpura haemorrhagica was severely decreased, thereby defining an important functional role for platelets in haemorrhagic (or bleeding) conditions, such as purpura. Whilst these landmark observations defined a physiological role for platelets, these blood cells remained neglected by researchers for many years following their initial discovery. The late 1950's and early 60's, saw a renewed interest in platelet function and revealed the critical role for platelets in initiating the early stages of thrombus formation. These studies demonstrated the unique ability of platelets to adhere to and aggregate at the site of vascular injury and arrest bleeding (Hughes, 1959; Lusher, 1960).

In 1961 it was demonstrated that it was the collagen component of connective tissue exposed during vascular injury that leads to platelet adhesion and aggregation "culminating in viscous metamorphosis" (Roskam, 1961). Subsequently the role of platelets in promoting the normal haemostatic and pathological thrombotic responses became an area of much interest and a number of *in-vitro* flow-based assays were developed to study the interaction of platelets with the vascular surfaces under physiological and pathophysiological flow conditions. Such studies demonstrated for the first time that patients presenting with various bleeding disorders, and prolonged bleeding times, often displayed defective platelet adhesion to the vascular subendothelium (Tschopp and Baumgartner, 1977; Tschopp et al., 1974; Weiss, 1975b). These studies also

demonstrated that rheological factors including the velocity of blood flow, vessel geometry and differences in viscosity due to the presence of other blood cells, could significantly influence the adhesion of platelets to the vessel wall (Baumgartner, 1973; Turitto and Baumgartner, 1974; Turitto and Baumgartner, 1975; Turitto, 1974; Virchow, 1856). Moreover, with the observation that von Willebrand's disease patients, who lack von Willebrand's factor (vWf), display significantly decreased platelet adhesion to the subendothelium, the critical role for vWf in mediating platelet adhesion to the subendothelium was recognised (Tschopp et al., 1974).

An improved understanding of the role of platelets in the formation of occlusive thrombi precipitating disease states such as acute myocardial infarctions and cerebrovascular accidents quickly developed. Studies by Baumgartner (1975) demonstrated that denudation of the vascular endothelium and exposure of the subendothelium to circulating platelets was not enough on it's own to induce the formation of occlusive thrombi. However, exposure of deeper more thrombogenic layers of the subendothelium enriched in collagen (van Zanten et al., 1994) resulting from atherosclerotic plaque rupture was a major factor contributing to the development of life-threatening thrombosis (Fuster and Chesebro, 1986).

More recent developments in the field of thrombosis and haemostasis have lead to a significant advance in our understanding of the important adhesive proteins, vWf, collagen and fibrinogen, (and their specific platelet adhesion receptors), in mediating the normal and pathological function of platelets under various physiological and pathophysiological flow conditions. These proteins appear to have distinct and complementary functions in the normal haemostatic process and support platelet adhesion by engaging one or more receptors on the platelet surface.

The introduction to this thesis presents an overview of the current knowledge of platelet functional responses at sites of vascular injury. Specifically, it will present the signalling mechanisms thought to regulate adhesion to the vessel wall and the relative contribution of the platelet adhesion receptors, GP Ib/V/IX, integrin $\alpha_{IIb}\beta_3$, integrin $\alpha_2\beta_1$, and GP VI, and their respective ligands (fibrinogen, vWf and collagen) to the haemostatic process.

1.2 Platelet Formation.

Platelets are produced by specialised parent cells called megakaryocytes. Megakaryocytes are large haematopoietic cells present in the bone marrow and in the lungs. Megakaryocytes produce their progeny, the platelets, using a unique mechanism of cytoplasmic fragmentation. As platelets are derived from the cytoplasm of megakaryocytes they are devoid of a nucleus (Holmsen, 1986) and usually lack ribosomes, yet they have a well defined cellular structure and possess numerous organelles that allow platelets to perform finely tuned functions in haemostasis and coagulation (Zucker-Franklin, 1996). During their maturation and development, megakaryocytes undergo a number of endomitoses during which their chromosomal DNA replicates several times to give megakaryocytes a ploidy of between 16N and 64N. This results in a significant enlargement of the nucleus and expansion of the cytoplasm, as well as an increase in the number of granules and mitochondria present within the cytoplasm, however the megakaryocyte cytoplasm does not divide (Zucker-Franklin, 1996). Instead, the cytoplasm of mature megakaryocytes fragments, and in so doing, gives rise to the megakaryocytic cell progeny, the platelet. Studies dating back to the early 1970's have suggested that at the end of their maturation, megakaryocytes residing in the bone marrow extend several processes that egress out of marrow sinusoids thus gaining access to the circulating blood. The membrane processes, filled with granules, microtubules and other structural features typical of platelets, eventually undergo fragmentation into the bloodstream to give rise to blood platelets (Tavassoli and Aoki, 1981).

1.2.1 Platelet Ultrastructure.

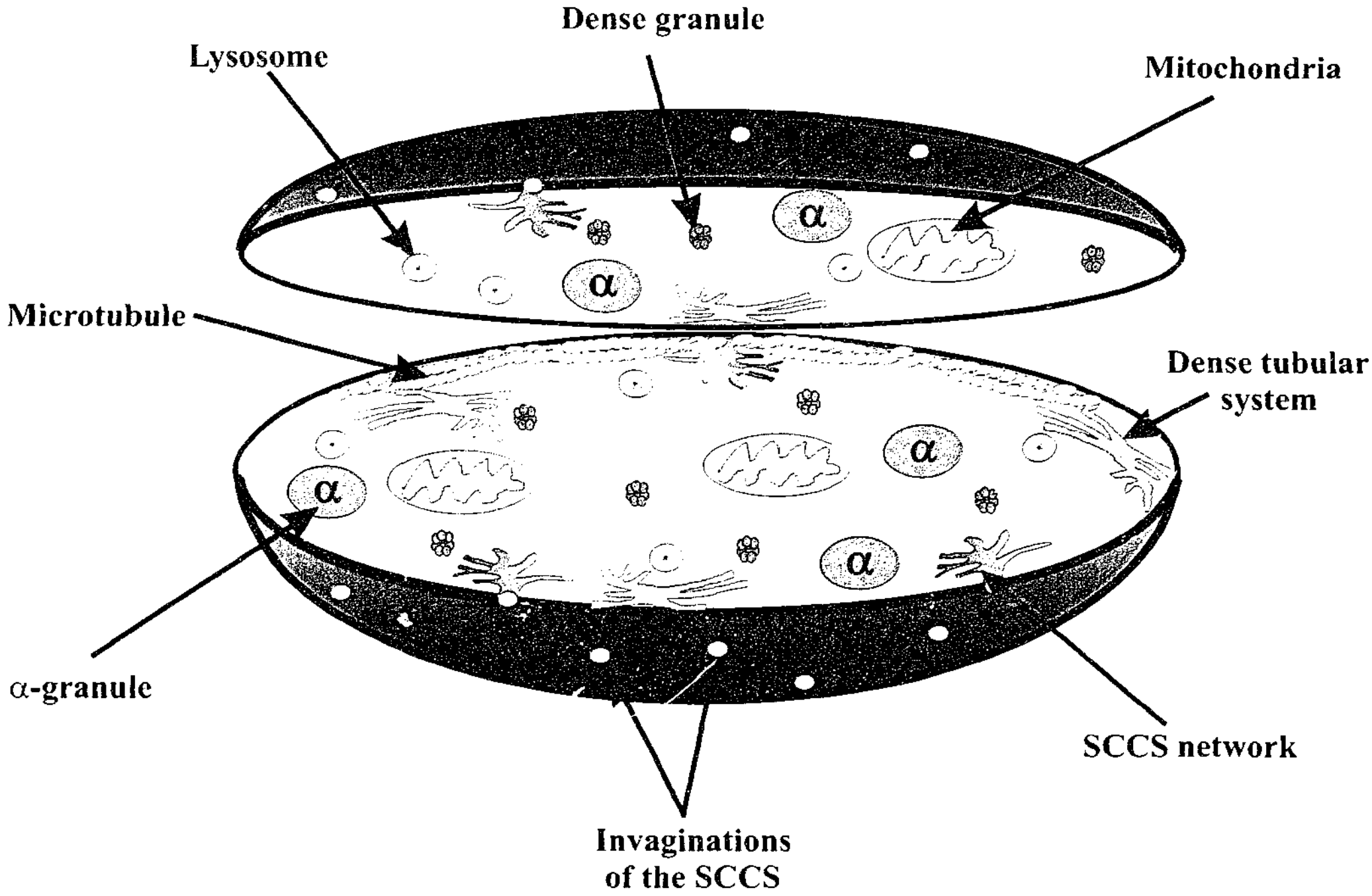
Resting platelets present themselves in the circulation as flat discoid shaped cells (White, 1981; White and Gerrard, 1982) (Fig 1.1). They are maintained in this discoid morphology by a cytoskeleton consisting of circumferential microtubules forming a tightly coiled bundle found just beneath the plasma membrane, and an underlying support of actin filaments (Sixma and Molenaar, 1966; White, 1987). Upon platelet activation there are dramatic alterations in the assembly of actin filaments and microtubules, which result in significant morphological changes.

Immediately beneath the periphery of the platelet plasma membrane lies an intricate system of endomembrane that forms a structure known as the surface connected canalicular system (SCCS). This structure is in fact a continuation of the platelet plasma membrane that invaginates into the cytoplasm to form a system of branching internal vesicles. The invaginations of the SCCS can be seen by electron microscopy as small openings on the platelet surface (Werner and Morgenstern, 1980). Two functions have been described for the SCCS. It is thought that the SCCS serves as a transport pathway for endocytic processes and for secretion of material from the platelet (holme, 1973; White and Clawson, 1980). It also forms an extensive reservoir of internal membrane that allows the dramatic increase in surface plasma membrane observed upon filopodia formation and spreading (Escolar et al., 1989).

Platelets also contain a second interconnected tubular network of narrow canuliculi known as the dense tubular system (DTS). The DTS is generally located in the centre of resting platelets and is not continuous with the plasma membrane. The many projections of the DTS come into close contact with every structural element within the platelet including the SCCS, dense granules, α-granules and the mitochondria (Werner and Morgenstern, 1980). This tubular system serves as a calcium storage organelle in the platelet and is able

Figure 1.1 Schematic representation of platelet ultrastructure (modified from Holmsen. 1986).

Platelets are maintained in their resting discoid morphology by a network of microtubules and an actin cytoskeleton lying directly beneath the plasma membrane (Note: the actin cytoskeleton is not shown in this schematic). Platelets also contain various intracellular granules and organelles including mitochondria, α-granules, dense granules and lysosomes, as well as two membrane complexes, the dense tubular system and the open canilicular system.


Dense granule
Lysosome
Mitochondria
α
α
Microtubule
Dense tubular system
α
α
α
α-granule
SCCS network
Invaginations of the SCCS

to regulate cytosolic calcium ion concentration in much the same way as the sarcoplasmic reticulum of nucleated cells (Cutler et al., 1978; Kaser-Glanzmann et al., 1977; White and Gerrard, 1976). The platelet dense tubular system has not only been implicated in the regulation of calcium flux, but has also been found to contain enzymes involved in prostaglandin (endoperoxide and thromboxane) synthesis (Gerrard and White, 1976; Gerrard et al., 1978; Kaser-Glanzmann et al., 1977).

Platelets have been found to contain a small number of mitochondria and various secretory organelles located centrally within the cytoplasm in an area called the organelle zone (White, 1987). Platelets contain three types of secretory granules, the α-granules, dense granules and lysosomes. The most prominent of the secretory organelles within the platelet are the α-granules (Stahl et al., 1978), which serve as a storage site for β-thromboglobulin, platelet factor 4, platelet-derived growth factor, Factor V and various adhesive proteins (fibrinogen, thombospondin, von Willebrand factor and fibronectin) (Kaplan et al., 1979). Dense granules are fewer in number and they contain adenine nucleotides (ADP and ATP), calcium and serotonin (Fukami and Salganicoff, 1977). Lysosomes contain hydrolytic enzymes and catalase-containing peroxisomes, however their physiological role remains unclear.

1.3 Platelet Physiology.

The process of haemostasis at sites of vascular injury can be divided into two distinct phases. The first phase, which is important for the immediate prevention of blood loss, is the formation of the primary haemostatic plug, mediated by adhesion and aggregation of platelets to the site of vascular injury. The second phase is the activation of the coagulation process, which culminates in the formation of a fibrin mesh that serves to reinforce the platelet plug.

Table 1.1 Platelet Adhesion Receptors
(Modified from Shattil *et al.*, 1994)

Receptor	Ligand	Recognition Sequence	Number/ Platelet
Integrin:			
$\alpha_{IIb}\beta_3$	fibrinogen fibronectin vitronectin vWf	RGD , dodecapeptide RGD RGD RGD	80,000
$\alpha_2\beta_1$	collagen	unknown	< 1,000
$\alpha_5\beta_1$	fibronectin	RGD	< 1,000
$\alpha_6\beta_1$	laminin	Not confined to single sequence	< 1,000
$\alpha_V\beta_3$	fibrinogen fibronectin vitronectin vWf	RGD RGD RGD RGD	250
Leucine-rich:			
GPIb/V/IX	vWf	Multiple recognition sequences	25,000
Selectin:			
P-selectin	CHO on white blood cells	Multiple recognition sequences	10,000
Immunoglobulin:			
GP VI	collagen	GPH	?
PECAM-1	PECAM-1 Glycosaminoglycans	Not confined to single sequence	5,000

RGD – Arginine-Glycine-Aspartic acid, vWf – von Willebrand factor, GPH – Glycine-Proline-Hydroxyproline, PECAM-1 – Platelet Endothelial Adhesion Molecule-1, CHO – sialylated and fucosylated carbohydrates

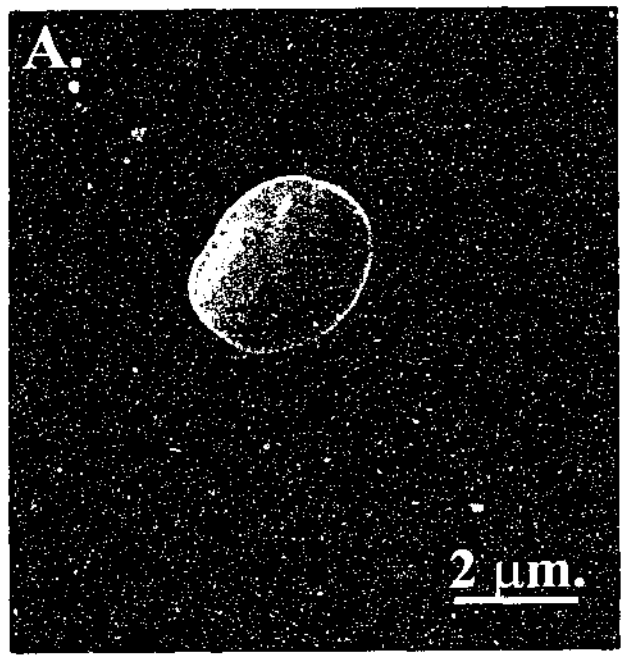
adhesion, spreading and aggregation is mediated by vWf binding to integrin $\alpha_{IIb}\beta_3$ (Savage et al., 1996).

Adhesion of platelets to exposed subendothelial matrix proteins, including vWf and collagen, results not only in rapid recruitment of platelets at the sites of injury, but also induces platelet activation. This is characterised by the dramatic conversion of platelets from resting discoid structures to spherical cells extending numerous membrane processes called filopodia (Allen et al., 1979; Mannucci and Sharp, 1967), giving them the appearance of spiny spheres. Activated platelets then form stable adhesion contacts with the matrix and ultimately extend lamellipodial sheets between the filopodial processes, which results in full platelet spreading thereby forming a platelet monolayer known as the pseudoendothelium (fig. 1.2) (Savage et al., 1992; Yuan et al., 1997). This platelet monolayer subsequently provides a reactive surface that recruits additional platelets from the circulation leading to the development of platelet aggregates, which form a primary haemostatic plug. Platelet adhesion is associated with the secretion of various platelet-activating factors such as adrenaline and ADP, and the generation of thromboxane A_2 (TXA_2), which participate in a positive feedback loop to further accelerate platelet activation (Holmsen and Weiss, 1979).

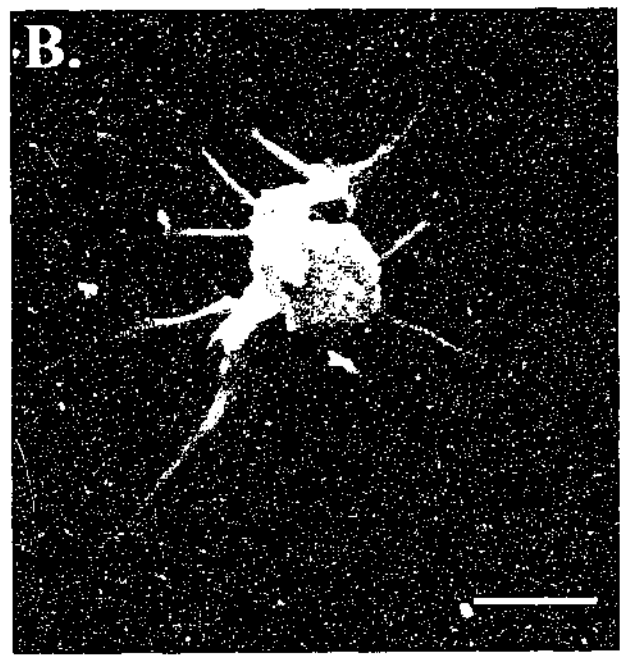
The same processes used by platelets during haemostatic plug formation are also involved in the development of pathological thrombi. Occlusive thrombi generally develop at sites of atherosclerotic plaque formation. Rupture of the plaques is believed to expose tissue factor and deeper more thrombogenic constituents of the subendothelial matrix to the circulation, leading to rapid platelet thrombus formation at the site of the lesion. It has been demonstrated that the likelihood of occlusive thrombus formation increases significantly as the level of stenosis increases (Falk, 1983; Forrester, 1991; Forrester et al., 1991; Maalej et al., 1998; Qiao and Fishbein, 1991). Occlusion of blood flow in the coronary and cerebral

Figure 1.2 Diagramatic representation of platelet shape change and spreading (modified from Hartwig and De Sisto, 1991; Hartwig 1992)

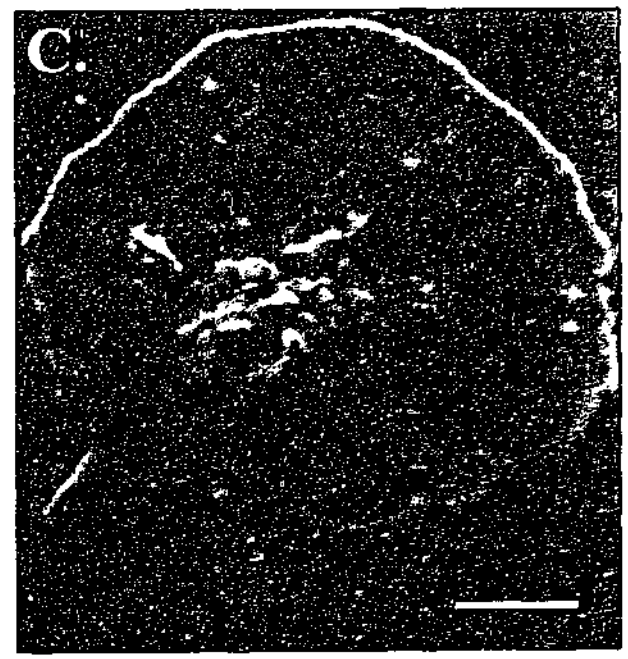
Scanning electron micrographs of the process of platelet spreading. In their resting state, platelets are found in a discoid morphology (a). Platelet adhesion and activation triggers platelet sphering, reorganisation of the actin cytoskeleton and bundling of actin polymers into membrane projections called filopodia (b). Further orthagonal actin elongation promotes the extension of lammelipodial sheets between the filopodia, which results in full platelet spreading (c). Scale bar = 2μm.



Resting discoid morphology



Filopodial Extension



Lamellipodial Formation

circulation results in a reduced oxygen supply to these tissues (Forrester, 1991; Verheugt and Brugada, 1991) leading to ischaemic injury precipitating myocardial infarction and stroke, respectively.

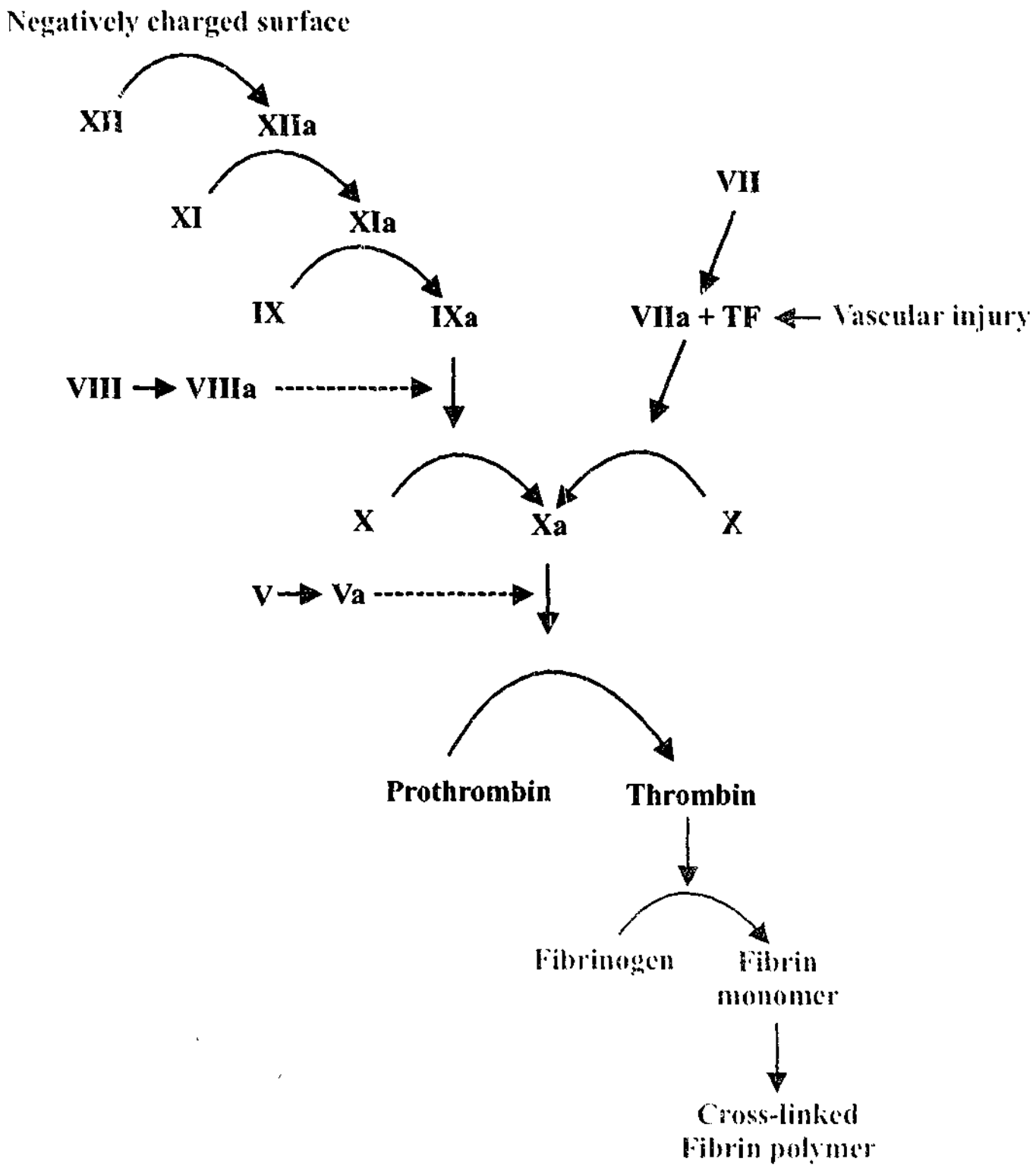
1.3.2 Blood Coagulation in Haemostasis.

Coagulation is a complex cascade of enzymatic reactions involving the conversion of numerous plasma coagulation proteins from their inactive precursor forms (zymogens), to their active enzymatic forms (Davie et al., 1991; Fiore and Deykin, 1994). The coagulation cascade can be triggered through two pathways; the intrinsic and extrinsic pathways (fig. 1.3). The extrinsic pathway, which is believed to be important physiologically, is initiated by the expression of tissue factor (factor III) on the surface of endothelial cells, smooth muscle cells and monocytes. The intrinsic pathway is initiated by the exposure of a clotting factor called factor XII, to negatively charged surfaces (such as glass or collagen), but the physiological relevance of this pathway is unclear (Collins et al., 1995; Holmsen, 1986). Both pathways ultimately lead to the activation of a common protein known as factor X. Conversion of factor X to its active form (factor Xa) then mediates the generation of thrombin at sites of vascular injury, through the cleavage of the inactive precursor, prothrombin (Bevers et al., 1987). Thrombin subsequently catalyses the conversion of soluble plasma fibrinogen to insoluble fibrin. The formation of a fibrin clot serves to stabilise and strengthen the primary haemostatic plug.

Coagulation is accelerated at sites of platelet deposition due to the procoagulant activity of platelets. The activation of factor Xa is facilitated by the assembly of an enzymatic complex at surface of active platelets called the tenase complex. This consists of calcium, factors VIIIa, IXa and X. Subsequent activation of prothrombin to thrombin by factor Xa is facilitated by yet another enzymatic complex that assembles at the platelet

Figure 1.3 The clotting cascade in humans.

Schematic representation of the clotting cascade illustrating the intrinsic and extrinsic pathways. The intrinsic pathway is activated by exposure of factor XII to negatively charged surfaces, such as glass or collagen, while the extrinsic pathway is activated by exposure of tissue factor (also known as factor III). The extrinsic pathway is the primary mechanism by which coagulation is initiated *in vivo*. The two pathways converge at the conversion of factor X to Xa (note: the active form of the clotting factors are designated by the letter –a). Factor Xa is responsible for the conversion of prothrombin to active thrombin, which is ultimately responsible for converting fibrinogen to fibrin.


Intrinsic pathway
Extrinsic pathway
Negatively charged surface
XII
XIIa
XI
XIa
IX
IXa
VIII
VIIIa
VII
VIIa + TF
Vascular injury
X
Xa
X
V
Va
Prothrombin
Thrombin
Fibrinogen
Fibrin monomer
Cross-linked Fibrin polymer

surface called the prothrombinase complex, which consists of calcium, factors Va, Xa and prothrombin. Active platelets express charged phospholipids such as platelet factor III (PF3) on their surface, which provides a favourable surface for the assembly of the tenase and prothrombinase complex, and for the conversion of prothrombin to thrombin by factor Xa (Bevers et al., 1987). Activated platelets also release other coagulation factors such as fibrinogen, factor V and vWf that further enhance the process of coagulation.

1.4 Adhesive Proteins.

Platelet adhesion and aggregation at sites of vessel damage is mediated by the interaction of multiple surface receptors with adhesive proteins present in the subendothelial matrix or in plasma. Though there are numerous proteins that platelets are capable of binding to, the substrates that appear to play the most important roles in platelet recruitment and activation at sites of vessel damage are vWf and collagen. Of the numerous proteins found circulating in the plasma, fibrinogen is known to be important for promoting platelet aggregation and reinforcing the primary haemostatic plug through the formation of a fibrin clot.

1.4.1 von Willebrand factor (vWf).

As mentioned above, vWf is arguably the most important protein required for the adhesion and aggregation of platelets at sites of vascular injury. In fact, lack of this adhesive protein, or expression of functionally defective vWf, leads to a severe bleeding disorder called von Willebrand's disease (vWD) (Ruggeri and Zimmerman, 1987). vWf is found within the vascular subendothelium, in Weible-Palade bodies of endothelial cells, in platelet α-granules and also in the circulation as a complex with coagulation factor VIII. At least two distinct roles have been described for vWf during normal haemostasis. The first

and most important is its ability to mediate platelet adhesion and aggregation at sites of injury under high shear conditions, through its interaction with the platelet surface receptor, GP Ib/V/IX. The second is its ability to act as a carrier for procoagulant factor VIII in the circulation (Weiss, 1975a). More recent studies of platelet adhesion and thrombus formation have revealed that vWf can also bind active integrin $\alpha_{IIb}\beta_3$ and plays an important role in supporting platelet aggregation and thrombus growth at high shear rates (Kulkarni et al., 2000; Ruggeri, 1999).

vWf is synthesised by megakaryocytes and endothelial cells as a 2,050 amino acid (250 kDa) monomer, however it is assembled into high molecular weight multimers ranging from dimers of 500 kDa up to 20,000 kDa in mass. Multimers are made up of subunits of vWf that are arranged in a repeating head-to-head, tail-to-tail, configuration that are linked together by disulfide bonds (Chopek et al., 1986; Marti et al., 1987). The vWf multimers that are synthesised by endothelial cells are stored in structures called Weible-Palade bodies, while vWf synthesised by megakaryocytes are stored in platelet α-granules. In general, the largest vWf multimers are found within the subendothelium (secreted from endothelial cells) or stored within platelets and endothelial cells. These larger vWf multimers are very effective in promoting platelet adhesion and aggregation (Meyer and Girma, 1993). On the other hand, plasma vWf multimers are characteristically smaller than subendothelial vWf, and are less efficient in promoting platelet adhesion (Dent et al., 1991).

Upon secretion into the blood stream the size of vWf multimers is reduced via the cleavage of large multimers by a plasma metalloprotease known as vWf cleaving protease or ADAMTS-13 (Fujikawa et al., 2001; Tsai, 1996; Zheng et al., 2001; Zheng et al., 2002). Patients who lack this protease have been shown to have excessive amounts of large vWf multimers and suffer from a condition called thrombotic thrombocytopenia purpura (TTP)

resulting from vWf-induced platelet clumping and microvascular thrombus formation (Moake et al., 1982).

The vWf molecule consists of a number of homologous domains through which it is able to interact with platelet adhesion receptors and with other subendothelial matrix proteins. There are three A-domains (A1-A3), two C-domains (C1-C2) and three D-domains (D1-D3) (fig. 1.3). The three A-domains are about 200 amino acid residues each, and have been implicated in a number of cell adhesion events (Colombatti and Bonaldo, 1991). The A1 and A3 domain, contain cysteine residues that participate in the formation of two intra-chain disulphide bridges. This causes the vWf molecule to fold back on itself and gives rise to large flexible loop structures in the A1 and A3 domains (fig. 1.4) (Marti et al., 1987). The A1 domain has been shown to perform a critical role in supporting platelet adhesion by mediating the interaction between vWf and GP Ib/V/IX receptors on the platelet surface. It contains three recognition sequences for the binding of GP Ibα (residues 474-488, 514-542 and 694-708) (Berndt et al., 1992; Mohri et al., 1988), however, it appears that this interaction requires a conformational change in the vWf molecule to expose these binding sites. This hypothesis is based on the fact that soluble vWf is unable to bind GP Ib/V/IX. Binding of artificial modulators, such as ristocetin and botrocetin, immobilisation onto subendothelial surfaces and pathological shear stresses are all believed to cause changes in the vWf molecule that enhance the affinity of vWf for GP Ib/V/IX (Howard et al., 1973; Scott et al., 1991; Sugimoto et al., 1991a; Sugimoto et al., 1991b; Weiss, 1995). It has also been shown that mutations occurring within the A1 domain of individuals with type II von Willebrand disease, result in spontaneous soluble vWf binding to platelet GP Ib/V/IX (Cooney et al., 1991; Randi et al., 1991; Ruggeri et al., 1980). Both the A1 and A3 domain have also been shown to contain binding sites for collagen, sulphatides and heparin (Mohri et al., 1989; Roth et al., 1986). The presence of these

Figure 1.4 The structure of von Willebrand factor (modified from Ruggeri and Ware, 1992; Meyer and Girma, 1993)

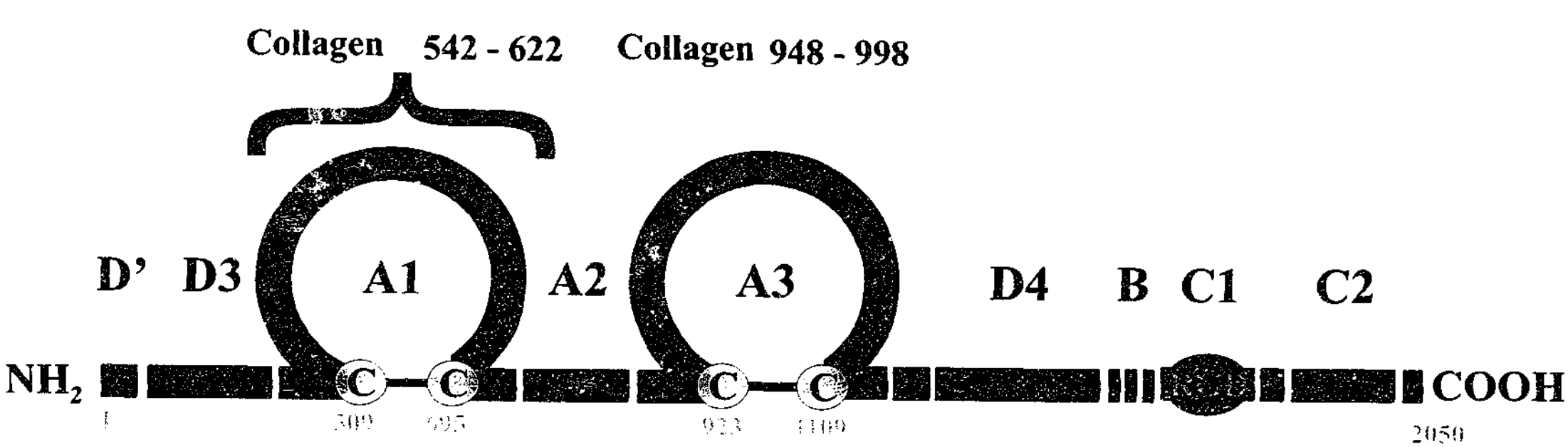
Schematic representation of a mature vWf molecule including its functional domains and the various subendothelial ligands and platelet receptors that it can potentially interact with (C-C, disulphide bonds).

GPIba 514 - 542

Heparin 565 - 587

Sulfatides 569 - 584
628 - 646

Botrocetin 539 - 553
569 - 583
629 - 643



Factor VIII
1 - 272

(GPIb)
Ristocetin
474 - 488
695 - 708

Integrin $\alpha_{IIb}\beta_3$
1744 - 1746

collagen-binding sites allows vWf to adsorb to the subendothelial matrix and mediate platelet recruitment to sites of vascular injury. The C1 domain of the vWf subunit is also important for platelet adhesion as it contains an Arg-Gly-Asp (RGD) motif at residues 1744 to 1746 of the carboxyl terminal C1 domain. This is a conserved recognition sequence for integrin binding and is critical for allowing platelet integrin $\alpha_{IIb}\beta_3$ to bind to vWf and mediate stable adhesion and aggregation.

1.4.2 Collagen.

Collagen has been recognised for many years as a key component of the subendothelium that determines the thrombogenicity of an injured vessel wall. It has been shown to play a major role in mediating stable platelet adhesion and induce platelet aggregation at sites of vascular injury. Up to 19 different types of collagens have been discovered to date and at least nine have been found to exist within the subendothelial matrix of human blood vessels (types I, III, IV, V, VI, VIII, XII, XIII, XIV) (Kehrel, 1995). Depending on their tertiary and quaternary structures, collagen proteins can present themselves as either monomeric or fibrillar forms. Particularly noted for their thrombogenicity are the fibrillar collagen types I and III which are present in the highest concentrations in the subendothelium and are able to induce both strong platelet adhesion and activation (Rauterberg et al., 1993).

All of the collagen proteins have similar molecular structures consisting of three α polypeptide chains that are arranged in a triple helical conformation, with each α chain containing repeating Glycine-Proline-Hydroxyproline (Gly-Pro-Hyp) sequences that serve to stabilise the triple helical structure (Morton et al., 1993). Synthetic peptides containing these Gly-Pro-Hyp repeats spontaneously arrange into a triple helix conformation are able

to support platelet adhesion and activation under static conditions (Morton et al., 1995), but cannot mediate stable adhesion under flow (Verkleij et al., 1998).

Platelets express at least four collagen receptors; GPVI, integrin $\alpha_2\beta_1$, GP IV and p65. The two most important and well-characterised collagen receptors are GP VI and $\alpha_2\beta_1$. For many years it was believed that integrin $\alpha_2\beta_1$ was the primary adhesive receptor for collagen (Morton et al., 1989; Santoro et al., 1991). This was highlighted by the fact that patients deficient in the α_2 integrin subunit display severely reduced platelet adhesion to collagen and that normal platelet adhesion to collagen under flow can be blocked using antibodies against $\alpha_2\beta_1$ (Nieuwenhuis et al., 1985). GP VI was believed to play a minor role in mediating platelet adhesion to collagen. Instead GP VI was thought to be responsible for collagen-induced signal transduction and platelet activation through its association with the FcRγ chain in platelets (Moroi et al., 1996; Nieswandt et al., 2000) (see section 1.8.3). This was highlighted by the finding that deletion of the FcRγ chain in mouse platelets resulted in a lack of GP VI expression and impaired activation in response to collagen stimulation (Nieswandt et al., 2000). More recent studies reported by Nieswandt et al., (2001), and Holtkotter (2002), suggest that GP VI is very important for promoting both platelet adhesion and activation on collagen.

Contrary to early reports suggesting a critical role for integrin $\alpha_2\beta_1$ in platelet adhesion to collagen, studies by Nieswandt et al., (2001), and Holtkotter (2002), have demonstrated that integrin $\alpha_2\beta_1$ does not play a central role platelet adhesion to collagen unless collagen binding to GP VI is impaired. These studies also suggested that integrin $\alpha_2\beta_1$ plays only a supportive role in promoting collagen induced platelet aggregation.

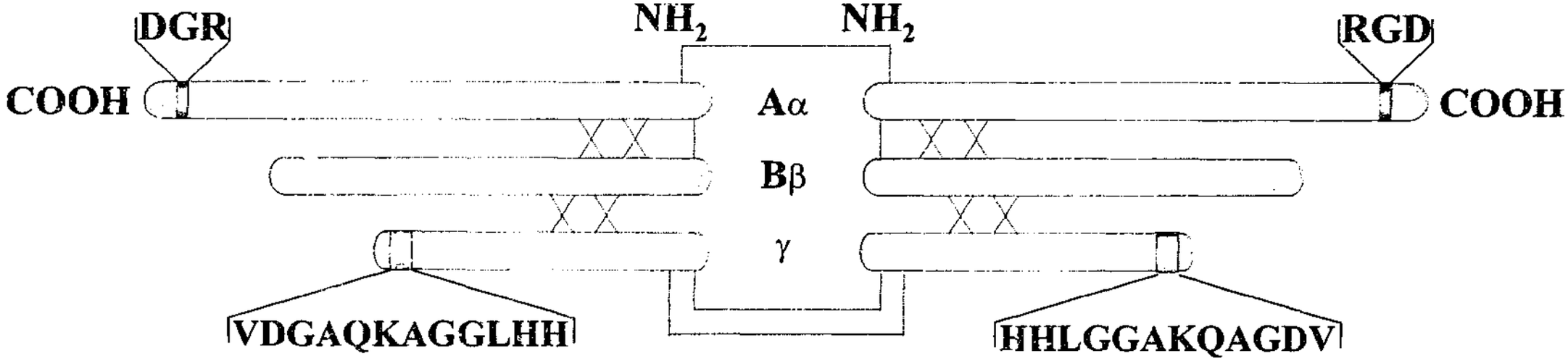
1.4.3 Fibrinogen.

Fibrinogen is a 340-kDa dimeric protein found in plasma that is critically involved in the process of platelet aggregation and clot stabilisation. Fibrinogen monomers are elongated 45 nm structures comprised of two sets of three polypeptide chains termed the Aα, Bβ, and γ polypeptide chains (Doolittle et al., 1979; Mosesson et al., 2001), that assemble in an anti-parallel fashion to form two outer D domains which are connected through a coiled-coil segment to a central E domain. The Aα chain contains 610 amino acid residues, the Bβ 461 and the γ chain contains 411 residues (Henschen, 1983). The N-terminal regions of the six polypeptides are joined to each other by disulfide bridges to form the E domain of the fibrinogen molecule. One pair of disulfide bonds are found at position Aα28, two pairs between positions Aα36 and Bβ65 and a third pair between the γ8 and γ9 positions (Blomback et al., 1976). The carboxy termini of the Bβ and γ polypeptides, and the central region of the Aα chains form the two distal D domains of the fibrinogen molecule (fig. 1.5).

The Aα chain of fibrinogen has two RGD containing sequences at position Aα 95-98 (RGDS) and position Aα 572-575 (RGDS), which are involved in mediating platelet adhesion through the integrin $\alpha_{IIb}\beta_3$ (Asakura et al., 1997; Dejana et al., 1985; Henschen, 1983). These two RGD sequences on the Aα chain appear to reside within a hidden region in the triple helical coiled-coil structure of the E domain. The C-terminus of the fibrinogen γ chain also contains a 12-residue sequence γ400-411, which is thought to be the major recognition sequence for integrin $\alpha_{IIb}\beta_3$ (Bennett and Vilaire, 1979; Farrell et al., 1992; Kloczewiak et al., 1983; Kloczewiak et al., 1984; Marguerie et al., 1979). It is believed that structural changes occur following integrin binding, or adsorption of fibrinogen onto

Figure 1.5 The structure of dimeric fibrinogen (modified from Ruggeri, 1993)

Schematic representation of a fibrinogen molecule showing the recognition sequences for integrin $\alpha_{IIb}\beta_3$ [RGDS and the dodecapaptide (HHLGGAKQAGDV)]. The fibrinogen monomer comprises of three chains, an Aα, Bβ and a γ chain linked to each other by intrachain disulphide bonds (broken lines). Linking of two monomers in an antiparallel fashion results in the formation of fibrinogen dimers.


DGR
NH2
NH2
RGD
COOH
Aα
COOH
Bβ
γ
VDGAQKAGGLHH
HHLGGAKQAGDV

surfaces such as glass or plastic, which can reveal the binding sites within the Aα and γ chains and allow integrin $\alpha_{IIb}\beta_3$-fibrinogen interactions to occur (Ugarova et al., 1993).

The importance of fibrinogen in mediating platelet aggregate and thrombus formation is highlighted by the fact that patients with a hereditary deficiency in fibrinogen, known as afibrinogenemia (Gugler and Luscher, 1965), suffer from a bleeding diathesis. It is thought that at low shear rates such as those typically found in the venous circulation, fibrinogen is the main ligand mediating platelet adhesion and aggregation. This concept is supported by studies demonstrating that aggregation in stirred platelet suspensions and adhesion to immobilised fibrinogen at low shear rates, is not inhibited by GP Ib/V/IX blocking antibodies (Phillips et al., 1991; Ruggeri, 1999). Recent studies by Ruggeri et al., (1999), have suggested that thrombi formed at high shear in the presence of vWf alone, are unstable, and that addition of fibrinogen to this system results in larger thrombi that are resistant to the destabilising effect of rapid blood flow. Similar finding have been shown *in vivo* using fibrinogen knockout mice in which developing thrombi were found to continuously detach from the subendothelium (Ni et al., 2000). Together these studies demonstrate that at high shear rates, fibrinogen acts in synergy with vWf to stabilise and strengthen developing thrombi, however it is unclear whether this effect is due to fibrinogen binding to integrin $\alpha_{IIb}\beta_3$, or due to the generation of a fibrin network.

1.5 Platelet Adhesion Receptors.

Platelets express several specific cell surface receptors, including members of the leucine-rich glycoprotein gene family, immunoglobulin gene family, integrins, and selectins (Table 1.1), that allow them to bind a multitude of subendothelial and plasma proteins.

1.5.1 Glycoprotein Ib/V/IX.

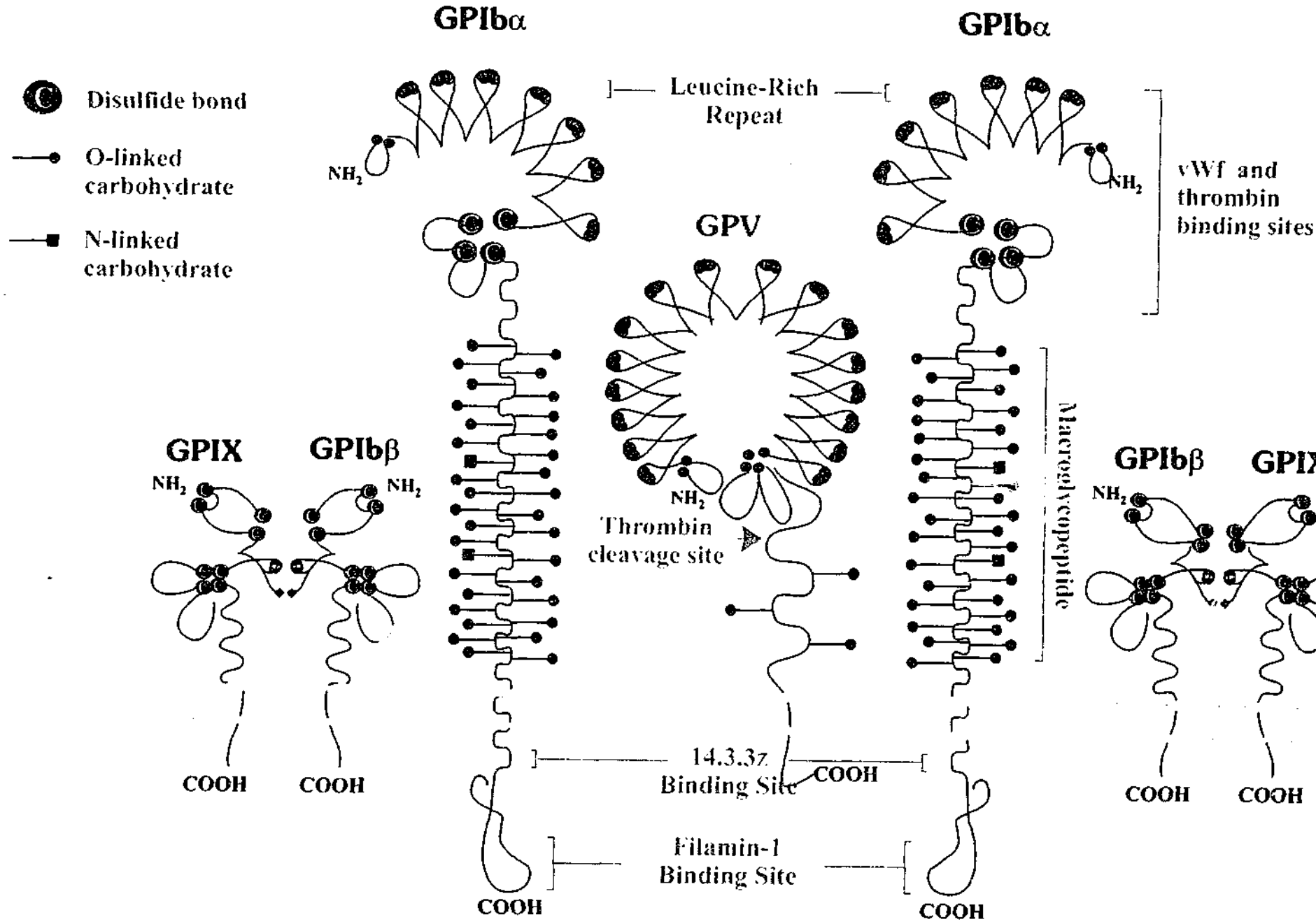
The vWf receptor complex, GP Ib/V/IX, is composed of four transmembrane subunits, GP Ibα, GP Ibβ, GP IX, GP V, (fig. 1.6) each of which is a member of the leucine-rich repeat superfamily (Lopez, 1994). The subunits of the GP Ib/V/IX complex are thought to combine in a 2:2:2:1 ratio, such that each receptor complex contains two copies of the GP Ibα protein, two of the GP Ibβ, two GP IX subunits and one GP V subunit. GP Ibα is disulfide linked to the GP Ibβ subunit via cysteine residues in the extracellular portion of the complex. This GP Ib complex is non-covalently associated with GP IX in a 1:1 ratio to produce a GP Ib/IX complex, and the GP V molecule links two GP Ib/IX complexes in a 1:2 ratio. GP Ibα is the most functionally important subunit of the GP Ib/V/IX complex as it contains the binding site for vWf (Handa et al., 1986; Harmon and Jamieson, 1986). The physiological role for GP Ibβ, GP IX and GP V subunits within the complex is not known.

The GP Ibα subunit is a 135 kDa glycoprotein that consists of several distinct structural domains. The extracellular portion of the subunit has a 300 residue N-terminal globular domain that contains seven leucine-repeats flanked by conserved N- and C-terminal flanking sequences. It is believed that one or more of the seven leucine repeat sequences contains binding sites for the vWf A1 domain, as well as a high affinity binding site for thrombin (De Marco et al., 1994; Gralnick et al., 1994; Jandrot-Perrus et al., 1992; Lopez et al., 1987; Shen et al., 2000; Titani et al., 1987). The globular amino terminus is followed by a long flexible rod-like stalk known as the macroglycopeptide domain that is rich in serine, threonine and proline residues and is highly O-glycosylated. Electron microscopy studies by Fox et al., (1988) have revealed that the macroglycopeptide domain of this subunit measures approximately 60 nm in length and allows the vWf binding domain within the globular amino terminus of GP Ibα to extend well above any other

Figure 1.6 The glycoprotein Ib/V/IX complex (modified from Lopez, 1994)

Diagram of the GP Ib/V/IX complex illustrating the functional and structural domains of the GP Ibα, GP Ibβ, GP V and GP IX subunits.

(NH_2, amino terminus; COOH, carboxy terminus; C-C, disulphide bond)


Disulfide bond
O-linked carbohydrate
N-linked carbohydrate
GPIbα
GPIbα
Leucine-Rich Repeat
NH2
NH2
vWf and thrombin binding sites
GPV
GPIX
GPIbβ
NH2
NH2
Thrombin cleavage site
Macroglycopeptide
GPIbβ
GPIX
NH2
NH2
14.3.3z Binding Site
COOH
COOH
COOH
COOH
Filamin-1 Binding Site
COOH
COOH
COOH

platelet surface receptor thus facilitating its interaction with exposed vWf (Fox et al., 1988). Directly following the macroglycopeptide domain, lying just above the plasma membrane, is a protease-sensitive domain. GP Ibα can be cleaved at the protease-sensitive domain *in vitro* by proteases such as calpain, trypsin, plasmin and elastase (Adelman et al., 1985; Berndt and Phillips, 1981a; Berndt and Phillips, 1981b; Brower et al., 1985; LaRosa et al., 1994; Michelson and Barnard, 1990; Wicki and Clemetson, 1985). Cleavage of GP Ibα at this site *in vivo* results in the release of a 120 kDa soluble fragment called glycocalicin (Clemetson et al., 1981). The significance of this cleavage *in vivo* is not clear, but it has been proposed to be a mechanism used by platelets to control the level of GP Ib/V/IX on the platelet surface, and hence limit platelet adhesion and thrombus formation.

The cytoplasmic tail of GP Ibα consists of 96 amino acid residues (Lopez et al., 1987) and it contains binding sites for actin binding protein and the signalling protein 14-3-3ζ (Andrews and Fox, 1991; Fox, 1985a; Fox, 1985b). The interaction with actin binding protein is thought to be important for maintaining the surface distribution of the complex in the platelet membrane (Dong et al., 1997), and to prevent membrane extraction under flow conditions (Williamson et al., 2002). The presence of binding sites for the signalling protein, 14-3-3ζ implies a role for GP Ibα in mediating signalling events following vWf engagement of GP Ib/V/IX (Andrews and Berndt, 1998; Calverley et al., 1998; Du et al., 1994).

The GP Ibβ subunit of GP Ib/V/IX contains similar structural features to GP Ibα. The amino-terminal region of the GP Ibβ subunit consists of a leucine-rich repeat flanked on either side by two disulphide loop structures. The GP Ibβ subunit is covalently linked to GP Ibα through a single cysteine residue immediately above the transmembrane region. Similar to the GP Ibα subunit, the carboxyl-terminal end of the GP Ibβ immediately following the transmembrane region also contains a distinct binding site for the signalling

protein 14-3-3ζ, implying a role for GP Ibβ during GP Ib/V/IX signal transduction (Andrews and Berndt, 1998; Calverley et al., 1998; Du et al., 1994). The amino-terminal region of the GP IX subunit of the GP Ib/V/IX complex is composed of a single leucine-rich repeat flanked again by two disulphide loops, followed by a transmembrane region and a short cytoplasmic region. GP V consists of a leucine-rich repeat region lying between two disulphide loops. This subunit has a cleavage site lying immediately above the transmembrane domain that is sensitive to cleavage by a range of proteases including endogenous platelet calpain.

Platelets express approximately 25,000 copies of the GP Ib/V/IX complex on their surface (Kroll et al., 1996). It is thought that in resting platelets, this receptor is evenly distributed across the platelet membrane. Due to its multimeric nature, vWf is able to bind numerous GP Ib/V/IX receptors. This is believed to induce clustering of the receptor complex, which has been proposed to facilitate signal transduction processes mediated by GP Ib/V/IX (Berndt et al., 1995; Clemetson, 1995; Clemetson and Clemetson, 1995). The clustering of GP Ib/V/IX is thought to increase the avidity of binding between GP Ib/V/IX and vWf, which may be critical for platelet adhesion to sites of vascular damage under high shear conditions (Berndt et al., 1995; Clemetson, 1995; Clemetson and Clemetson, 1995; Ruggeri, 1991).

1.5.2 Integrins.

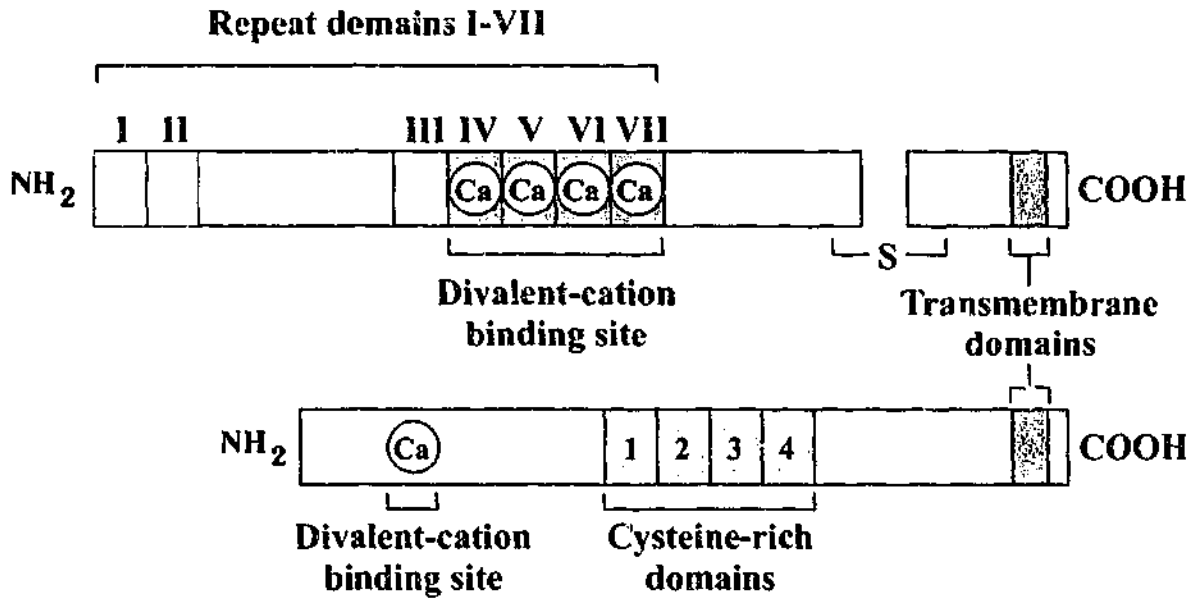
Integrins are a family of cell surface glycoproteins that play a major role in regulating cell adhesion to the extracellular matrix (ECM), and also in regulating cell-cell interactions. All integrins are transmembrane heterodimeric glycoproteins that are composed of one α and one β subunit (Hynes, 1987; Ruoslahti, 1991). To date, 16 individual α subunits and 8 β subunits have been identified, and at least 20 different

combinations of these α and β subunits have been described. The unique combination of α and β subunits defines individual integrin receptors and confers ligand-binding specificity to each integrin (Calvete, 1995; Hynes, 1992; Parise, 1989; Springer, 1990). Integrin receptors provide a physical link between the extracellular matrix (ECM) and intracellular cytoskeletal proteins. Integrins play an important function in cell signalling and the regulation of numerous cell processes such as cytokinesis, migration, cytoskeletal reorganisation, protein phosphorylation, calcium flux and gene expression (Calderwood et al., 2000; Coppolino and Dedhar, 2000; Hughes and Pfaff, 1998). Integrin α and β subunits consist of a large extracellular N-terminal domain, a transmembrane region and short cytoplasmic tail (fig. 1.7A). The amino-terminal extracellular region of the α subunit contains a stretch of seven homologous amino acid sequence repeats. It has been shown that repeats IV-VII contain sequence motifs that resemble those of the EF-hand structure responsible for the binding of divalent cations Ca^{2+} or Mg^{2+}, found in proteins such as calmodulin. These four calcium-binding domains are important for maintaining the integrity of the αβ dimers and for their interaction with ligands (Ruoslahti, 1991). Most integrins interact with their extracellular ligands though a common sequence called the Arg-Gly-Asp (RGD) motif, found in many ECM proteins (Humphries, 1996; Mould et al., 1996). Several different integrin receptors, including $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, are expressed on the platelet surface. The most abundant (80,000 copies per platelet), and most important integrin in terms of platelet haemostatic function is the fibrinogen and vWf receptor, integrin $\alpha_{IIb}\beta_3$ (Payrastre et al., 2000).

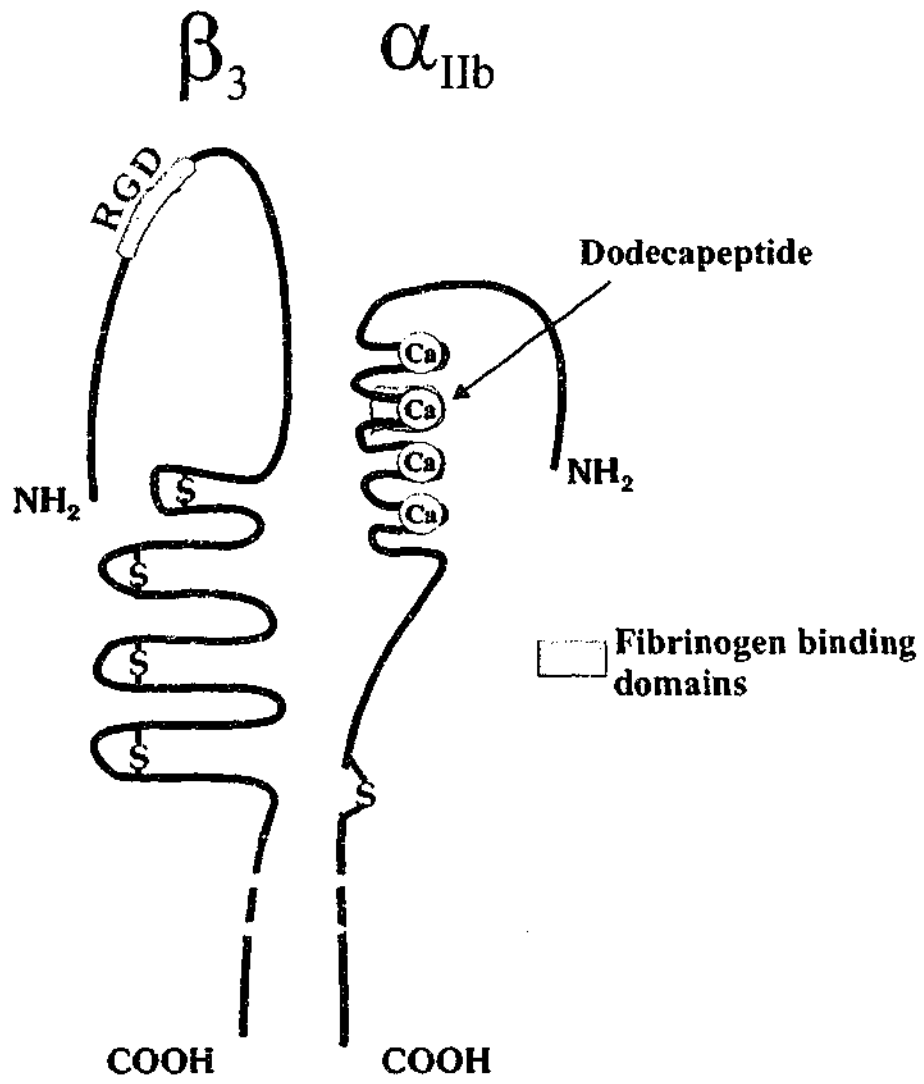
Figure 1.7 Structure of integrin $\alpha_{IIb}\beta_3$ (modified from Phillips et al., 1991; Kuhn and Eble, 1994; Leftkovitz et al., 1995)

Schematic representation depicting the functional domains of the α– and β– subunits of integrin receptors (a). Diagram of integrin $\alpha_{IIb}\beta_3$ structure including the fibrinogen binding domains of the receptor (b).

a.


α-subunit
Repeat demains I-VII
I II III IV V VI VII
NH2
Ca Ca Ca Ca
COOH
S
Divalent-cation binding site
Transmembrane domains
NH2
Ca
1 2 3 4
COOH
Divalent-cation binding site
Cysteine-rich domains
β-subunit

b.


β3
αIIb
RGD
Dodecapeptide
Ca
Ca
Ca
Ca
NH2
NH2
S
S
S
S
Fibrinogen binding domains
COOH
COOH

1.5.2.1 Integrin $\alpha_{IIb}\beta_3$.

Integrin $\alpha_{IIb}\beta_3$ is important for promoting stable platelet adhesion, spreading, aggregation and clot retraction at sites of vascular damage. In resting platelets, integrin $\alpha_{IIb}\beta_3$ does not bind soluble ligands, but upon platelet activation conformational changes occur in the integrin $\alpha_{IIb}\beta_3$ molecule that allow it to bind a number of adhesive ligands including vWf, fibrinogen, fibronectin, and vitronectin. Integrin $\alpha_{IIb}\beta_3$ binds to at least two recognition sequences, the common integrin binding motif, RGD, that is present within all of the integrin $\alpha_{IIb}\beta_3$ ligands (Ruoslahti and Pierschbacher, 1986), and also to the dodecapeptide sequence located within the carboxy-terminus of the fibrinogen γ-chain (fig. 1.7b) (Kloczewiak et al., 1984). The integrin $\alpha_{IIb}\beta_3$, also known as the GP IIb/IIIa receptor, consists of an α_{IIb} (GP IIb) and a β_3 (GP IIIa) subunit. The α_{IIb} subunit is a 140-kDa protein that consists of an extracellular heavy chain, which is disulfide bonded to a light chain that comprises of three domains; a short extracellular N-terminal domain, a transmembrane region and a short intracellular C-terminal domain (fig. 1.7b). The β_3 subunit is a single polypeptide of 105-kDa, which also has an extracellular region, a transmembrane domain and a short cytoplasmic tail (Naik and Parise, 1997). Membrane proximal cytoplasmic sequences of the α (KxGFFKR) and β (LLVXIHDR) subunits, are thought to participate in the formation of a salt bridge that maintains the receptor in a default inactive conformation in the resting platelet (Hughes et al., 1996). It is believed that upon platelet activation, intracellular signalling events induce disruption of the salt bridge between the α_{IIb} and β_3 subunits. This results in a conformational change that converts the integrin $\alpha_{IIb}\beta_3$ into a high affinity state capable of binding soluble or immobilised ligands, and mediating platelet spreading and aggregation. The precise molecular mechanisms that induce disruption of this salt-bridge are still unknown (Coppolino and Dedhar, 2000;

Hughes et al., 1996; Sims et al., 1991). Activation of integrin $\alpha_{IIb}\beta_3$, and subsequent ligand binding, induces clustering of the receptor and attachment to the platelet cytoskeleton. Activation also results in the recruitment of a number of structural and signalling proteins including; $pp60^{c\text{-}src}$, $pp125^{FAK}$ and PI 3-kinase to the cytoskeleton giving rise to integrin signalling complexes (Calvete, 1994; Fox, 1993; Fox et al., 1993; Grondin et al., 1991).

1.5.2.2 Other Integrin Receptors.

Platelets have the ability to adhere to several of the proteins found in the subendothelial matrix due to the expression of a large number of integrin receptors on their surface, including those that bind to collagen ($\alpha_2\beta_1$), fibronectin ($\alpha_5\beta_1$), laminin ($\alpha_6\beta_1$) and vitronectin ($\alpha_v\beta_3$). Like integrin $\alpha_{IIb}\beta_3$, all of these integrin receptors are typical $\alpha\beta$ dimers but the different combination of α and β subunits results in varied degrees of ligand specificity. Most of these integrins, such as $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$, bind to the typical integrin-binding motif (RGD), contained in a variety of adhesive ligands including fibrinogen, vWf, vitronectin and fibronectin. In contrast, the $\alpha_2\beta_1$ and $\alpha_6\beta_1$ receptors specifically bind to collagen and laminin respectively and do not recognise the RGD motif. Notwithstanding the large number of integrin receptors expressed by platelets and the various adhesive proteins found in the subendothelium, integrin $\alpha_{IIb}\beta_3$ is absolutely crucial for maintaining the normal haemostatic function of platelets (Lerea et al., 1999; Nieuwenhuis et al., 1986; Weiss et al., 1989; Weiss et al., 1993).

1.6 Platelet Adhesion Under Flow.

The association of platelet surface receptors with the various adhesive proteins present in plasma and the vascular subendothelium is crucial for allowing platelet adhesion to the damaged vessel wall. Under physiological conditions, these adhesive interactions

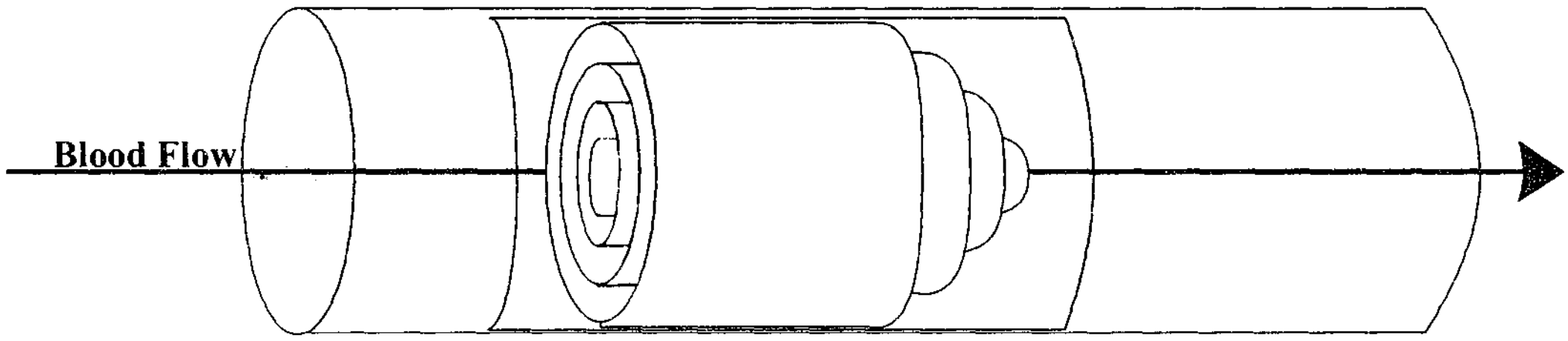
occur in the presence of fluid shear stresses generated by local blood flow conditions and are influenced by alterations in blood constituents, changes in the local blood flow conditions and changes in the vessel wall itself.

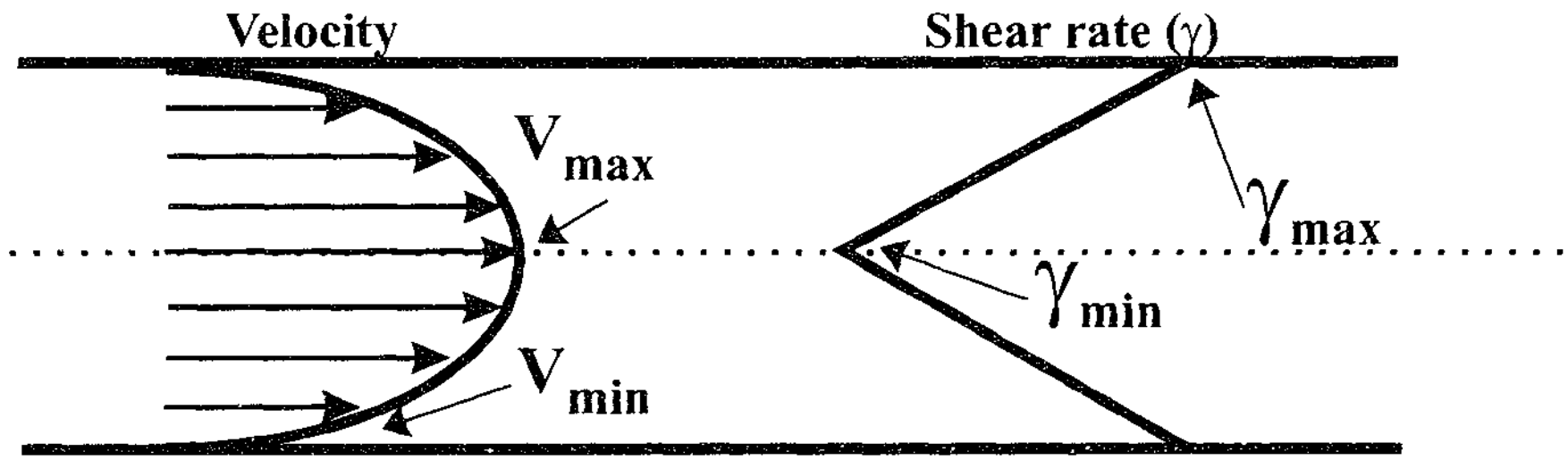
1.6.1 Effect of Blood Flow on Platelet Function.

Throughout the circulation, both the blood and the blood vessels are subjected to a variety of mechanical forces that have a significant influence on the process of thrombosis and haemostasis. Pressure changes within the vessel lumen due to the pumping of blood through the vessels at different velocities, and transmural pressure changes that cause the blood vessel to expand or contract during the cardiac cycle, generate mechanical forces known as shear stress and tensile stress. The flow of a viscous fluid, such as blood, through a vessel, creates a force per unit area known as fluid shear stress that is measured in dynes per square centimetre. Shear forces arise in the circulation as a result of differential blood flow velocities across the vessel diameter. The velocity of blood flow is generally greatest at the centre of the vessel and tends to decrease progressively as the vessel wall is approached because of the viscous drag exerted by the vessel wall on the flowing blood (Bird, 1960; Kroll et al., 1996; Lowe, 1999). This results in a parabolic velocity profile across the vessel lumen which creates a shearing effect between adjacent layers of fluid moving across each other at different speeds. The greatest shearing effect occurs at the vessel wall, which is stationary with respect to flowing blood, and decreases to zero at the centre of the vessel where blood flow is fastest (fig. 1.8). Poiseuille law relates the flow of a simple fluid through a straight tube to the flow rate along the tube, the radius of the tube and the viscosity of the fluid (Lowe, 1999). The wall shear rate is measured in cm/s per cm or s^{-1} and can be derived using the following equation:

Figure 1.8 Schematic representation of blood flow velocity profile through a cylindrical vessel (modified from Goldsmith and Turitto, 1986; Kroll et al., 1996)

The flowing of a Newtonian fluid through a vessel resembles a series of concentric cylinders flowing across each other at different velocities (a). The layer in contact with the vessel wall is at rest and the velocity of each adjacent layer increase to a maximum velocity at the centre of the vessel. This creates a parabolic velocity profile with maximum velocity (V_{max}) and minimal shear (γ_{min}) at the centre of the vessel (b). The differential flow velocities across the vessel are responsible for generating shearing forces in blood vessels.


Blood Flow


Velocity
Shear rate (γ)
V max
V min
γ max
γ min

$$\text{Wall shear rate } (\gamma) = \frac{4.Q}{\pi.R^3}$$

where Q is the flow rate (ml/sec) and R is the radius of the vessel (mm).

Fluid shear stress generated by the blood flow exerts tensile stress on cells adherent to the vessel wall, this is known as wall shear stress (τ). If we assume that the viscosity of blood remains constant with respect to changes in shear rate, that is, if blood is assumed to be a Newtonian fluid, then the shear stress (measured in Pascals-Pa) is directly proportional to the shear rate and the viscosity of blood:

$$\text{wall shear stress } (\tau) = \eta.\gamma$$

where (η) is the blood viscosity (Pa.s) and γ is the wall shear rate (s^{-1}).

Since blood cells experience a variety of shear forces, the ability of these cells to adhere to the vessel wall depends on the efficiency of bond formation between adhesive proteins and their receptors, and also on the ability of those bonds to resist the mechanical forces generated by flowing blood. Platelets are required to adhere and aggregate at sites of vascular injury over a broad range of shear rates (from 150 s^{-1} up to 10,000 s^{-1}) (table 1.2). It is important therefore, that platelets are equipped with appropriate adhesive mechanisms for the various shear environments encountered in the circulation.

1.6.2 Effect of Blood Constituents on Platelet Function.

A number of factors are known to influence shear forces in the circulation, one of which is the viscosity of the blood which can vary depending on plasma protein concentration, red blood cell number and red blood cell deformability (Blann and Lip, 2001; Lowe, 1999; Pearson, 1997). Calculations of shear forces in the vasculature are

Table 1.2 Typical Ranges of Wall Shear Rates & Stresses Found in the Vasculature* (Modified from Kroll *et al.*, 1996)

Blood Vessel	Wall Shear Rate (s^{-1})	Wall Shear Stress (Pa)
Veins	20 – 200	0.076 – 0.76
Large Arteries	300 – 800	1.14 – 3.04
Arterioles	500 – 1,600	1.9 – 6.08
Stenotic Vessels	800 – 10,000	3.04 – 38.0

* Wall shear stresses are calculated on the assumption that the viscosity of whole blood is 0.038 Poise.

usually based on the assumption that blood behaves as a "Newtonian fluid" whose viscosity is constant throughout the vasculature and between individuals. However, *in vitro* studies of blood viscosity have revealed that blood is in fact, a non-Newtonian fluid that behaves very differently depending on the shear environment.

Blood is essentially a highly concentrated suspension of cells (approximately 45% red blood cells by volume) and proteins (60 – 80 mg/ml). The concentration of red blood cells (RBC) in the blood (haematocrit) is thought to have the greatest influence on blood viscosity and platelet adhesion. Platelets flowing through blood vessels in the absence of RBC's tend to stream towards the centre of the vessel lumen where the flow velocity is greatest, and do not interact efficiently with the vessel wall. With increasing haematocrit levels, a rise in blood viscosity and RBC collisions result in an outward displacement of the small and relatively rigid platelets towards the vessel wall. The RBC's on the other hand, migrate away from the vessel wall towards the centre of the vessel. This inwards displacement of RBC's is aided by their ability to deform and squeeze past each other (Chien et al., 1970).

1.6.3 Effect of the Blood Vessel on Platelet Function.

As discussed in section 1.2.1, in the normal healthy vasculature, platelets interact passively with the vessel wall because of an endothelial cell layer that functions as a non-thrombogenic barrier between the circulating platelets and the underlying subendothelium (Gryglewski et al., 1976; Schror, 1991; Yurchenco, 1990; Yurchenco and Schittny, 1990). At sites of vascular injury where the endothelial cell layer is breached, a number of highly reactive subendothelial matrix structures become exposed, resulting in rapid platelet adhesion and aggregation. The subendothelium consists of a number of highly thrombogenic matrix proteins that associate to form distinct structures. The region

immediately underlying the endothelial cell layer is called the basement membrane and is rich in type IV collagen and laminin (Yurchenco, 1990; Yurchenco and Schittny, 1990). The collagen and laminin proteins of the basement membrane form a thin network interconnected by the sulphated glycoprotein entacten/nidogen. Heparin sulphate proteoglycan complexes are also found anchored to the collagen/laminin networks. Beneath the basement membrane lies a structure called the strima. The strima is perhaps the most platelet reactive structure in the subendothelium as it consists of several, highly thrombogenic proteins including collagen types I and III fibres, multimeric vWf, elastin, fibronectin, vitronectin and thrombospondin.

1.7 Platelet Adhesion and Aggregation Under Flow.

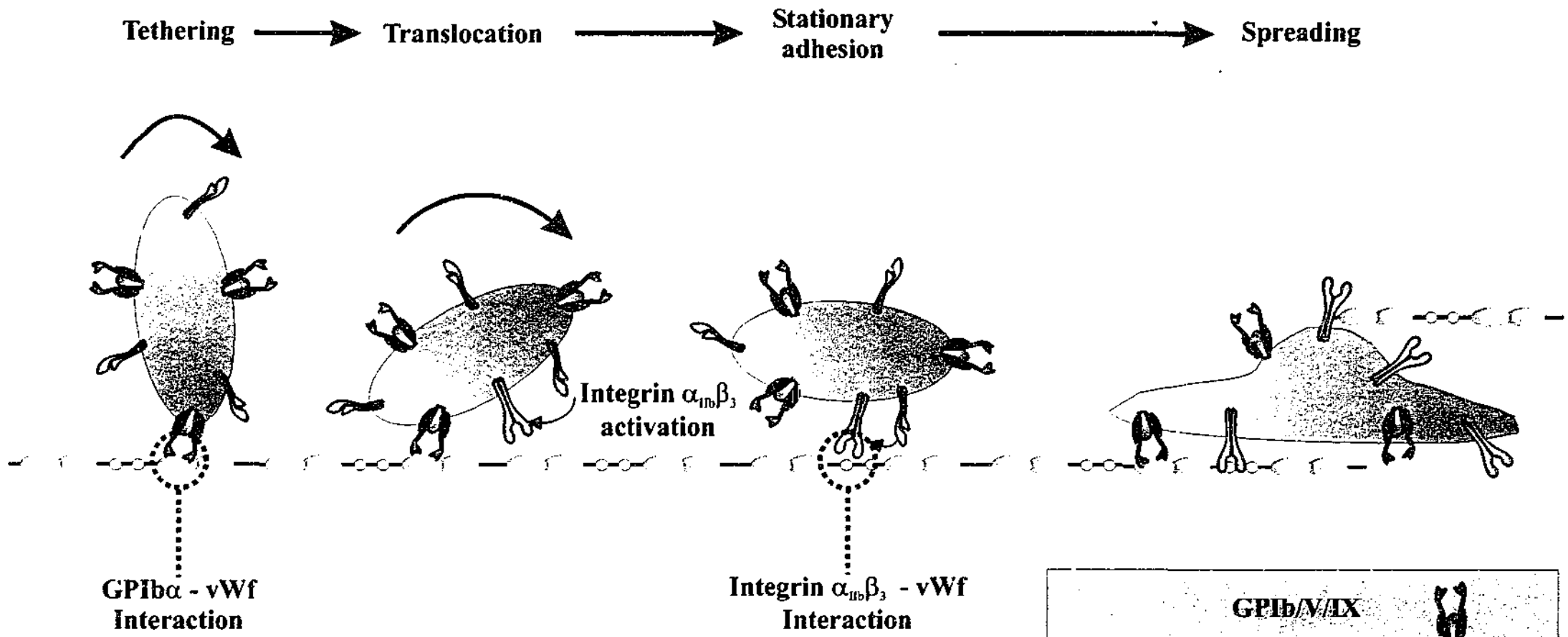
Platelet adhesion to the vessel wall under the flow conditions encountered in the circulation occurs through a multi-step process involving several platelet adhesion receptors and subendothelial matrix proteins (fig. 1.9) (Savage et al., 1996). The first step in this process is mediated by GP Ib/V/IX binding to vWf, which allows initial platelet recruitment and translocation at the site of vascular injury. This is followed by a phase of irreversible adhesion mediated by one or more platelet integrins, and a third phase of platelet-platelet adhesion (aggregation) that is critically dependent on the activation of the major platelet integrin $\alpha_{IIb}\beta_3$.

1.7.1 Platelet Capture or Tethering.

The initial tethering of platelets to exposed subendothelium under high shear conditions is mediated by the interaction between vWf and GP Ib/V/IX (Andrews et al., 1997; Lopez, 1994; Lopez and Dong, 1997; Savage et al., 1996; Weiss, 1995). This interaction is characterised by rapid association and dissociation rates, and high tensile

Figure 1.9 The mechanism of platelet adhesion and spreading on immobilised vWf under flow (modified from Ruggeri, 1997)

Schematic representation of the multistep mechanism promoting tethering and stable adhesion of circulating platelets to vWf under high shear. The initial tethering of circulating platelets to immobilised vWf is mediated through GP Ib/V/IX binding to the AI domain of vWf. This interaction is characterised by a high mechanical strength as well as fast association and dissociation rates making the GPIb-vWf binding resistant to high shear and allowing platelets to roll across the vWf surface (translocation). Engagement of the GP Ib/V/IX receptor also results in intracellular signal transduction leading to the activation of integrin $\alpha_{IIb}\beta_3$. Integrin $\alpha_{IIb}\beta_3$ activation then supports the formation of stationary adhesion contacts and platelet spreading.


Tethering
Translocation
Stationary adhesion
Spreading
Integrin $\alpha_{IIb}\beta_3$ activation
GPIbα - vWf Interaction
Integrin $\alpha_{IIb}\beta_3$ - vWf Interaction
GPIb/V/IX
von Willebrand Factor
Inactive $\alpha_{IIb}\beta_3$
Active $\alpha_{IIb}\beta_3$

strength. The rapid association rate allows binding to occur efficiently even under high shear conditions, and the rapid dissociation rate makes the binding reversible. The high tensile strength of this interaction makes tethered platelets inherently resistant to detachment even under high shear conditions such as those present in arterioles and stenosed arteries. As a result, platelets that tether to subendothelial vWf through GP Ib/V/IX tend to translocate or roll on the matrix in the direction of blood flow (Savage et al., 1998; Savage et al., 1996). The ability of the GP Ib/V/IX-vWf interaction to resist the effects of high shear stresses can be attributed to the high density of GP Ib/V/IX on the platelet surface and the multivalent nature of vWf (Ruggeri, 1993b; Ruggeri et al., 1983; Ruggeri and Ware, 1993). vWf binding to GP Ib/V/IX also results in signal transduction events that induce platelet activation and morphological changes in these cells. Consequently, upon adhesion to vWf, platelets rapidly transform from disc shaped cells to spherical cells extending multiple filopodia, a morphology that may facilitate platelet rolling (Yuan et al., 1999).

1.7.2 Integrin-Mediated Arrest.

Platelet tethering to vWf serves to decrease their velocity with respect to flowing blood, allowing other receptors with relatively slow binding kinetics such as integrins, to interact with subendothelial matrix proteins and stabilise adhesion. As opposed to the reversible interaction between GP Ib/V/IX and vWf, integrin receptors are able to mediate irreversible adhesion due to their characteristically slow rate of bond dissociation. The two major integrin receptors that promote stationary platelet adhesion to the subendothelium are integrin $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ (Ni et al., 2001; Savage et al., 1998).

In resting platelets, integrin $\alpha_{IIb}\beta_3$ adopts a resting conformation, unable to bind vWf. During platelet translocation *in vitro*, the binding of vWf to GP Ib/V/IX triggers

intracellular signalling events that lead to the activation of integrin $\alpha_{IIb}\beta_3$, enabling it to recognise the RGD motif found within the C1 domain of the vWf molecule and to mediate irreversible adhesion and spreading (fig. 1.9) (Kroll et al., 1991). This process is believed to be potentiated *in vivo* by the synergistic effects of soluble platelet agonists (ADP, thrombin and epinephrine) generated at the site of injury, and to the presence of other highly thrombogenic subendothelial components such as collagen (Baumgartner, 1974; Baumgartner, 1977b; Baumgartner et al., 1977; Kroll and Schafer, 1989).

Unlike $\alpha_{IIb}\beta_3$, for many years integrin $\alpha_2\beta_1$ was not believed to require activation in order to bind to immobilised collagen following platelet tethering. However, recent studies have shown that integrin $\alpha_2\beta_1$ can become activated in response to soluble agonist stimulation (Jung and Moroi, 1998; Jung and Moroi, 2000a; Jung and Moroi, 2000b). These studies demonstrate that $\alpha_2\beta_1$ can be converted from a resting state to at least two active conformations capable of binding soluble collagen with increasing affinities. Platelet adhesion to collagen through integrin $\alpha_2\beta_1$ allows a second collagen receptor, GP VI to bind to the triple helical repeat sequences (Gly-Pro-Hyp) within the collagen molecule (Morton et al., 1995). While $\alpha_2\beta_1$ is responsible for platelet adhesion to collagen, collagen induced platelet activation is primarily mediated by GP VI (Watson et al., 2001). GP VI binding to collagen induces a signalling pathway that is dependent on dimerisation and phosphorylation of the FcR γ-chain that ultimately leads to integrin $\alpha_{IIb}\beta_3$ activation (Gibbins et al., 1997; Inoue et al., 1999; Nakamura et al., 1999; Tsuji et al., 1997).

1.7.3 Aggregation and Thrombus Formation.

The conversion of integrin $\alpha_{IIb}\beta_3$ into a high affinity conformation is essential for platelet aggregation and thrombus formation. Much of our current understanding of platelet aggregate formation and thrombus growth has come from suspension based aggregation

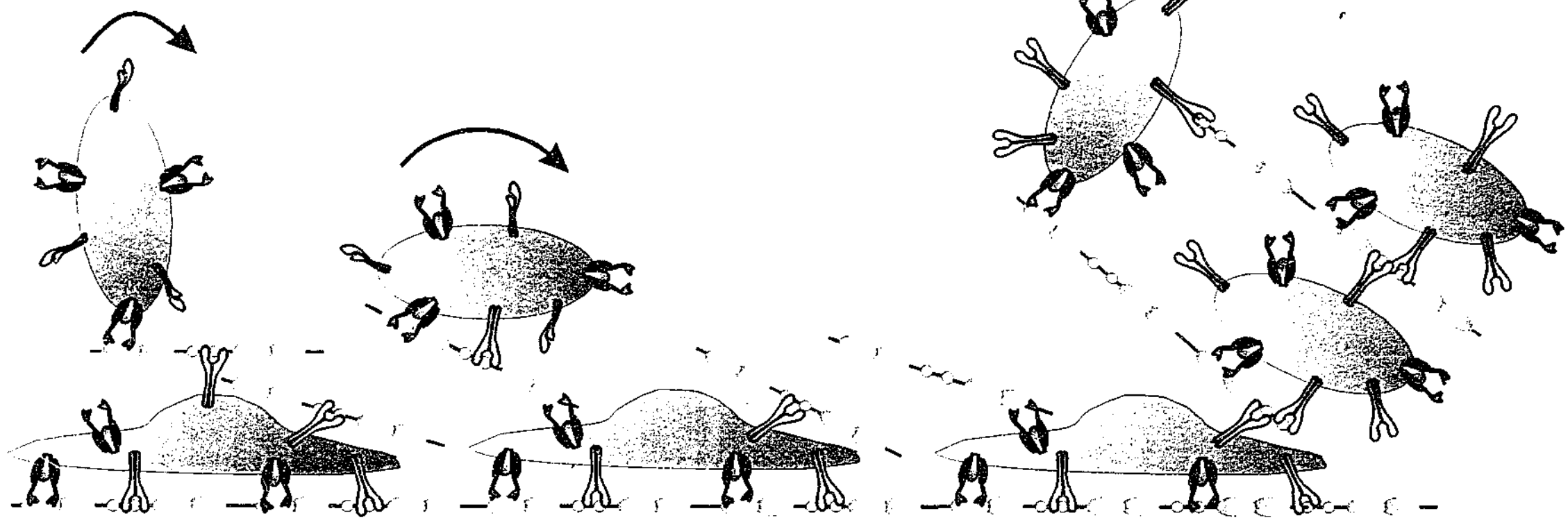
studies performed at low shear and in the presence of excess soluble platelet agonists. These studies demonstrated that platelet aggregation is mediated by the binding of fibrinogen to activated integrin $\alpha_{IIb}\beta_3$ receptors expressed on adherent platelets following platelet activation with soluble agonists (Holmsen 1986; Lefkovits et al., 1995). Later studies using suspension based, cone and plate viscometer assays, revealed that vWf binding sequentially to GP Ib/V/IX and integrin $\alpha_{IIb}\beta_3$, can also support aggregate formation. The binding of integrin $\alpha_{IIb}\beta_3$ to vWf and subsequent platelet aggregation was believed to occur only at abnormally high shear conditions of over 5000 s^{-1} generated by the cone and plate viscometer (Goto et al., 1998; Ikeda et al., 1991; Peterson et al., 1987). Recent studies examining thrombus growth using *in vivo* and *in vitro* thrombosis assays, have demonstrated that vWf can also support thrombus growth on immobilised adhesive substrates (Kulkarni et al., 2000; Ni et al., 2000; Ruggeri, 1999; Ruggeri et al., 1999). These studies have shown that circulating platelets interacting with a developing thrombus initially tether and translocate over the thrombus surface through GP Ib/V/IX binding to vWf which is expressed on the thrombus surface following α-granule release (fig. 1.10) (Kulkarni et al., 2000). Subsequent irreversible platelet adhesion and aggregation is mediated by active integrin $\alpha_{IIb}\beta_3$ binding to the RGD motif within the vWf molecule (Ni et al., 2000; Ruggeri, 1993b; Ruggeri, 1999; Ruggeri et al., 1999).

1.8 Signalling Processes Regulating Platelet Adhesion and Activation.

Activation of platelet integrin $\alpha_{IIb}\beta_3$ is a crucial functional response required for normal platelet adhesion, spreading and aggregation. Integrin $\alpha_{IIb}\beta_3$ is normally found on the platelet surface in an inactive conformation unable to bind adhesive ligands such as soluble fibrinogen and vWf. Ligand binding to integrin $\alpha_{IIb}\beta_3$ requires conversion of the integrin from a low affinity binding state (inactive conformation), to a high affinity binding

Figure 1.10 The mechanism of platelet-platelet cohesion (aggregation) under flow (modified from Kulkarni et al., 2000)

Platelets that become activated through adhesion to vWf, spread over the vWf surface to form a monolayer, and release their granule contents. During this process, vWf adsorbed from plasma and released from α-granules, is expressed on the surface of spread platelets and serves to recruit more circulating platelets through the binding of GP Ib/V/IX, these platelets also become activated and adhere irreversibly to the platelet expressed vWf through integrin $\alpha_{IIb}\beta_3$. This results in the formation of platelet aggregates or thrombi.


GPIb/V/IX
von Willebrand Factor
Inactive $\alpha_{IIb}\beta_3$
Active $\alpha_{IIb}\beta_3$
Tethering
Translocation
Stationary & Aggregation adhesion

state (active conformation) through a process known as inside-out signalling. Inside-out signalling pathways that lead to integrin $\alpha_{IIb}\beta_3$ activation are stimulated by the binding of soluble platelet agonists such as adenosine diphosphate (ADP), thrombin, epinephrine, serotonin and TXA_2, or by platelet adhesion to subendothelial proteins such as vWf or collagen (Brass et al., 1991; Shattil, 1995). The specific signalling mechanisms stimulated by individual receptors are incompletely understood but several studies have demonstrated that common signalling pathways can be activated by structurally unrelated platelet surface receptors (Shattil et al., 1998). In fact, platelet stimulation with soluble agonists has been shown to result in similar protein phosphorylation events induced by adhesion receptors such as the vWf or collagen receptors, GP Ib/V/IX and GP VI (Bachelot et al., 1992; Golden et al., 1990; Jackson et al., 1994; Nakamura and Yamamura, 1989; Razdan et al., 1994; Yuan et al., 1997). Furthermore, most platelet agonists appear to demonstrate similar calcium and protein kinase C (PKC) requirements to induce integrin $\alpha_{IIb}\beta_3$ activation (Gu et al., 1999; Yap et al., 2000).

The majority of platelet agonists capable of initiating inside-out signalling processes, including thrombin, ADP, TXA_2 and serotonin, are known to bind to G-protein coupled receptors on the platelet surface. Binding of ligands to these receptors induces the activation of the associated heterotrimeric G proteins. These proteins consist of three subunits α, β and γ. A number of different G-proteins have been identified, each differing in the specific α subunit that they contain. These G-proteins are membrane associated through their $\beta\gamma$ subunits. Upon activation the α subunits dissociate from the membrane bound $\beta\gamma$ subunits. Both the α subunit and the $\beta\gamma$ subunits can initiate multiple inside-out signalling pathways by interacting with various downstream effectors. To date, platelets have been shown to express six different G-proteins; $G\alpha_s$, $G\alpha_q$, $G\alpha_{12/13}$, $G\alpha_z$, $G\alpha_{i2}$, and $G\alpha_{i3}$ (Brass et al., 1997; Oda et al., 2000; Ohlmann et al., 1995; Williams et al., 1990).

These G-proteins appear to regulate different signalling processes in the cells. The G_s protein has been found to mediate prostacyclin-dependent platelet inhibition by stimulating adenylate cyclase and elevating cAMP levels in the cytosol. Conversely, G_z, is known to inhibit adenylate cyclase leading to reduced cAMP levels in platelets. The α subunits of G_{i2} and G_{i3} have also been shown to inhibit cAMP formation whereas the $\beta\gamma$ subunits of these proteins are thought to be able to activate phospholipase Cβ or other signalling molecules on their own accord (Brass et al., 1997; Offermanns, 2000). Recent evidence has suggested a critically important role for G_q for platelet activation by most physiological platelet agonists (Offermanns et al., 1997). G_q has been shown to activate phospholipase C, resulting in release of intracellular calcium and activation of protein kinase C (see section 1.9.1). Platelets from mice that lack G_q have been found to be defective in calcium mobilisation and aggregation by all agonists (Offermanns et al., 1997). Recent studies examining platelet responses to ADP have demonstrated that the G_i pathway is also an important signalling molecule required for platelet activation.

1.9 ADP.

ADP is recognised as one of the most important factors regulating platelet activation and thrombosis (Gachet et al., 1997; Mills, 1996). Despite being the first soluble agonist known to stimulate platelet aggregation, the molecular mechanisms regulating ADP-induced platelet activation have only recently been elucidated. ADP is present at near molar concentrations in the platelet dense granules and is secreted during platelet stimulation by other agonists, such as thrombin or collagen, and appears to reinforce aggregation in response other platelet agonists (Moritz et al., 1983). Its role in thrombosis and haemostasis has been highlighted by recent studies showing that pharmacological inhibitors of ADP-induced platelet aggregation are very effective antithrombotic drugs

(Humphries et al., 1995; Schror, 1995; Schror, 1998). Moreover, patients with congenital ADP receptor defects, and those lacking endogenous ADP altogether, suffer from bleeding diatheses (Cattaneo and Gachet, 1999; Cáttaneo et al., 1992; Rao, 1993).

Platelets express three purinergic receptors on their surface that act in synergy to promote platelet aggregation. The ionotropic $P2X_1$ receptor is a ligand gated ion channel that appears to be responsible for rapid influx of ionised calcium into the cytosol. The $P2Y_1$ metabotropic receptor, linked to the Gαq protein, which is responsible for mobilisation of ionised calcium from internal stores, and a third metabotropic receptor, $P2Y_{12}$, is coupled to G_i, and is essential for the full platelet aggregation in response to ADP. The latter receptor is the molecular target of the ADP-selective antithrombotic drugs ticlopidine and clopidogrel, and is known to be defective in patients with a bleeding diathesis that is characterised by selective impairment of platelet responses to ADP (Gachet, 2001a; Gachet, 2001b). All studies to date suggest that all three ADP receptors are involved in the complex process of platelet activation and aggregation.

1.9.1 ADP Induced Platelet Activation.

In platelets ADP stimulation activates multiple signal transduction pathways. These result in the inhibition of adenylate cyclase, through activation of the Gi linked $P2Y_{12}$ receptor, and also a concomitant transient rise in free cytoplasmic calcium, due to both calcium influx through the P2X receptor, and mobilisation of internal calcium stores through $P2Y_1$ (Gachet et al., 1997; Mills, 1996). Full aggregation in response to ADP is only achieved when ADP concomitantly binds to the G_i coupled $P2Y_{12}$ receptor and the G_q coupled $P2Y_1$ receptor.

Recent studies have demonstrated impaired ADP aggregation in platelets from mice lacking the heterotrimeric Gαq (Offermanns et al., 1997). This suggests that activation of

the PLCβ pathway leading to intracellular calcium mobilisation after ADP stimulation is essential for platelet aggregation. However, blockade of the G_i-linked $P2Y_{12}$ receptor, has been found to impair ADP induced platelet aggregation without inhibiting platelet shape change. Similar results were found in platelets from mice deficient in $G_{\alpha i}$, suggesting that binding of ADP to the $P2Y_1$ receptor alone is sufficient to induce platelet shape change, but it is not enough to support full platelet aggregation (Fagura et al., 1998; Geiger et al., 1998; Hechler et al., 1998a; Hollopeter et al., 2001; Jantzen et al., 1999; Jantzen et al., 2001; Jin et al., 1998; Jin and Kunapuli, 1998; Savi et al., 1998). In both cases, ADP-dependent aggregation could be rescued by epinephrine, which lowers cAMP levels through a G_i-independent pathway (Hechler et al., 1998a; Jin and Kunapuli, 1998). The exact mechanism by which G_i-dependent, inhibition of adenylate cyclase mediates integrin $\alpha_{IIb}\beta_3$ activation remains unclear as reduced cAMP levels do not appear to have a direct causal relationship to ADP induced platelet aggregation (Gachet et al., 1997; Mills, 1996). The role of the $P2X_1$ ionotropic receptor during platelet activation remains to be established.

1.10 Signalling Through Platelet Adhesion Receptors.

It has become increasingly clear that the platelet adhesion receptors GP Ib/V/IX, $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$ and GP VI, not only mediate adhesion of platelets but are also capable of transducing signals leading to platelet activation.

1.10.1 Signalling Through GP Ib/V/IX.

Several studies over the past decade have demonstrated that GP Ib/V/IX is capable of transducing intracellular signals leading to platelet shape change, integrin $\alpha_{IIb}\beta_3$ activation and aggregate formation (De Marco et al., 1985a; De Marco et al., 1985b;

Grainick et al., 1985; Kroll et al., 1991; Savage et al., 1996; Savage et al., 1992). The ability of GP Ib/V/IX to initiate signal transduction was first described in the mid-1980's following studies using desialylated vWf (asialo vWf). Asialo vWf spontaneously binds to GP Ib/V/IX in solution inducing granule release, TXA_2 production and integrin $\alpha_{IIb}\beta_3$ activation (De Marco et al., 1985a; Grainick et al., 1985). Subsequent shear-induced platelet activation (SIPA) studies using a device called a cone and plate viscometer, demonstrated that induction of vWf-GPIb binding under pathological shear conditions could also result in integrin $\alpha_{IIb}\beta_3$ activation (Moake et al., 1988; Moritz et al., 1983). Studies of SIPA have provided significant insight into the mechanism by which vWf binding to GP Ib/V/IX may induce integrin $\alpha_{IIb}\beta_3$ activation (Ikeda et al., 1991; Peterson et al., 1987). These studies highlighted the critical requirement for ADP release (Moake et al., 1988; Oda et al., 1995), protein kinase C (PKC) activation and transmembrane calcium influx during shear induced platelet activation (Chow et al., 1992; Ikeda et al., 1993; Kroll et al., 1993). Furthermore, shear induced platelet aggregation was also found to be associated with the activation of tyrosine kinases, further supporting a signalling role for GP Ib/V/IX (Oda et al., 1995; Razdan et al., 1994; Yanabu et al., 1997). Similar suspension-based studies of vWf-induced platelet aggregation using artificial modulators such as ristocetin and botrocetin have demonstrated increased cytosolic calcium and PKC activation (Kroll et al., 1991), as well as the activation of PI 3-kinase (Jackson et al., 1994), and tyrosine kinases such as $p72^{syk}$ (Asazuma et al., 1997). Furthermore, inhibition of PKC was found to abolish stationary adhesion to immobilised vWf supporting a role for PKC in vWf-induced signalling in adhesion based assays (Savage et al., 1992). Together these studies have established that GP Ib/V/IX is capable of promoting intracellular signalling pathways leading to integrin $\alpha_{IIb}\beta_3$ activation following vWf engagement. It is unclear however whether integrin $\alpha_{IIb}\beta_3$ activation induced by GP Ib/V/IX signalling occurs

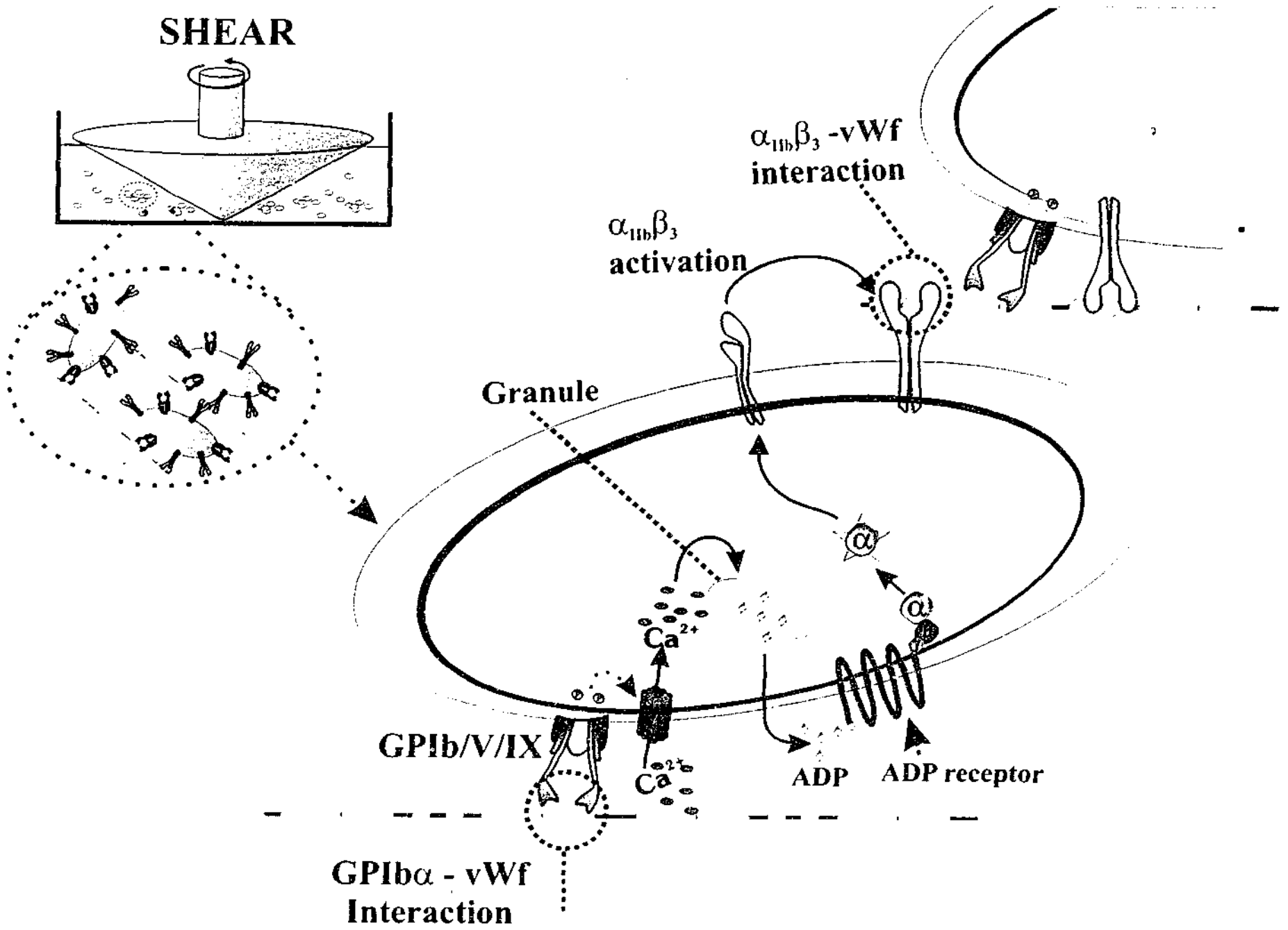
directly as a result of intracellular second messenger generation, or indirectly through the release of endogenous agonists such as ADP and TXA_2.

Studies of shear induced platelet activation have provided evidence to support an indirect mechanism by which GP Ib/V/IX induced signalling promotes integrin $\alpha_{IIb}\beta_3$ activation. The current dogma derived from these studies proposes that vWf binding to GP Ib/V/IX under high shear conditions results in calcium influx through the plasma membrane and the activation of PKC and other tyrosine kinases (Ikeda et al., 1993; Kroll et al., 1993; Razdan et al., 1994). The spike in cytosolic calcium concentration is thought to promote the secretion of endogenous platelet agonists, such as dense granule ADP and the generation of TXA_2 (fig. 1.11) (Chow et al., 1992). These soluble agonists feed back onto their respective platelet surface receptors (Daniel et al., 1998; Hechler et al., 1998a; Hechler et al., 1998b; Jin et al., 1998; MacKenzie et al., 1996), and stimulate inside-out signalling pathways that ultimately lead to integrin $\alpha_{IIb}\beta_3$ activation (Gachet et al., 1997).

While GP Ib/V/IX is known to induce signal transduction, it has not been shown to have any intrinsic enzymatic activity, nor has it been shown to couple to G proteins or to be phosphorylated by tyrosine kinases. Despite intense research, little is known about the exact signalling mechanisms through which GP Ib/V/IX mediates platelet activation. However, it appears likely that the GP Ib/V/IX complex induces signal transduction by recruiting several signalling proteins from the cytosol. For example, it has been demonstrated that the GP Ibα and GP Ibβ subunits of the GP Ib/V/IX complex associate with the 14.3.3ζ signalling protein (Du et al., 1994), which can potentially link GP Ib/V/IX to a variety of intracellular signalling pathways. The members of the 14.3.3 protein family are known to bind and regulate the activity of several key cytosolic signalling proteins that are believed to be involved in platelet activation such as PI 3-Kinase, PKC, Raf-1/B-Raf protein kinases, Ber or Ber-Abl kinases, the BCL-2 family member BAD and the adaptor

Figure 1.11 GP Ib/V/IX-induced activation of integrin $\alpha_{IIb}\beta_3$ under pathological shear conditions (modified from Yap et al., 2000)

Schematic representation of a model of GP Ib/V/IX-induced integrin $\alpha_{IIb}\beta_3$ activation derived from shear-induced platelet aggregation studies. In this model, GP Ib/V/IX binding to vWf is induced by high shear forces. This results in transmembrane calcium influx, which leads to secretion of endogenous ADP from platelet dense granules. The binding of ADP to purinergic receptors expressed on the platelet surface results in inside-out signalling processes leading to integrin $\alpha_{IIb}\beta_3$ activation, binding of multimeric vWf and platelet aggregation. The top left-hand panel illustrates shear-induced platelet aggregation occurring in a cone and plate viscometer.


SHEAR
$\alpha_{IIb}\beta_3$ -vWf
interaction
$\alpha_{IIb}\beta_3$
activation
Granule
GPIb/V/IX
Ca^{2+}
Ca^{2+}
ADP
ADP receptor
GPIbα - vWf
Interaction

protein Cbl (Bonnefoy et al., 2000; Fantl et al., 1994; Freed et al., 1994a; Freed et al., 1994b; Fu et al., 1994; Isobe et al., 1992; Liu et al., 2000; Reuther et al., 1994; Tanji et al., 1994; Toker et al., 1990). Binding of 14.3.3ζ to the cytoplasmic tail of GP Ibα has been shown to require phosphorylation of serine residue 609 (Bodnar et al., 1999), and binding of 14.3.3ζ to GP Ibβ appears to be regulated by protein kinase A (PKA) phosphorylation of Ser^{166} (Andrews and Berndt, 1998; Calverley et al., 1998). The functional importance of the interaction between the 14.3.3ζ protein and GP Ib/V/XI in integrin $\alpha_{IIb}\beta_3$ activation is unclear. One recent study demonstrated that disruption if the 14.3.3ζ binding site of GP Ibα results in impaired vWf induced integrin $\alpha_{IIb}\beta_3$ activation (Gu et al., 1999), while others have suggested that disrupting this interaction does not effect integrin $\alpha_{IIb}\beta_3$ activation at all (Zaffran et al., 2000).

Another protein interaction of potential importance for GP Ib/V/IX signalling is the interaction between GP Ib/V/IX and platelet Fc Receptors. The potential involvement of FcR γ-chain and/or FcγRIIa was suggested following the demonstration of tyrosine phosphorylation and activation of the non-receptor tyrosine kinase, $p72^{syk}$ upon vWf stimulation of platelets (Asazuma et al., 1997; Yanabu et al., 1997). $p72^{syk}$ has been found to play a crucial role in collagen-induced platelet activation (Asselin et al., 1997; Ezumi et al., 1998; Gibbins et al., 1997; Ichinohe et al., 1997; Poole et al., 1997; Yanaga et al., 1995). Activation of $p72^{syk}$ requires engagement of its tandem Src homology 2 (SH2) domains with proteins containing phosphorylated immuno-receptor tyrosine containing activation motif (ITAM). The only platelet proteins that contain such ITAM motifs are the FcγRIIa and FcR γ-chain (Asselin et al., 1997; Chacko et al., 1996; Gibbins et al., 1996), both of which have been shown to associate with the GP Ib/V/IX complex (Falati et al., 1999; Marshall et al., 2002; Sullam et al., 1998; Sun et al., 1999). This raises the possibility that GP Ib/V/IX can also induce signal transduction through a Fc receptor

dependent mechanism (Falati et al., 1999; Sullam et al., 1998). In this model of GP Ib/V/IX-induced $\alpha_{IIb}\beta_3$ activation, vWf binding to GP Ib/V/IX results in the phosphorylation of Fc receptors on their ITAM motifs. This drives the formation of large signalling complexes and activation of a number of non-receptor tyrosine kinases including $p72^{syk}$, $p59^{lyn}$ and $p53/56^{lyn}$, resulting in a cascade of events leading to phospholipase Cγ2 (PLCγ2) activation in a similar process as described for collagen induced signalling (Falati et al., 1999).

1.10.2 Integrin $\alpha_{IIb}\beta_3$-Mediated Signalling Events.

The platelet integrin $\alpha_{IIb}\beta_3$ is able to transduce biochemical signals across the plasma membrane in a bi-directional manner, through inside-out and outside-in signalling pathways. It is well established that initially upon integrin $\alpha_{IIb}\beta_3$ activation, ligand binding is reversible, but becomes progressively more stable as time goes on (Peerschke, 1995; Shattil et al., 1998). Integrin $\alpha_{IIb}\beta_3$ activation has been shown to result not only in affinity modulation requiring conformational changes in the receptor, but also in avidity modulation through clustering of the receptor (Hato et al., 1998).

Affinity modulation is believed to be important for the initial reversible phase of integrin $\alpha_{IIb}\beta_3$ activation, and is thought to be mediated by the association of the cytoplasmic portions of integrin $\alpha_{IIb}\beta_3$ with regulatory proteins in the cytoplasm. This results in conformational changes in the extracellular portion of the integrin and exposure of encrypted ligand binding sites allowing the integrin to interact with soluble fibrinogen or vWf. Naturally occurring mutations in the cytoplasmic domain of integrin $\alpha_{IIb}\beta_3$, have been found to result in both defective integrin $\alpha_{IIb}\beta_3$ activation and also in constitutive activation of this receptor (Ambo et al., 1998a; Ambo et al., 1998b; Chen et al., 1992; Liu et al., 1998; Shattil et al., 1998; Wang et al., 1997). Numerous regulatory proteins have

been shown to associate with the cytoplasmic tails of both the α_{IIb} and β_3 subunits of integrin $\alpha_{IIb}\beta_3$, eg. calcium-and-integrin binding protein (CIB), which associates with the α_{IIb} cytoplasmic tail, and β_3-endonexin, which binds to the cytoplasmic region of integrin β_3 as well as to a number of structural proteins (Naik and Parise, 1997; Shattil, 1995; Shattil et al., 1995), however, the role of such proteins in affinity modulation is unclear.

Avidity modulation is believed to promote the irreversible phase of integrin $\alpha_{IIb}\beta_3$ activation. Ligand binding and clustering of integrin $\alpha_{IIb}\beta_3$ induces a host of outside-in signalling events. These integrin mediated signalling processes lead to the activation of protein tyrosine kinases and cytoskeletal reorganisation which ultimately determine the extent of platelet aggregation, spreading, secretion and clot retraction (Abrams et al., 1994; Clark and Brugge, 1995; Narumiya, 1996; Peerschke, 1995; Shattil and Brugge, 1991). Similar to GP/Ib/V/IX, the integrin $\alpha_{IIb}\beta_3$ receptor does not possess intrinsic catalytic activity. Following activation and clustering, integrin $\alpha_{IIb}\beta_3$ recruit various structural proteins (talin, vinculin, α-actinin, tensin, paxillin) and signalling molecules (FAK, Src kinases, PI 3-kinase, PKC, PLCγ2, Calpain) to form large cytoskeletal signalling complexes called focal complexes (Burridge and Chrzanowska-Wodnicka, 1996; Chrzanowska-Wodnicka and Burridge, 1996; Hemler, 1998).

The β_3 subunit of the $\alpha_{IIb}\beta_3$ receptor is thought to play a major role in integrin-mediated signalling and focal contact formation. The cytoplasmic domain of this subunit becomes tyrosine phosphorylated during platelet activation and has been shown to associate with several cytosolic proteins including, talin (Knezevic et al., 1996), Shc and Grb2 (Law et al., 1996), β_3-endonexin (Shattil, 1995; Shattil et al., 1995) and calcium- and integrin binding protein (CIB) (Naik and Parise, 1997; Naik et al., 1997).

Ligand binding to integrin $\alpha_{IIb}\beta_3$ induces the activation of two independent signalling pathways leading to two distinct waves of protein tyrosine phosphorylation. The first is an early outside-in signalling event linked to the activation of Syk by a Src family tyrosine kinase (Gao et al., 1997) following $\alpha_{IIb}\beta_3$ receptor clustering. This pathway coincides with filopodial extension and the rapid tyrosine phosphorylation of various cytosolic proteins ranging between 50 – 72 kDa and 140 kDa (Hartwig, 1992; Hartwig et al., 1996; Huang et al., 1993; Shattil et al., 1994; Yuan et al., 1997), and requires intact α_{IIb} and β_3 cytoplasmic domains (Clark et al., 1994; Gao et al., 1997; Hato et al., 1998; Miranti et al., 1998). Following this first wave of phosphorylation is a second signalling event coinciding with maximal platelet spreading and/or aggregation involving Src, and the activation of pp125FAK. This results in the tyrosine phosphorylation of the as yet unidentified proteins of 95 kDa, 97 kDa, 101 kDa and 105 kDa, as well as the phosphorylation of the tyrosine kinases, Tec kinase, $pp125^{FAK}$ and the 5-phosphatase SHIP (Giuriato et al., 2000; Laffargue et al., 1997; Shattil et al., 1994). Eventually, the initial increase in protein tyrosine phosphorylation is followed by a down-regulation of tyrosine kinase activity due to proteolysis of protein tyrosine kinases by the cysteine protease calpain (Cooray et al., 1996; Oda et al., 1993), and the recruitment of a number tyrosine phosphatases such as PTP-1B and SHIP-1 (Ezumi et al., 1995; Frangioni et al., 1993; Li et al., 1994).

Signalling events other than protein phosphorylation that have been shown to occur post-integrin $\alpha_{IIb}\beta_3$ activation, include calcium mobilisation, activation of PKC and PI 3-kinase, and arachidonic acid metabolism. Of particular importance, is the role of the second messenger calcium during integrin signalling. Calcium release from intracellular stores by the action of inositol triphosphate (IP_3) is important for mediating platelet activation in response to various agonists. Cytosolic calcium mobilisation plays a critical

role in inside-out signalling events leading to platelet shape change, secretion and integrin to $\alpha_{IIb}\beta_3$ activation (Berridge, 1984; Berridge and Irvine, 1984; Gerrard and Carroll, 1981; Rink and Sanchez, 1984; Yoshida et al., 1988), and also in the subsequent outside-in signalling events generated through the active integrin (Heemskerk et al., 1994; Heemskerk et al., 1992; Heemskerk et al., 1993; Kuwahara et al., 1999; Sage et al., 1993; Yap et al., 2000). More recently it has been demonstrated that cytosolic calcium is not only required for initial integrin $\alpha_{IIb}\beta_3$ activation, but it is also involved in a positive feedback mechanism required for amplifying and maintaining the activation status of this receptor (Nesbitt et al., 2002; Yap et al., 2000).

1.10.3 Collagen Receptor Signalling.

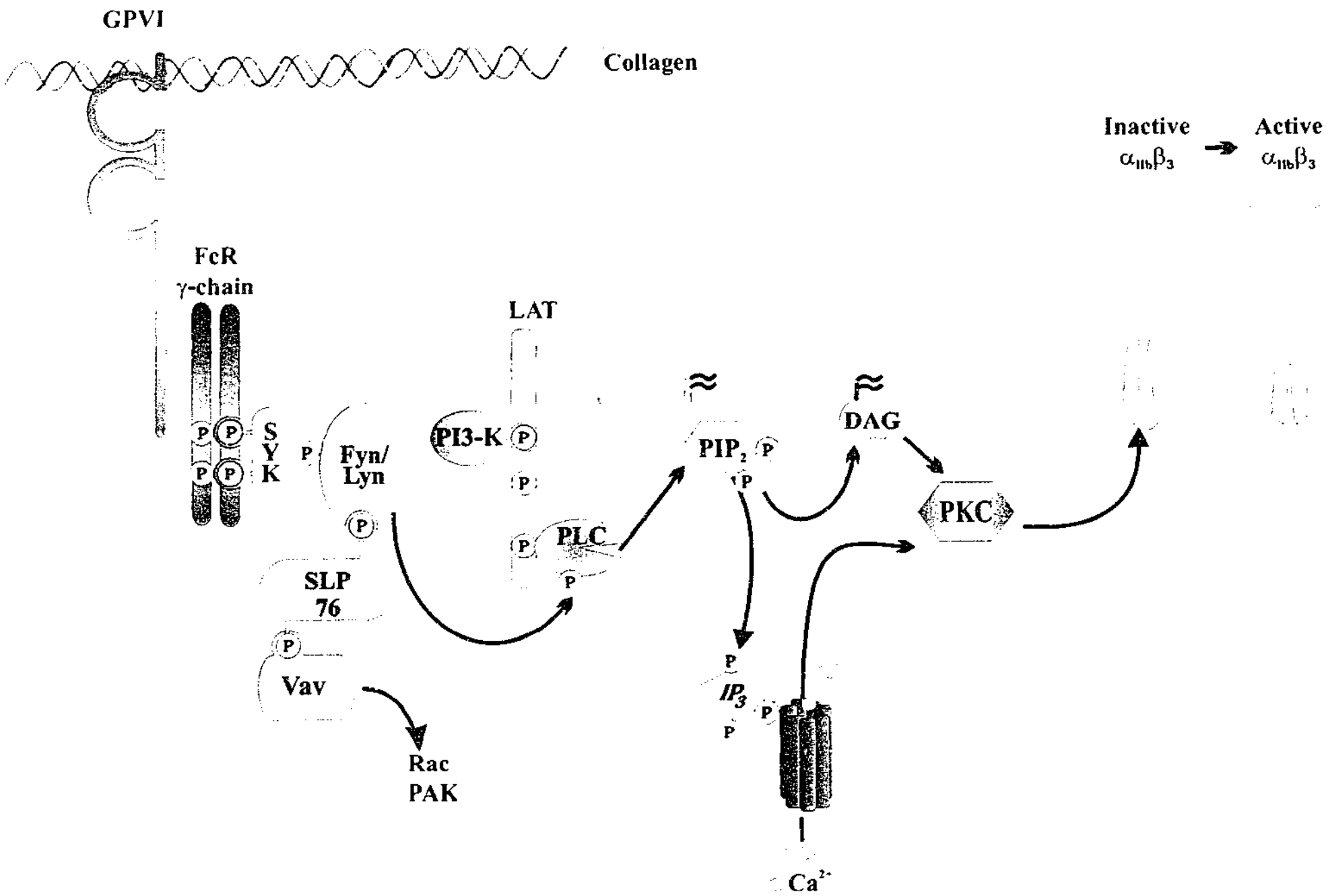
Platelet adhesion and activation on a collagen matrix under flow requires the synergistic contribution of integrin $\alpha_2\beta_1$ and the GPVI/FcRγ complex (Nieswandt et al., 2001a; Savage et al., 1998), with recent evidence supporting a major role for the GPVI/FcRγ complex in the collagen induced signalling process (Moroi et al., 1996; Tsuji et al., 1997). The initial interaction of platelets with collagen is mainly mediated by integrin $\alpha_2\beta_1$, while subsequent platelet activation and firm adhesion is mediated by the GP VI/FcRγ complex (Moroi et al., 1996). Signalling through the GP VI collagen receptor occurs through a similar pathway to immune receptor signalling (Watson et al., 2001), ie. through association of GP VI with the ITAM motif of the Fc receptor γ-chain (Gibbins et al., 1996). Data supporting an important role for GPVI/FcRγ-derived activating signals was derived from studies using FcRγ-deficient mice (Poole et al., 1997; Tsuji et al., 1997). The FcR γ-chain is co-expressed with GP VI in platelets. Binding of GP VI to collagen results in clustering of GP IV/FcRγ complex, leading to autophosphorylation of tyrosine residues

within the ITAM domain of the FcRγ chain (fig. 1.12) (Gibbins et al., 1996; Gibbins et al., 1997; Nieswandt et al., 2000; Quek et al., 1998; Tsuji et al., 1997). Phosphorylation of the FcR γ-chain triggers a signalling cascade resulting in the activation of non-receptor tyrosine kinases including $p72^{syk}$, $p59^{fyn}$ and $p53/56^{lyn}$ (Briddon and Watson, 1999; Ezumi et al., 1998). This is believed to promote the downstream activation of PLCγ2, via the action of adaptor proteins such as SLP-76 and LAT (linker for activation of T cells), and this subsequently leads to calcium mobilisation from intracellular stores (Clemetson, 1999; Pasquet et al., 1999b).

Unlike most soluble platelet agonists that promote calcium mobilisation through G-protein coupled signalling mechanisms leading to PLCβ activation, GP VI-induced calcium mobilisation and protein kinase C (PKC) activation occurs through a PLCγ2-dependent mechanism and is independent of G-protein activity. The central role for SLP-76 for the activation of PLCγ2 was demonstrated in studies using SLP-76 knockout mice, which showed impaired PLCγ2 activation and calcium mobilisation in response to collagen stimulation (Clements et al., 1999; Gross et al., 1999). Apart from promoting PLCγ2 activation, the adaptor protein SLP-76 is also known to regulate other proteins involved in GP VI signal transduction including Vav, SLAP-130, Fyn and Lyn (Gross et al., 1999), however the importance of these molecules in collagen induced activation in platelets is unknown. There is also some evidence supporting a role for PI 3-kinase in the regulation of PLCγ2. It has been shown that a PI 3-kinase lipid product, phosphatidylinositol (3,4,5)-triphosphate (see section 1.9), can bind to PLCγ2 and induce its activation (Gratacap et al., 1998). Furthermore, in studies using murine megakaryocytes, microinjection of peptides containing pleckstrin homology domains that interact with phosphatidylinositol (3,4,5)-triphosphate, results in impaired PLC-mediated calcium mobilisation (Pasquet et al., 1999a).

Figure 1.12 The mechanism of collagen induced platelet activation (modified from Watson, 1999)

Collagen binding to the major collagen receptor GP VI, results in the association of this receptor with the FcR γ-chain. This leads to clustering of and autophosphorylation of the FcR γ-chain, which results in the subsequent activation of Syk and LAT, and the recruitment of several signalling proteins including PI 3-Kinase (PI 3-K), PLCγ2 (PLC), SLP-76 and the Src family kinases Fyn/Lyn to the platelet membrane. Membrane associated PLC is then phosphorylated by the Src family kinases Fyn/Lyn, leading to mobilisation of intracellular calcium and activation of PKC. This signalling cascade ultimately promotes integrin $\alpha_{IIb}\beta_3$ activation.


GPVI
Collagen
Inactive
$\alpha_{IIb}\beta_3$
Active
$\alpha_{IIb}\beta_3$
FcR
γ-chain
LAT
SYK
Fyn/
Lyn
PI3-K
PIP2
DAG
PKC
PLC
SLP
76
Vav
Rac
PAK
IP3
Ca2+

1.11 Role of Phospholipids and Phosphoinositide (PI) Turnover in Platelet Signalling.

The propagation of cytosolic signals upon agonist stimulation generally occurs through the generation of intracellular second messengers such as 1,2-diacylglycerol (DAG) and inositol triphosphate (IP_3). In platelets, IP_3 binds to its receptor on the dense tubular system resulting in calcium release from intracellular stores, while DAG is a key intermediate for PKC activation. These molecules are responsible for activating a multitude of downstream effector pathways that regulate platelet function, including shape change, aggregation and secretion (Castagna et al., 1982; Kajikawa et al., 1983; Kuwahara et al., 2002; Kuwahara et al., 1999; Nunn and Watson, 1987; O'Rourke et al., 1987).

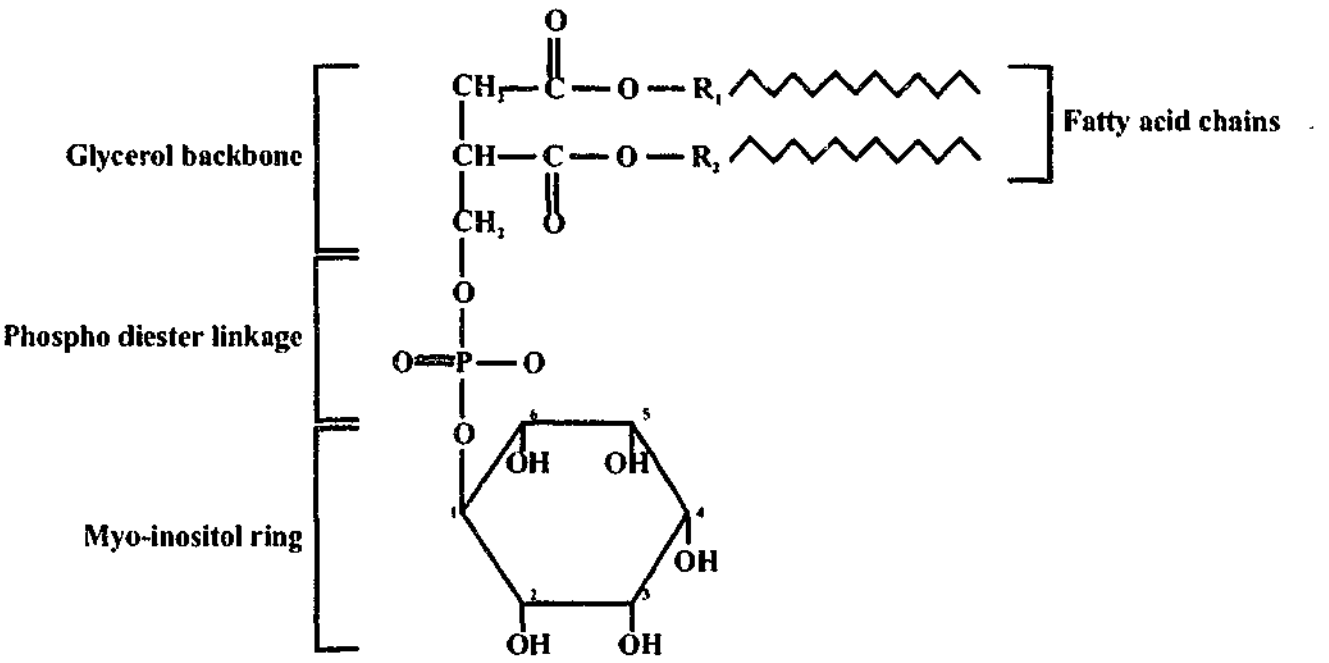
The generation of such second messengers is critically dependent on a particularly important class of membrane phospholipid called the phosphatidylinositols (PtdIns) (Majerus et al., 1984). These phospholipids constitute about 8% of total membrane phospholipids (Esko and Raetz, 1980; Henry et al., 1977), and are found in abundance in the inner leaflet of the platelet plasma membrane (Mauco et al., 1987). Phosphatidylinositols are believed to function by acting as precursor molecules for a variety of second messengers including DAG and IP_3, and the turnover of phosphoinositides has been shown to be an important signalling mechanism for platelet activation (Bell and Majerus, 1980; Mauco et al., 1979; Rittenhouse, 1983).

Phosphatidylinositols (PtdIns) consist of a myo-inositol ring containing five hydroxyl (OH) groups that provide reactive sites for substitution with phosphate groups. This is linked to a glycerol backbone through a phosphodiester bond at the 1-hydroxyl position of the ring. In addition, the glycerol backbone is ester linked to two fatty-acid chains (fig. 1.13). There are three major myo-inositol-containing phospholipids expressed in the platelet membrane that vary in their extent of phosphorylation. They are phoshatidylinositol [PtdIns], which contains only hydroxyl groups on the myo-inositol

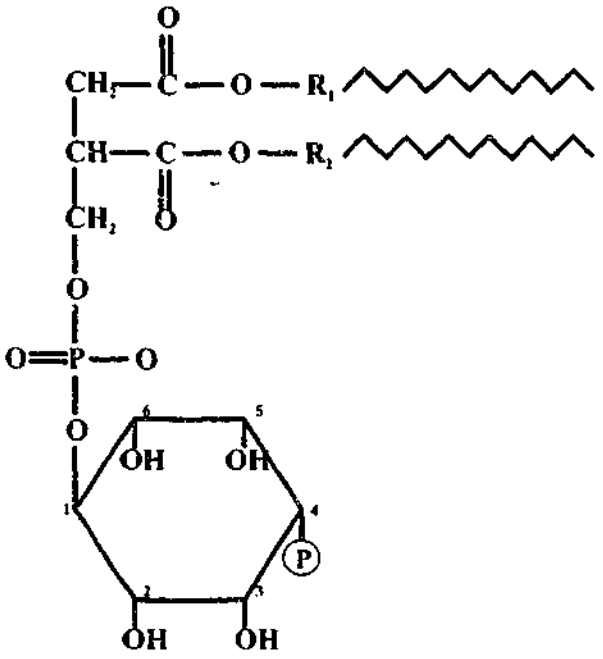
Figure 1.13 Schematic representation of membrane phosphoinositides

The molecular structure of PtdInt, PtdInt(4) P, PtdInt(4,5) P_2, are illustrated highlighting the myo-inositol ring, glycerol backbone and fatty acid tails.

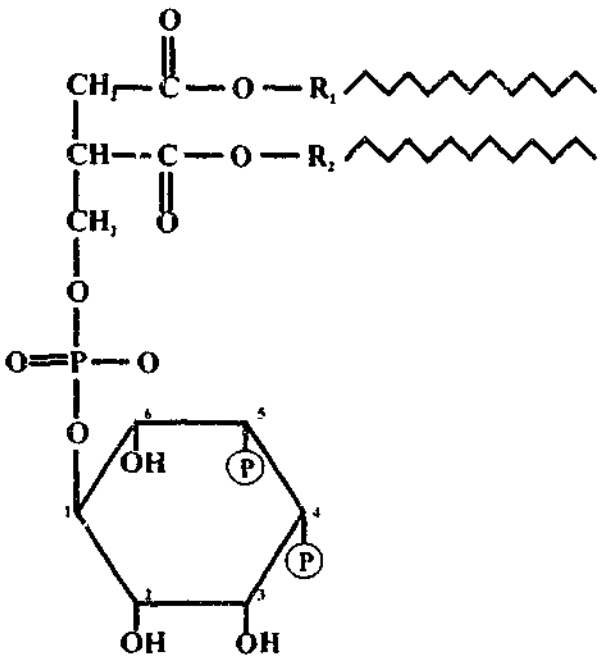
PtdIns



PtdIns (4) P



PtdIns (4,5)P_2



ring, phoshatidylinositol 4-phosphate [PtdIns(4)P] which contains a phosphate group at position 4 of the myo-inositol ring, and phoshatidylinositol 4,5-bisphosphate [PtdIns (4,5)P_2], which contains two phosphate groups at position 4 and 5 of the ring. In platelets, PtdIns accounts for up to 7% of total membrane phospholipids, PtdIns (4)P accounts for 1% and PtdIns (4,5)P_2 for 0.4% (Majerus et al., 1984). The levels of these phospholipids are regulated in resting platelets through a dynamic process of phosphorylation and dephosphorylation events mediated by specific kinases and phosphatases (Berridge, 1984; Michell, 1975).

The mechanism by which phosphatidylinositides promote cell activation is known as phoshoinositide (PI) turnover. This process is dependent on the activation of PLC and the subsequent hydrolysis of membrane bound phosphoinositides. Al. three phosphoinositides can be hydrolysed by PLC following platelet stimulation, however PtdIns (4,5)P_2 is considered to be the preferred substrate *in vivo*. The hydrolysis of PtdIns(4,5)P_2 results in the generation of lipid soluble DAG, and the water soluble IP_3 (Agranoff, 1983; Billah and Lapetina, 1982a; Billah and Lapetina, 1982b; Kuwahara et al., 1999; Rittenhouse, 1983).

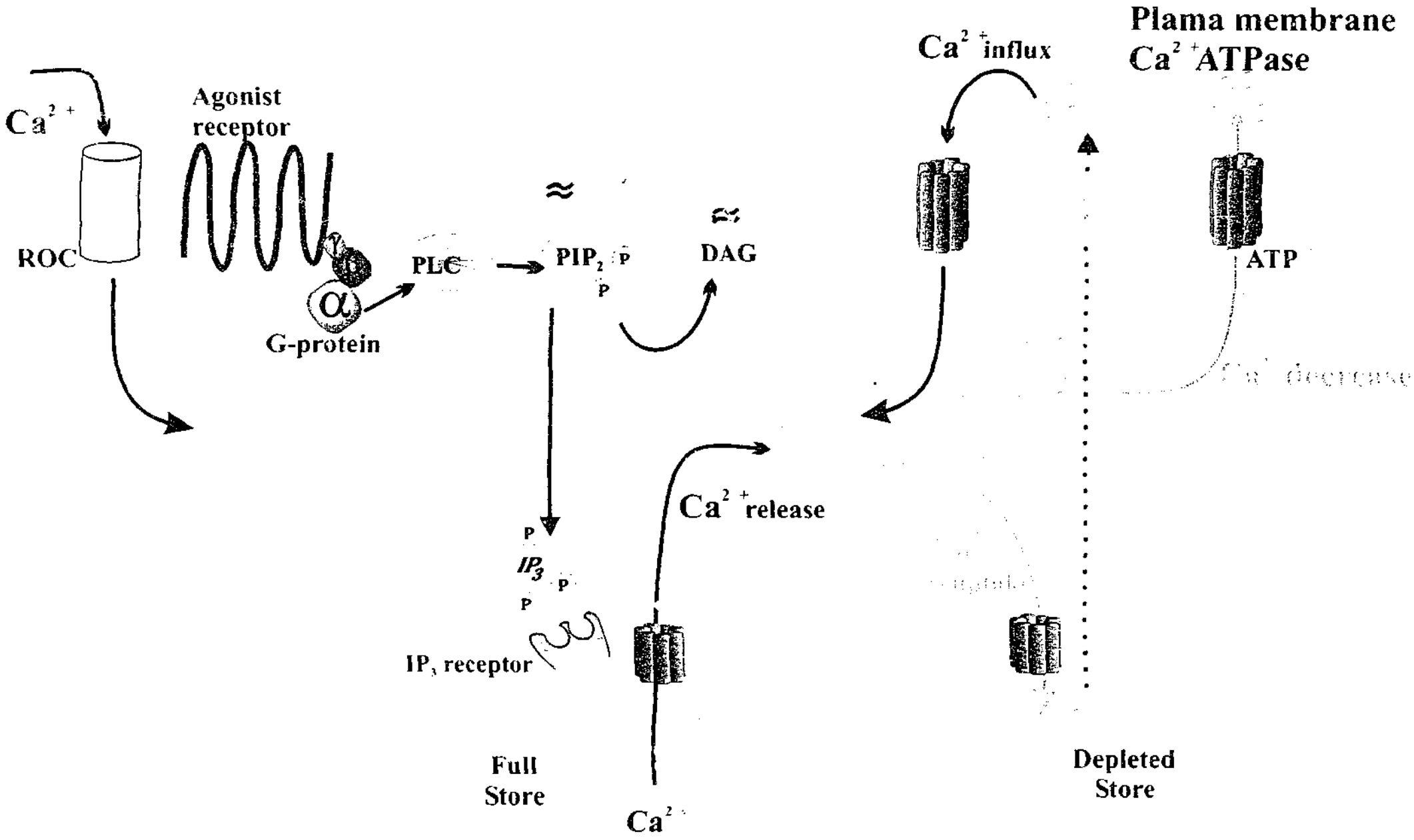
1.11.1 Role of Calcium in Platelet Function.

Calcium is a key second messenger involved in many biological processes including the control of gene transcription (Dolmetsch et al., 1998; Li et al., 1998), cell proliferation, organ development and muscle contraction (Clapham, 1995). Platelet stimulation by most platelet agonists is accompanied by a rise in cytosolic calcium levels (Saitoh et al., 1989). The role of calcium in platelet function has been the subject of intense investigation for many years (Heemskerk et al., 1994; Kroll and Schafer, 1989). There are two main sources of calcium in the platelet, (i) internal calcium stored within the platelets

dense tubular system, and (ii) calcium channels in the plasma membrane that allow calcium to enter the cell from the extracellular environment. Following platelet stimulation with an activating agonist, the initial rise in cytosolic calcium is mainly derived from mobilisation of calcium from the platelet dense tubular system (ie. from intracellular calcium stores). It is believed that depletion of intracellular calcium stores triggers the opening of membrane calcium channels allowing calcium influx from the extracellular environment through a process known as store-regulated calcium entry (SRCE) (fig. 1.14) (Alonso et al., 1991; Rosado et al., 2000a; Rosado et al., 2000b; Sage et al., 1990). An alternative mode of cytosolic calcium elevation is through calcium entry facilitated by surface receptors directly opening calcium channels in the plasma membrane, an example of which is the ionotropic ADP receptor, P2X which is believed to allow calcium influx upon ADP binding (Gachet, 2001a; Gachet, 2001b). Recent studies have also proposed a role for PI 3-kinase in regulating both calcium mobilisation and influx in platelets. PI 3-kinase is thought to promote transmembrane influx through the generation of the PI 3-kinase lipid product PtdIns(3,4,5)P_3. This hypothesis is supported by studies demonstrating that platelets derived from mice that lack the Src-homology 2 (SH2)-containing inositol 5' phosphatase (SHIP), display elevated levels of PtdIns(3,4,5)P_3 and enhanced calcium influx (Huber et al., 1998a; Huber et al., 1998b; Pasquet et al., 2000). The mechanism by which PtdIns(3,4,5)P_3 promotes influx is not understood. In platelets, inhibition of PI 3-kinase has been found to reduce the level of IP_3 generation leading to a reduction in calcium mobilisation (Pasquet et al., 1999b). The role of PI 3-kinase in promoting calcium mobilisation also seems to involve PtdIns (3,4,5)P_3 as microinjection of pleckstrin homology (PH) domains that bind to this lipid product has been shown to reduce IP_3 levels and calcium mobilisation in COS-1 cells and megakaryocytes (Falasca et al., 1998; Pasquet et al., 1999a; Pasquet et al., 1999b).

Figure 1.14 Schematic representation of the mechanisms regulating cytosolic calcium levels in platelets

Two processes are believed to result in elevated cytosolic calcium levels in activated platelets. Platelets contain receptor operated calcium channels (ROCs) on their surface. Binding of agonists to these receptors (eg. ADP binding to P2X ionotropic receptor), results in transmembrane calcium influx. Also, agonists binding to G-protein-linked platelet surface receptors leads to PLC activation and the subsequent hydrolysis of the membrane phospholipid, phosphatidylinositol (4,5)-bis phosphate (PIP_2). The products of this hydrolysis are membrane bound diacylglycerol (DAG) and water soluble inositol triphosphate (IP_3). IP_3 binds to its receptor on the dense tubular system and induces release of calcium from intracellular calcium stores. Depletion of intracellular calcium from the dense tubular system is believed to induce further calcium influx through unidentified calcium channels in the plasma membrane. Decreases in cytosolic calcium are achieved by the re-uptake of calcium into intracellular stores (calcium re-uptake) and also by active calcium efflux through membrane calcium ATPase pumps.


Plama membrane
Ca2+ ATPase
Ca2+ influx
Ca2+
Agonist
receptor
ROC
PLC
PIP2
P
P
DAG
G-protein
ATP
P
IP3
P
P
IP3 receptor
Ca2+ release
Full
Store
Depleted
Store
Ca2+

Prolonged periods of elevated calcium are toxic to most cell types therefore, cytosolic calcium levels in all cell systems tend to be very tightly regulated to ensure effective functional responses without cytotoxicity. Following platelet activation, basal cytosolic calcium levels are restored in platelets through the action of calcium ATPases. These are found on the dense tubular system, which help to re-sequester calcium back into the intracellular storage organelles, and also on the plasma membrane, which actively pump calcium out of the cell through the plasma membrane (fig. 1.14) (Gerrard, 1986).

1.12 Protein Kinase C (PKC).

The Protein kinase C (PKC) family includes 12 structurally related serine/threonine kinases that are differentially expressed in many cell types (Nishizuka, 1988a; Nishizuka, 1988b). Since their discovery by Nishizuka et al., (1977), these enzymes have been shown to regulate such diverse cellular functions as differentiation, proliferation, secretion and metabolism (Blobe et al., 1996; Mellor and Parker, 1998; Newton, 1997; Slater et al., 2000; Toker, 1998; Vaughan et al., 1998). In platelets these proteins have been shown to play central roles in regulating platelet adhesion, shape change, secretion, and aggregation (Siess and Lapetina, 1989a; Toullec et al., 1991). The PKC superfamily can be subdivided into three groups based on their isoform structure (described in detail in section 1.5.1), and on their co-factor requirements for activation. The three groups are, (i) the conventional/classical PKC group (cPKC) including α, βI/βII and γ isoforms, (ii) a novel PKC group (nPKC) including δ, ε, η and θ isoforms; and (iii) an atypical PKC group (aPKC) including ζ, λ, and μ. The conventional PKC isoforms are activated by the concerted efforts of calcium, diacylglycerol (DAG) or phorbol esters (eg. PMA and TPA), and membrane phospholipids such as phosphatidyl serine (PS) and phoshatidyl ethanloamine (PE). The novel PKC isoforms lack a calcium-binding domain. They are

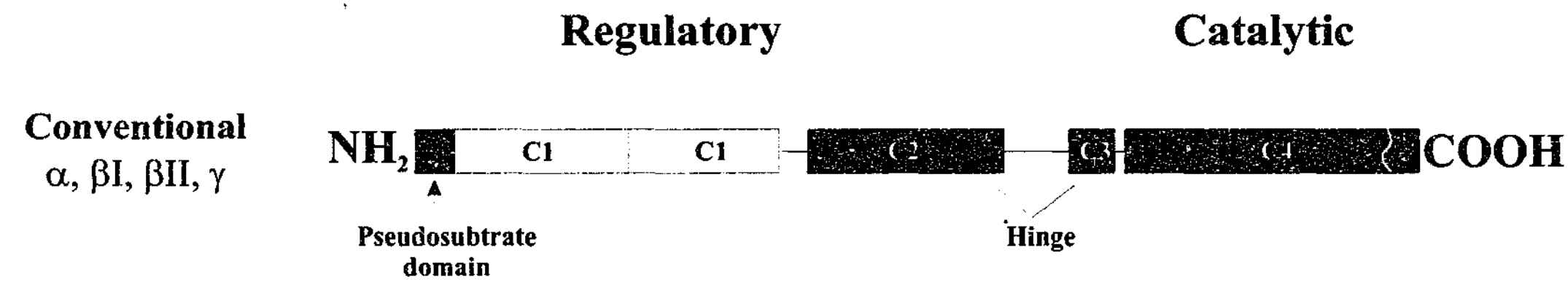
therefore calcium independent and can be activated by phorbol esters and phospholipids alone. The atypical PKC isoforms lack both a calcium binding and phorbol ester-binding domain and are therefore insensitive to either calcium or phorbol esters. The mechanism of activation of the atypical PKC isoforms remains unknown. To date only six of the twelve known PKC isoforms have been found in platelets, they are the α, βI/βII, ζ, δ, η and 0 isoforms (Baldassare et al., 1992; Crabos et al., 1991; Selbie et al., 1993).

1.12.1 Structure of PKC.

Protein kinase C isozymes consist of a single polypeptide chain that contains an amino-terminal regulatory region (of 20-70 kDa) and a carboxy-terminal kinase domain (of approximately 45 kDa) (fig. 1.15) (Nishizuka, 1988b). The regulatory half of the conventional PKC molecules contains an autoinhibitory domain called the pseudosubstrate domain, followed immediately by one or two membrane targeting domains called, the C1 and C2 domains. The C1 domain contains the phorbol ester and DAG-binding site, while the C2 domain contains the phospholipid binding and calcium binding domains. The novel PKC isoforms contain a C1 domain but lack a functional C2 domain making them calcium independent isoforms, and the atypical isoforms lack both the C1 and C2 domain and are therefore unresponsive to calcium, phorbol esters or phospholipids. The catalytic half of the PKC enzymes contains the C3 and C4 domains, which comprise the ATP- and substrate-binding lobes of the kinase core. The catalytic domain is separated from the regulatory domain by a short hinge region that allows the enzyme to fold back on itself in its resting conformation. In resting cells, PKC is maintained in its inactive state by the interaction of the pseudosubstrate domain with the catalytic domain of the PKC isozymes (Newton, 1997).

Figure 1.15 Structure of protein kinase C (modified from Newton, 1997)

Schematic representation of the structures of conventional, novel and atypical forms of PKC. The diagrams highlight the pseudosubstrate domain, the C1 domain which binds to DAG and phorbol esters, the C2 domain which binds acidic lipids (such as phosphatidyl serine) and calcium, and the C3 and C4 domains which contain the ATP- and substrate-binding sites of the active subunit of the enzyme. The novel isoforms lack a C2 (calcium binding) domain making them insensitive to calcium, and the atypical isoforms lack both a C1 and a C2 domain making them insensitive to both calcium and phorbol esters (note: the members of each isoform class are listed on the left).


Regulatory
Catalytic
Conventional
α, βI, βII, γ
NH2
C1
C1
C2
C3
C4
COOH
Pseudosubtrate domain
Hinge
Novel
δ, ε, θ, η
NH2
COOH
Atypical
ζ, ι, λ
NH2
COOH

1.12.2 PKC Activation.

It is well established that PKC activation is mediated by the concerted efforts of calcium, phospholipids and diacylglycerol, though the requirements for these co-factors differ widely between PKC isoforms as discussed above. Studies by Hannun et al., in the mid 1980's, using mixed micelle assays, helped elucidate the precise interactions between calcium, phospholipids and diacylglycerol during PKC activation (Hannun and Bell, 1986; Hannun et al., 1985; Hannun et al., 1986a; Hannun et al., 1986b). These studies suggested a two step mechanism of PKC activation whereby calcium and/or phospholipid binding to the C2 domain is sufficient to induce PKC translocation to the plasma membrane and low affinity association between the PKC enzyme and the membrane phospholipids. The enzyme however, remains catalytically inactive until phorbol ester or diacylglycerol binds the C1 domain. This induces a conformational change that causes the pseudosubstrate region to dissociate from the catalytic domain and also increases the affinity of binding between PKC and membrane phospholipids.

1.12.3 Physiological Function of PKC in Platelets.

In platelets, PKC regulates multiple responses including shape change, secretion and aggregation (Crouch and Lapetina, 1988; Salari et al., 1990; Siess and Lapetina, 1989a; Siess and Lapetina, 1989b; Toullec et al., 1991; Walker et al., 1990). Several independent lines of evidence support an important role for PKC in both inside-out and outside-in signalling leading to integrin $\alpha_{IIb}\beta_3$ activation. For instance, PKC activation, as measured by enzyme translocation from the cytosol to the membrane, has been demonstrated in platelets stimulated by all agonists known to induce $\alpha_{IIb}\beta_3$ activation (Siess and Lapetina, 1989b; Crouch and Lapetina, 1988; Salari et al., 1990;Walker et al.,

1990). Moreover, direct evidence for PKC involvement comes from studies using PKC-specific activators and inhibitors. Exogenous phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), directly activate PKC by acting as a non-labile substitute for DAG. Addition of PMA to platelets results in $\alpha_{IIb}\beta_3$ activation, leading to platelet aggregation and spreading on fibrinogen and vWf matrices (Marguerie et al., 1980; Shattil and Brass, 1987). Conversely, inhibition of PKC with specific inhibitors, such as bisindoylmaleimide (BIM), blocks $\alpha_{IIb}\beta_3$ activation, platelet aggregation, and platelet spreading on fibrinogen and vWf matrices (Shattil and Brass, 1987). Finally, activation of $\alpha_{IIb}\beta_3$ and subsequent platelet aggregation correlates with the phosphorylation of a 47-kDa protein called pleckstrin. This protein is exclusively expressed in haematopoietic cells and is a major PKC substrate in platelets.

Several translocation studies have demonstrated that different platelet agonists are able to preferentially activate different PKC isozymes. Thrombin and PMA have been shown to activate PKC α, β and ζ, PAF activates η and θ, and the Mab-F11 activating antibody has been found to cause transient activation of α and ζ and prolonged activation of α, β, η, and θ (Baldassare et al., 1992; Crabos et al., 1991; Wang et al., 1993; Wang et al., 1995). While these studies indicate specific isozyme activation following platelet activation with different stimuli, definitive information is lacking on the identity of the specific PKC isozymes responsible for $\alpha_{IIb}\beta_3$ regulation. Furthermore, although several signalling molecules have been identified as downstream targets of PKC in platelets, including pleckstrin and myrisoylated alanine-rich C kinase substrate (MARKS), their role in inside-out signalling has yet to be identified.

In addition to the wealth of evidence implicating PKC as a critical component of the inside-out signalling cascade in platelets, there is increasing evidence to support a role for PKC in downstream (outside-in) signalling events linked to integrin $\alpha_{IIb}\beta_3$ ligation and

platelet aggregation (Shattil et al., 1994). For example, platelet integrin $\alpha_{IIb}\beta_3$ engagement is known to mediate platelet spreading on fibrinogen and to induce tyrosine phosphorylation of the focal adhesion kinase, pp125FAK. PKC has been shown to play an important role in both of these processes. Pharmacological inhibitors of PKC activation were found to inhibit both platelet spreading and FAK phosphorylation on fibrinogen (Haimovich et al., 1996). It has also been demonstrated that platelet spreading on fibrinogen and FAK phosphorylation can be blocked using the ADP scavenging enzyme, apyrase (Haimovich et al., 1993). Treatment of platelets with PMA, which directly activates PKC, was found to effectively restore platelet spreading on fibrinogen and FAK phosphorylation in apyrase treated platelets (Haimovich et al., 1996). Furthermore, it was recently demonstrated that PKC inhibition abrogates platelet adhesion and spreading on a vWf matrix under both static and flow conditions thus establishing a critical role for PKC in promoting integrin $\alpha_{IIb}\beta_3$ activation on the surface of platelets adhering to vWf (Yap et al., 2000).

1.12.4 Pleckstrin (p47) and MARCKS.

Pleckstrin is a major PKC substrate found in platelets and other haematopoietic cells. In most cases, phosphorylation of the 47 kDa substrate of PKC (p47) accompanies platelet secretion and aggregation (Haslam et al., 1979). Two functionally important domains have been identified in pleckstrin, a pleckstrin homology (PH) domain, which is believed to target pleckstrin binding to phosphoinositides, G-proteins, and PKC; and the DEP-domain which is thought to regulate GTP-GDP exchange by Ras-like molecules (Ponting and Benjamin, 1996). Following platelet activation, pleckstrin is thought to interact directly with the activated forms of PKC through its PH domain (Zhang et al., 1996). Once bound, PKC may phosphorylate pleckstrin on three closely localised residues (Ser113, Thr114, and Ser117), enabling it to localise to membranes where its DEP domain

is proposed to regulate GTP-GDP exchange on one or more Ras-like molecules. While Ras proteins appear to have a role in $\alpha_{IIb}\beta_3$ activation, it is unclear if they have a positive or negative effect on $\alpha_{IIb}\beta_3$ activation process. For example, in $\alpha_{IIb}\beta_3$ transfected CHO cells R-Ras activates $\alpha_{IIb}\beta_3$ whereas H-Ras suppresses activation (Gabbeta et al., 1996; Hughes et al., 1997). The strongest evidence to date that pleckstrin can promote $\alpha_{IIb}\beta_3$ activation is derived from the study of a patient with a mucutaneous bleeding disorder who showed impaired platelet aggregation and secretion associated with a reduced pleckstrin phosphorylation (Gabbeta et al., 1996). Another PKC substrate in platelets is a protein called myristoylated alanine-rich C kinase substrate (MARCKS). MARCKS has been shown to bind and cross-link actin filaments in platelets (Elzagallaai et al., 2000), and although to date MARCKS has only been implicated in the platelet secretion pathway, this does not exclude a role for MARCKS in inside-out signalling. MARKS and pleckstrin have different phosphorylation profiles, implying different functions for the two signalling proteins.

CHAPTER 2:

METHODS

Chapter 2.

Methods.

2.1 Blood Collection and Preparation.

For all studies blood was collected by venesection from the antecubital vein of healthy volunteers who had not taken any anti-platelet medication in the preceding fortnight. For washed platelet preparations, blood was collected into acid-citrate-dextrose (ACD) anticoagulant containing 100 mM theophylline. For whole blood studies, blood was collected into either 15 mM trisodium citrate or 200 U/ml of the α-thrombin inhibitor, hirudin. Blood was kept at room temperature without agitation and was used within the four hours following collection.

2.1.1 Preparation of Washed Human Platelets.

Whole blood was drawn into syringes containing a 1:10 ratio (v/v) of acid-citrate-dextrose (ACD) anticoagulant containing theophylline [90 mM sodium citrate, 70 mM citric acid, 140 mM dextrose, 100 mM theophylline, pH 4.6]. Platelet Rich Plasma (PRP) was isolated from whole blood by centrifugation of anticoagulated blood at 200 x *g* for 30 minutes. Platelets were isolated by further centrifugation of PRP at 2,000 x *g* for 10 minutes. The platelet pellet isolated by this procedure was then resuspended in one-tenth of the original whole blood volume in Platelet Washing Buffer (PWB) [4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, pH 6.5, 113 mM NaCl, 5.5 mM glucose, 0.5% (w/v) bovine serum albumin (BSA) and 10 mM theophylline]. Immediately prior to use, platelets in PWB were pelleted by centrifugation at 2,000 x *g* for 10 minutes, resuspended in modified Tyrode's buffer [10 mM HEPES, pH 7.5, 12 mM $NaHCO_3$, 137 mM NaCl, 2.7 M KCl, 5 mM

glucose] supplemented with ! mM $CaCl_2$ or 1 mM EGTA and 1 mM $MgCl_2$, where indicated, and equilibrated for 10 minutes at 37°C.

2.1.3 Preparation of Washed Red Blood Cells.

After isolation of PRP (section 2.1.1), the remaining red blood cell (RBC) pellet was washed three times in RBC washing buffer [140 mM NaCl, 10 mM HEPES, pH 7.4, 5 mM glucose]. The RBC's were resuspended in an equal volume of RBC washing buffer and pelleted by centrifugation at 2000 x *g* for 10 minutes. The washed RBC suspension was supplemented with 0.025 U/ml apyrase (ADPase activity), and 1 U/ml of the α-thrombin inhibitor, hirudin, to remove and ADP and/or thrombin that may have been generated during RBC washing procedure.

2.1.5 Preparation of Human Serum.

Whole blood was collected into glass tubes in the absence of anticoagulant or platelet inhibitors, and allowed clot at 37°C for 3 hours. The fibrin rich clot was then removed manually from the tube using a thin wooden taper, and the supernatant was clarified of any remnant cells and debris by centrifugation at 2000 x *g* for 10 minutes. The resulting serum was heat-inactivated at 56°C for 30 minutes, to denature complement enzymes and thrombin. Heat inactivated human serum was cooled and stored in small aliquots at –20°C. Adhesive matrices in all experiments were blocked with 10% serum in Tyrode's buffer supplemented with 50 µg/ml of phenylmethylsulphonyl fluoride (PMSF) to inhibit residual thrombin activity.

2.2 Preparation of Adhesion Proteins.

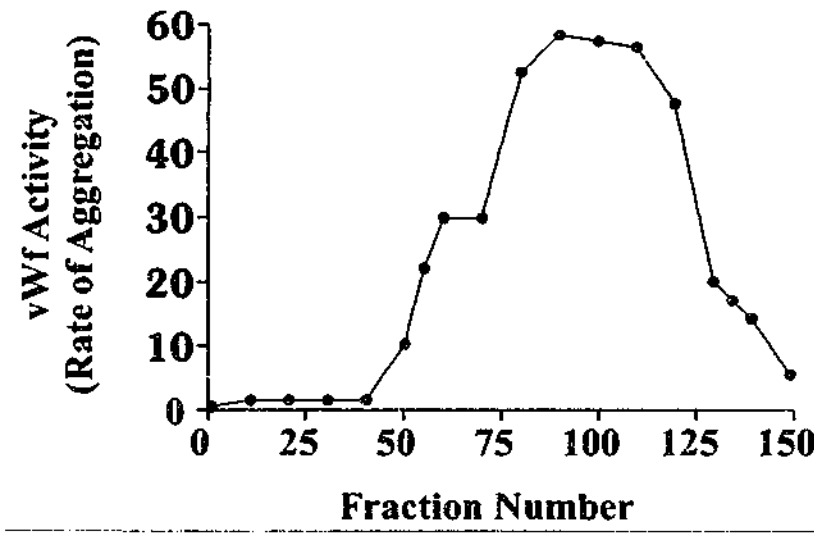
2.2.1 Purification of von Willebrand Factor (vWf).

von Willebrand factor was purified from human plasma according the method of (Montgomery and Zimmerman, 1978). Cryoprecipitate was produced from 20 litres of pooled human plasma, by initially freezing the plasma at –70°C and then allowing it to thaw at 4°C over 24 – 36 hours. The insoluble cryoprecipitate formed at 4°C was pelleted by centrifugation at 15000 x *g* for 1 hour at 4°C and dissolved at one-tenth of the original plasma volume in Tris-saline buffer containing protease inhibitors [20 mM Tris, pH 7.2, 150 mM NaCl, 1 mM EDTA, 10 mM benzamidine]. Lipoproteins were removed from the cryoprecipitate by density centrifugation, 25% (w/v) sucrose was added to the resuspended cryoprecipitate and this was then centrifuged at 100,000 x *g* for 60 minutes at room temperature. Using this method, the relatively low-density lipoprotein fraction was concentrated in the upper layer of the supernatant and was easily decanted from the clarified cryoprecipitate. The clarified solution was loaded onto a Tris-saline equilibrated Sepharose CL-6B size exclusion column and eluted with Tris-saline buffer. Multiple 20 ml fractions of eluent were collected and analysed for the presence of vWf antigen using ristocetin-induced washed platelet aggregations (fig. 2.1a) followed by SDS-PAGE analysis (fig. 2.1b). The purest fractions (determined by SDS-PAGE analysis) showing the greatest vWf activity were pooled and concentrated using a YM-30 membrane to a final concentration of approximately 100 µg/ml. Small aliquots (1 ml) were stored at -20°C prior to use. The multimeric composition of different purified vWf preparations was periodically assessed by native SDS-agarose gel to exclude any differences between preparations.

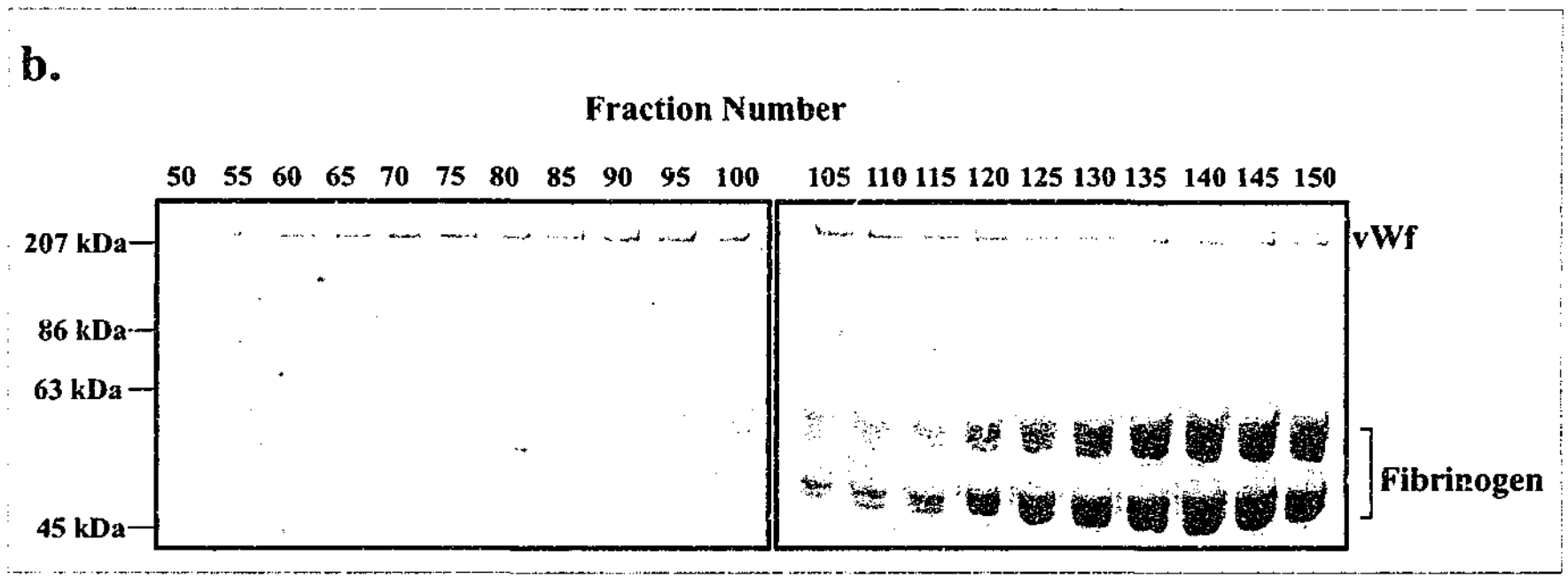
Figure 2.1 Analysis of purified vWf

vWf was purified from clarified human cryoprecipitate by size-exclusion chromatography using Tris-saline equilibrated sepharose CL-2B. 200 x 20 ml fractions were collected and the functional activity of eluted fractions was determined in ristocetin-induced platelet aggregation assays (a). The purity of active fractions was ascertained by SDS-PAGE (b). Purified vWf bands and contaminating fibrinogen bands are indicated.

a.


vWf Activity
(Rate of Aggregation)
60
50
40
30
20
10
0
0
25
50
75
100
125
150
Fraction Number

b.


Fraction Number
50 55 60 65 70 75 80 85 90 95 100
105 110 115 120 125 130 135 140 145 150
207 kDa
86 kDa
63 kDa
45 kDa
vWf
Fibrinogen

2.2.2 Purification of Fibrinogen.

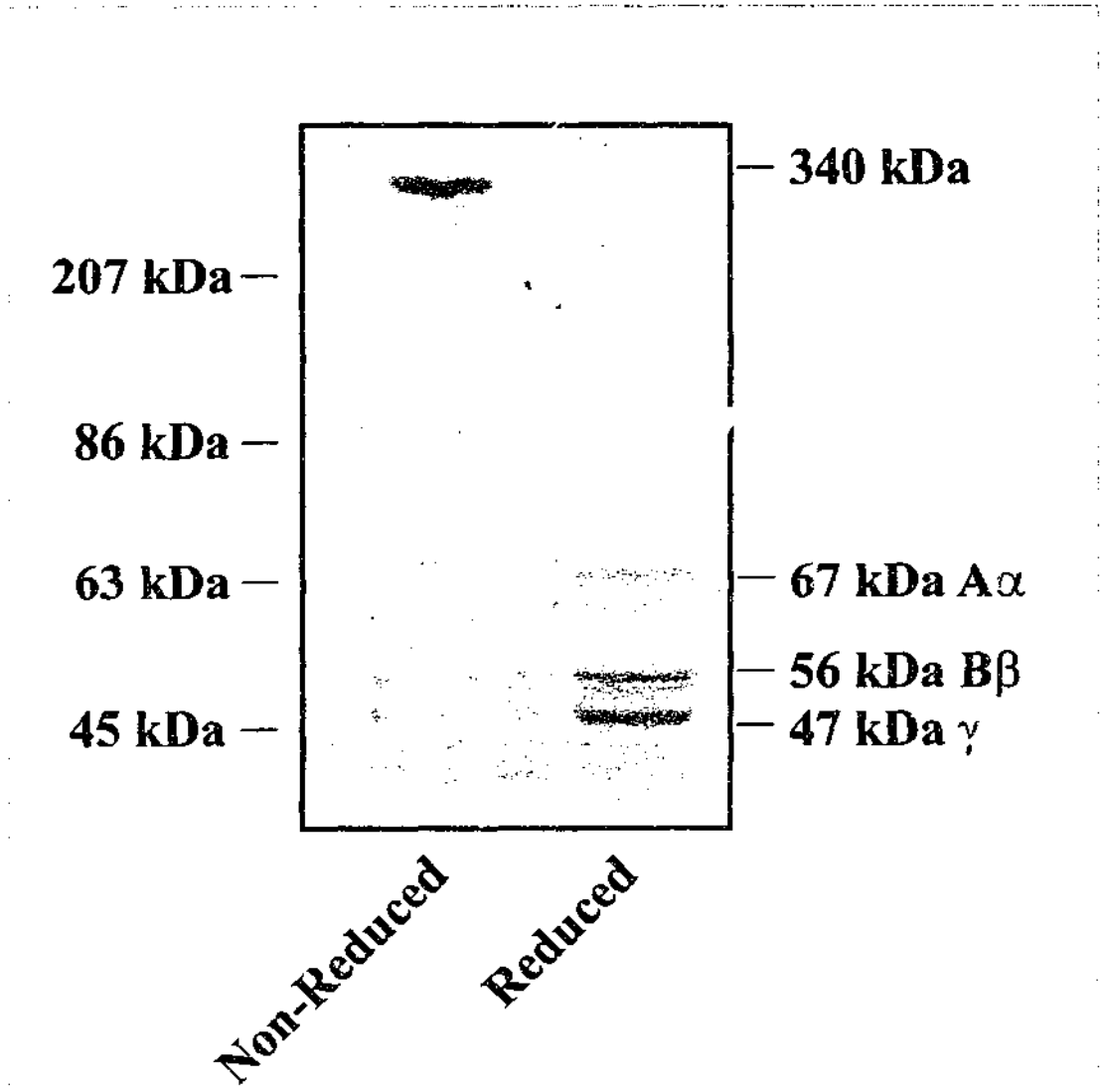
Fibrinogen was purified from human Plasma using the method of (Jackobsen, 1970). Initially vWf and fibronectin were removed by adding 250 ml of 6 M β-alanine drop-wise to 1 litre of human plasma while stirring continuously for 60 minutes at room temperature. The resulting precipitate was then pelleted by centrifugation at 2,000 x *g* for 30 minutes. The pellet was discarded and a further 375 ml of 6 M β-alanine was stirred into the remaining supernatant for 30 minutes to precipitate fibrinogen from the solution, and this was then centrifuged at 5,000 x *g* for 30 minutes. The supernatant was discarded and the fibrinogen enriched pellet was resuspended in 250 ml of Buffer A [20 mM Tris, pH 7.4, 150 mM NaCl, 25 µg/ml PMSF, 1 mM benzamidine, 10 mM ε-amino-n-caproic acid]. Fibrinogen was re-precipitated from the Buffer A solution by adding 750 ml of 6 M β-alanine and stirring overnight at 4°C. The fibrinogen was again pelleted by centrifugation at 5,000 x *g* for 30 minutes, and resuspended in another 250 ml of Buffer A. Prior to use, the fibrinogen preparation was dialysed against Buffer B [20 mM Tris, pH 7.4, 150 mM NaCl] for 24 hours at 4°C. The purity of the fibrinogen was assessed by SDS-PAGE, and the functional activity of the preparation was determined using a clotting assay as well as platelet spreading and aggregation assays. SDS-PAGE of the purified fibrinogen showed three bands with molecular weights of 66.5, 52.5 and 46.5 kDa corresponding to the Aα, Bβ and γ subunits of fibrinogen respectively (fig. 2.2) (Doolittle et al., 1979).

2.2.3 Preparation of Purified Collagen.

Type I fibrillar collagen was prepared using a modified method of (Cazanave, 1983). 2.5 mg/ml (w/v) of lyophilised type I fibrillar collagen derived from equine tendon was reconstituted in 3% acetic acid (v/v) overnight at 4°C, and the resulting highly particulate solution was homogenised on ice using a tissue homogeniser equipped with a

Figure 2.2 Analysis of purified fibrinogen

Fibrinogen was purified from pooled plasma samples. Image of purified fibrinogen subjected to SDS-PAGE under reduced and non-reduced conditions illustrating bands corresponding to intact fibrinogen molecule and the individual Aα, Bβ and γ chains.


340 kDa
207 kDa
86 kDa
63 kDa
67 kDa Aα
56 kDa Bβ
45 kDa
47 kDa γ
Non-Reduced
Reduced

fine cutting blade. The homogenate was centrifuged at 3000 x g at room temperature to remove remnant particulate material and the supernatant stored at 4°C until further use.

2.3 Analysis of Purified and Cellular Proteins.

2.3.1 Estimation of Protein Concentration.

Protein concentrations were measured using the Bio-Rad protein assay described by (Bradford, 1976). Absorbance readings at a visible light wavelength of 595 nm were measured using spectrophotometry, and compared to standard curves prepared from known concentrations of BSA.

2.3.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE protein analysis was performed according to the method of (Laemmli, 1970) using a Bio-Rad Mini-Protean II dual slab gel apparatus. A 7.5 % resolving gel consisting of; 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 7.5% (w/v) acrylamide:bis-acrylamide (30:0.8), 0.7 mg/ml ammonium persulphate and 1.0% (v/v) TEMED, was prepared and poured into the gel casting apparatus. This was overlaid with distilled water and allowed to set for 30 minutes. A stacking gel consisting of; 0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 4% (w/v) acrylamide:bis-acrylamide (30:0.8), 0.7 mg/ml ammonium persulphate, 1.0% (v/v) TEMED, was poured over the preset resolving gel in the presence of a gel comb and allowed to set for 30 minutes. Protein samples were mixed with an equal volume of 2X reducing buffer [0.5 ml of 0.25 M Tris-HCl, pH 6.8, 0.2 ml glycerol, 0.6 ml of 25% SDS (w/v), 0.04 ml of 0.05% bromophenol blue, 0.1 ml β-mercaptoethanol], and boiled at 100°C for 5 minutes to denature the proteins. The boiled protein samples were then loaded into individual wells of the stacking gel and were resolved in the presence of

SDS-PAGE running buffer [25 mM Tris, pH 8.7, 192 mM glycine and 0.1% (w/v) SDS] at a constant voltage of 200 volts (variable current), for 45 minutes.

2.3.3 Coomassie Brilliant Blue Staining of SDS-PAGE Gels.

To visualise protein bands, SDS-PAGE gels were bathed in Coomassie Brilliant Blue staining solution [10% (v/v) acetic acid, 25% (v/v) methanol, 0.2% (w/v) Coomassie Brilliant Blue] for 15 minutes. Following Coomassie Brilliant Blue staining, the gels were washed in a destain solution [10% (v/v) acetic acid, 25% (v/v) methanol] until only protein bands were stained with Coomassie Brilliant Blue and areas of the gel not containing protein material were clear. The gels were washed in water to remove acetic acid and dried onto blotting paper under vacuum.

2.4 Studies with Platelets in Suspension.

2.4.1 Platelet Aggregation Assays.

Washed human platelets were resuspended in 400 µl Tyrode's buffer containing 1 mM $CaCl_2$ at a final concentration of 3 x 10^8/ml prior to stimulation. For vWf aggregations, 20 µl of each fraction eluted from the size exclusion column were added to the final platelet suspension and aggregation was initiated by the addition of 1 mg/ml ristocetin in the presence of stirring (950 rpm) at 37°C. Aggregation was monitored for 10 minutes. In agonist-induced aggregation studies, a final concentration of 0.5 mg/ml of purified fibrinogen was included in the washed platelet suspension and aggregation was initiated by stimulation with 12.5 µM ADP, 200 nM PMA or 5 µg/ml acid soluble type I collagen. Thrombin (1 U/ml) induced platelet aggregation studies were performed in the absence of fibrinogen. The extent of platelet aggregation was expressed arbitrarily as the

percentage change in light transmission, as measured by an automated platelet aggregation analyser, were an increase in light transmission correlates with a reduction in single platelet count in the platelet sample.

2.4.2 Pleckstrin Phosphorylation Assays.

Washed platelets were resuspended in phosphate free Tyrode's buffer supplemented with 10 mM theophylline and loaded for 2 hours with 0.5 µCi of [^{32}P]-P_i. They were then washed three times to remove unincorporated radioactive label. [^{32}P]-P_i-labelled platelets were then incubated with the indicated concentrations of c7E3 Fab, GF109203X or Apyrase, before stimulation. In suspension based assays, [^{32}P]-P_i-labelled platelets were exposed for 5 minutes to PMA or thrombin as indicated, and in adhesion based assays, [^{32}P]-P_i-labelled platelets were applied to a purified vWf matrix for 60 minutes as described below under static adhesion assays. Whole platelet lysates were prepared by directly lysing the platelets in reducing buffer (supplemented with 2 mM EDTA), and these were subjected to SDS-PAGE and autoradiography for detection of [^{32}P]-Pi incorporation. Protein standards of known molecular weight were electrophoresed along-side the platelet protein samples, and the sizes of unknown protein bands were estimated in comparison to the relative mobility of the known protein standards according to the method of (Shapiro, 1976).

2.5 Static Adhesion Assays.

Static adhesion assays were performed using a modified method of (Yuan et al., 1997). Round glass coverslips were coated with human vWf (10 µg/ml) or fibrinogen (100 µg/ml) or type I fibrillar collagen (2.5 mg/ml) overnight at 4°C. Unbound proteins were washed from the coverslips with Tyrode's buffer and any exposed glass was blocked with

10% human serum for 30 minutes at room temperature. Excess blocking solution was also removed by washing with Tyrode's buffer. Washed platelets (1 x 10^7/ml) were allowed to adhere and spread on the matrix for 60 minutes under static conditions in Tyrode's buffer supplemented with 1mM $CaCl_2$ + 1 mM $MgCl_2$ and 1μg/ml PAC-1. In some studies, platelets were incubated with the anti β3 antibody, c7E3 Fab (20 μg/ml), the membrane permeable calcium chelators EGTA-AM (100 μM), BAPTA-AM (100 μM) or DM-BAPTA.AM (30 or 100 μM), or the protein kinase C inhibitors GF109203X (0 – 500 nM) or Calphostin (0 – 500 nM), for 30 minutes prior to exposure to vWf or collagen coated cover slides. In experiments examining the activation of integrin $\alpha_{IIb}\beta_3$, platelets were allowed to adhere and spread in the presence of the activation specific integrin $\alpha_{IIb}\beta_3$ antibody, PAC-1 (1 mg/ml). Non-adherent platelets were aspirated at the end of the incubation period and the remaining adherent platelets fixed for 1 hour in 3.7 % formaldehyde. Fixed platelets were then stained for 30 minutes with FITC-conjugated secondary antibody and then mounted in Permafluor. The morphology of adherent platelets and the expression of platelet proteins were examined by DIC microscopy and fluorescence microscopy, respectively, using a Leica TCS-SP confocal microscope (63x water objective). For static adhesion assays involving calcium measurement, platelets were loaded with the calcium indicator dyes Oregon Green 488 BAPTA-1, AM (1 μM) and Fura Red, AM (1.25 μM), for 30 minutes at 37°C, and monitored by confocal microscopy as described in section 2.7.1 below.

2.5.2 Indirect Immunofluorescence Studies.

To examine the expression of surface proteins in suspended platelets, washed platelets were resuspended in Tyrode's buffer containing either 1 mM $CaCl_2$ or 1 mM EGTA/$MgCl_2$ and primary antibodies against the active conformation of $\alpha_{IIb}\beta_3$ (PAC-1, 2

μg/ml), or the GPIbα subunit (WM-23, 1 μg/ml), and were then stimulated with ADP, thrombin, PMA or collagen. After 5 minutes platelets were fixed in 3.7% formaldehyde for 30 minutes and washed twice with Tyrode's buffer to remove excess PAC-1. They were then incubated with FITC-conjugated sheep anti-rabbit or goat anti-mouse secondary antibody (1 μg/ml) for 30 minutes at room temperature. After removal of unbound secondary antibody, platelets were washed in phosphate buffered saline (PBS) + 1% BSA before being subjected to FACS analysis using a Becton Dickinson FACScalibur™ flow cytometer (BD Immunocytometry systems, San Jose, CA).

2.6 *In Vitro* Perfusion Studies.

2.6.1 Coating of Microcapillary Tubes with Adhesive Proteins.

For *in vitro* flow studies, glass microcapillary tubes were coated with 100 μg/ml of human vWf (hvWf) or with 2.5 mg/ml type I fibrillar collagen overnight at 4°C. Unbound protein was removed by flushing the microcapillary tubes with Tyrode's buffer. Any remaining uncoated glass surface was blocked with 10 % human serum for 30 minutes at room temperature. Excess human serum was again washed away by flushing the microcapillary tube with Tyrode's buffer. For whole blood studies, microcapillary tubes were left unblocked as it has been previously shown that platelets in whole blood do not adhere to glass under flow (Savage et al., 1998).

2.6.2 Labelling Platelets with $DiOC_6$ in Whole Blood.

$DiOC_6$ is a non-specific lipid dye belonging to the family of short (C_1-C_6) alkyl chain carbocyanines. When added to whole blood it incorporates into all phospholipid-containing membranes and emits green fluorescence when excited at 488 nm. However,

the haemoglobin found in RBCs acts as a fluorescence quencher (Savage et al., 1998) and thus, in whole blood, only platelets and leukocytes appear fluorescently labelled. In our experiments, anticoagulated whole blood was incubated with 1 μM of $DiOC_6$ for at least 10 minutes prior to use.

2.6.3 Platelet Adhesion to Purified Matrices.

In vitro flow-based adhesion studies were performed according to a modified method of (Cooke et al., 1993). Washed platelets reconstituted in 50 % RBC's and 50 % Tyrode's buffer (supplemented with $CaCl_2$ or EGTA/$MgCl_2$ and with platelet protein specific antibodies where indicated), or $DiOC_6$ labelled whole blood was perfused through microcapillary tubes coated with either human vWf or type I collagen at a defined shear rate of 1800 s^{-1}. Non-adherent cells were removed by washing the tubes with excess supplemented Tyrode's buffer. Platelet adhesion was visualised in real time by differential interference contrast (DIC) microscopy using a 63x water objective (DMIRB Leica microscope), and video-recorded for off-line analysis. In the indicated experiments, washed platelets were incubated with DM-BAPTA,AM (100 μM), NP-EGTA,AM (10 μM) or GF109203X (0.2-0.5 μM) for 30 minutes at 37°C, prior to reconstitution with red blood cells.

In control experiments, the effect of blocking integrin $\alpha_{IIb}\beta_3$ (with a 20 μg/ml of c7E3 Fab or 500 nM aggrastat, for 10 minutes) on the ability of platelets to tether to vWf was also examined. In all cases inhibition of integrin $\alpha_{IIb}\beta_3$ abolished stationary adhesion formation but did not affect the ability of platelets to tether to vWf.

2.6.4 Examination of Platelet Thrombus formation.

For thrombus studies, washed platelets were reconstituted with autologous RBC's (50% haematocrit) and plasma (anticoagulated with hirudin 200 units/ml), prior to perfusion through collagen-coated microcapillary tubes at 1800 s^{-1} for 5 minutes. Non-adherent cells were removed by washing the microcapillary tubes with Tyrode's buffer, and thrombi fixed for 1 hour with 4% formaldehyde. Fixed thrombi were stained with $DiOC_6$ overnight and imaged by fluorescence confocal microscopy. To examine the effect of cytosolic calcium chelation on thrombus growth, washed platelets were incubated with membrane permeable calcium chelators EGTA-AM, BAPTA-AM or DM,BAPTA-AM for 30 minutes prior to perfusion. Platelet adhesion and thrombus growth was measured by capturing 1 μm confocal sections in five randomly selected fields (x63; Leica TCS SP; Leica, Heidelberg, Germany). 3-D reconstruction of confocal images was performed using VoxBlast image analysis software, (Vaytek Inc., Fairfield, Iowa, USA) and the height and volume of thrombi in an individual field (26,192 μm^2) calculated using Image Tool software (University of Texas Health and Science Centre of San Antonio, University of Texas, San Antonio, Texas, USA).

2.6.5 Off-line Analysis of Platelet-matrix Interactions.

Analysis of platelet adhesion to the surface of immobilised vWf or collagen matrices, was performed by counting the total number of platelets in five, randomly chosen, visual fields at the indicated time points. The number of platelets tethering to the immobilised surface was analysed frame-by-frame (50 frames/sec) over the first 60 seconds of flow. Any cell forming an adhesion contact for greater than 40 milliseconds was scored as a tethered platelet. Stationary adhesion was defined as cells not moving more than a single cell diameter over a 10 to 30-second time period. Translocation was

defined as platelet movement greater than one cell diameter from the point of initial attachment.

2.7 Analysis of Cytosolic Calcium.

2.7.1 *In Vitro* calcium assay.

For platelet calcium studies, washed platelets in PWB (1.5 x 10^9/ml) were loaded for 30 minutes at 37°C with the membrane permeable calcium indicator dyes (OG) Oregon Green BAPTA-1 (1μM) and (FR) Fura Red (1.25 μM) according to (Yap et al., 2000). The platelets were washed twice with PWB and reconstituted in Tyrode's buffer containing either 1 mM $CaCl_2$ or 1 mM EGTA/$MgCl_2$ and 50 % washed red blood cells prior to their application to coverslides under static conditions or perfusion through vWf-coated microcapillary slides. Both of these compounds are calcium binding fluorescent dyes. The excitation wavelength of both dyes is 488 nm but each has a different emission spectra, 500 – 570 nm for OG and 600 – 710 nm for FR which allows detection of emitted fluorescence in two different channels. Increased calcium binding to OG as a result of elevated cytosolic calcium levels leads to an increase in green fluorescence intensity, while FR decreases in fluorescence intensity upon calcium elevation. The resulting ratio of OG relative to FR fluorescence can be calculated and used as an indication of cytosolic calcium levels in adherent cells. Basal calcium levels in resting platelets were obtained by analysing ~200 dye-loaded platelets resuspended in Tyrode's buffer containing 1 mM $CaCl_2$ prior to adhesion to reactive surfaces.

Platelet calcium flux in adherent platelets was monitored by recording the first 3 minutes of flow as sequential 37.5-second series (0.586 frames per second capture rate) by confocal microscopy. The fluorescence intensities in the OG and FR channels were analysed in a population of cells using MCID image analysis software OG/FR ratios were

calculated. Fluorescence ratios were then converted to relative cytosolic calcium concentration units according to equation 1,

$$\Delta[Ca^{2+}]_c = 170 \times (R\text{-}R_{min})/(R_{max}\text{-}R) \times (F_{max}/F_{min}) \quad \text{(Eq. 1)}$$

where 170 = K_d of Oregon Green Ca^{2+} binding; R = fluorescence ratio; R_{max} and R_{min} represent the fluorescence ratio of platelets that have been incubated with 50 μM A23187 + 10 mM $CaCl_2$ or 70 μM DM-BAPTA,AM + 2 mM EGTA, respectively; F_{min} and F_{max} represent the mean fluorescence values (arbitrary units) of Oregon Green BAPTA-1 fluorescence for R_{max} and R_{min} respectively. The calculated calcium flux are designated $\Delta[Ca^{2+}]_c$ to indicate that all calcium concentration estimates are relative to a zero point set by DM-BAPTA calcium chelation. Changes in OG/FR ratios were also analysed in single adherent platelets using TCS-NT Leica confocal software. In the indicated experiments, washed platelets were incubated with DM-BAPTA,AM (100 μM) or GF109203X (0.2-0.5 μM) for 30 minutes at 37°C, or with the sarco-endoplasmic reticulum calcium ATPase inhibitor, thapsigargin (25 nM) for 2 minutes prior to reconstitution with red blood cells.

2.7.2 NP-EGTA 'Caged Calcium' Assay.

In experiments examining the effect of transient calcium release on platelet adhesion, Oregon Green BAPTA-1/Fura-Red loaded platelets were incubated for a further 30 minutes at 37°C with the UV-sensitive caged calcium chelator, NP-EGTA (10μM) (Molecular Probes) as per (Nesbitt et al., 2002). These platelets were perfused through vWf-coated microcapillary tubes as described above, and the release of caged calcium induced by transient exposure to near UV (300-400 nm) light generated by a 100W Hg lamp, directed through the optical path of a Leica DMIRBE confocal microscope, for an

interval of 0.6seconds as described by (Nesbitt et al., 2002). The calculated calcium flux are designated $\Delta[Ca^{2+}]_i$ to indicate that all calcium concentration estimates are relative to a zero point set by DM-BAPTA calcium chelation. Prior to UV stimulation, NP-EGTA loaded platelets exhibited translocation and adhesive properties equivalent to untreated controls. Control studies were carried out with unloaded control platelets and demonstrated that the brief (0.6-s) UV exposure did not lead to photodynamic damage or activation of the platelets under flow.

2.7.3 Determination of Calcium in Platelets Interacting With Preformed Thrombi.

Microcapillary tubes were coated with type I equine tendon fibrillar collagen (2.5 mg/ml) overnight at 4°C as described above. Platelet thrombi were allowed to form on collagen following perfusion of washed platelets reconstituted with autologous red blood cells (50% haematocrit) and plasma for 10 minutes at 1800 s^{-1}. Washed platelets loaded with the calcium responsive dyes Oregon BAPTA and Fura Red were then perfused through the microcapillary tubes subsequent to thrombus formation. A single confocal section approximately 1 µm deep was taken at least 10 µm from the collagen matrix surface. The first 3 minutes of platelet flow within this focal plane was captured as a sequential 37.5-s series (0.586 frames per second capture rate) and analysed off-line using Leica Physiology software.

CHAPTER 3:

ABSOLUTE REQUIREMENT FOR CYTOSOLIC CALCIUM FLUX FOR PLATELET INTEGRIN $\alpha_{IIb}\beta_3$ ACTIVATION

Chapter 3.

Absolute Requirement for Cytosolic Calcium Flux for Platelet Integrin $\alpha_{IIb}\beta_3$ Activation.

3.1 Introduction.

The level of platelet activation at sites of vascular injury is influenced by three main determinants: the nature of the adhesive substrate; the presence of soluble co-stimuli; and the level of haemodynamic force experienced by the cell. Regardless of the nature of the primary platelet stimulus, a characteristic feature of platelet activation is an increase in the cytosolic calcium concentration, which has been shown to correlate with platelet secretion, spreading and aggregation. Despite the vast number of enzymatic pathways that can contribute to the process of platelet activation, most platelet agonists known to induce platelet aggregation generally induce some level of calcium mobilisation (Cavallini and Alexandre, 1994; Crabos et al., 1992; Dalla Via et al., 1996; Rosado and Sage, 2001; Sloan and Haslam, 1997). Despite the wealth of evidence supporting a key role for cytosolic calcium in regulating integrin $\alpha_{IIb}\beta_3$ activation, there are a number of reports suggesting the involvement of calcium-independent pathways in this process. For example, several studies utilising intracellular calcium chelators (Haimovich et al., 1996; Jen et al., 1996; Kuwahara et al., 1999; Quinton et al., 2002a; Rotondo et al., 1997), have concluded that cytosolic calcium is not essential for integrin $\alpha_{IIb}\beta_3$ activation induced by soluble agonists. Moreover, calcium-independent integrin $\alpha_{IIb}\beta_3$ activation has been described in several studies following stimulation with various combinations of Gi and Gq-protein coupled receptor agonists (Pulcinelli et al., 1998; Rotondo et al., 1997; Watson and Hambleton, 1989). More recently it has been demonstrated that platelets from $G_{\alpha q}$-knockout mice

undergo shape change and microaggregate formation in the absence of a measurable calcium response (Ohlmann et al., 2000). In this chapter a confocal-based imaging technique was used to examine the hypothesis that cytosolic calcium is absolutely critical for integrin $\alpha_{IIb}\beta_3$ activation in response to soluble agonist and adhesive matrix stimulation *in vitro*.

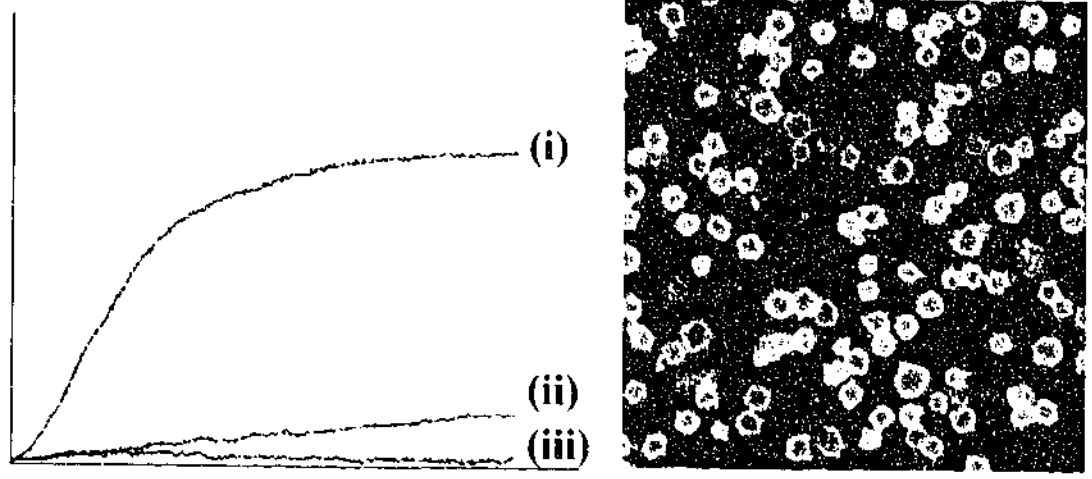
3.2 Effect of Cytosolic Calcium Chelation on Agonist-Induced Integrin $\alpha_{IIb}\beta_3$ Activation.

The absolute requirement for cytosolic calcium to support integrin $\alpha_{IIb}\beta_3$ activation was investigated initially in suspension-based platelet activation assays. In these studies, platelets were pre-treated with dimethyl-BAPTA,AM (DM-BAPTA,AM), a membrane permeable calcium chelating compound that has previously been shown to be a potent inhibitor of integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation (Jen et al., 1996; Paul et al., 1999). These platelets were then stimulated with soluble agonists in a four-channel aggregometer. Initially, the concentrations of DM-BAPTA,AM required to inhibit integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregate formation induced by weak (ADP and low dose (0.01 U/ml) thrombin) or strong soluble agonists (high dose (1 U/ml) thrombin) were examined. Integrin $\alpha_{IIb}\beta_3$ activation was assessed in two ways: indirectly, by the ability of platelets to form macroscopic and microscopic platelet aggregates, and directly, by the ability of platelets to bind the activation-specific monoclonal antibody to integrin $\alpha_{IIb}\beta_3$, PAC-1. As demonstrated in figure 3.1a and b, 30 μM DM-BAPTA,AM inhibited platelet aggregation induced by ADP (12.5μM) and low dose (0.01 U/ml) thrombin by >90%, but had minimal effect on platelet aggregation induced by high dose (1 U/ml) thrombin (fig. 3.1c). Increasing the concentration of DM-BAPTA,AM to 100μM completely abolished macroscopic and microscopic platelet aggregation induced by ADP and low dose thrombin

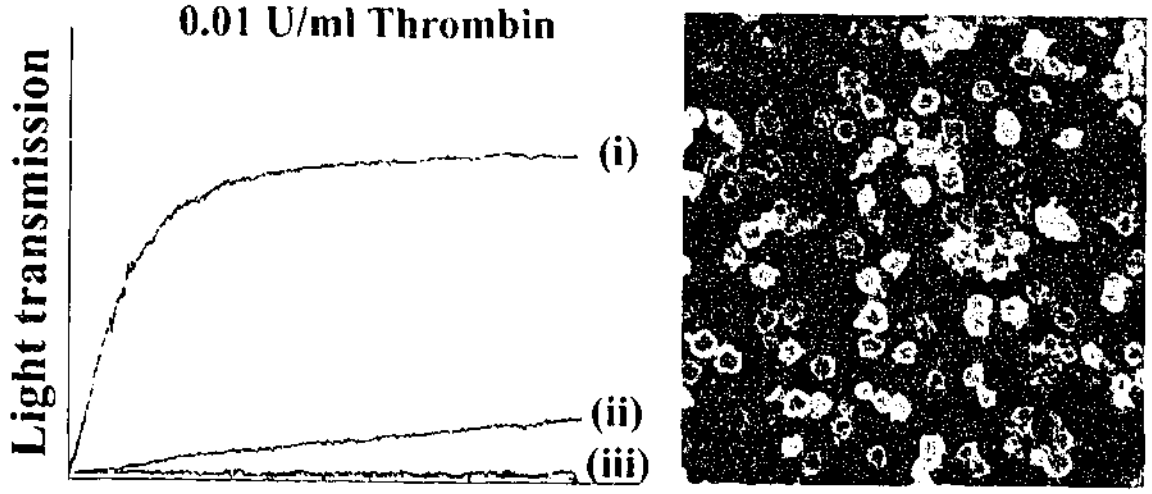
Figure 3.1 Effect of chelating cytosolic calcium on thrombin and ADP induced integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation.

Washed platelets were treated with (i) vehicle (0.25% Me_2SO), (ii) 30μM DM-BAPTA,AM (dm-B) or (iii) 100μM DM-BAPTA,AM, prior to stimulation with (a) 12.5 μM ADP, (b) 0.01 U/ml thrombin or (c)1 U/ml thrombin. Platelets were stirred continuously for 10 minutes at 37°C and the rate and extent of aggregation monitored in real-time using a four-channel platelet aggregometer. The aggregation tracings are from one experiment representative of four individual experiments. At the end of 10 minutes stimulation, platelets treated with 100μM DM-BAPTA,AM were fixed in 1% paraformaldehyde and imaged by fluorescence confocal microscopy. Red staining indicates the binding of WM23 mAb to GPIb and green staining indicates binding of PAC-1 mAb to activated $\alpha_{IIb}\beta_3$.

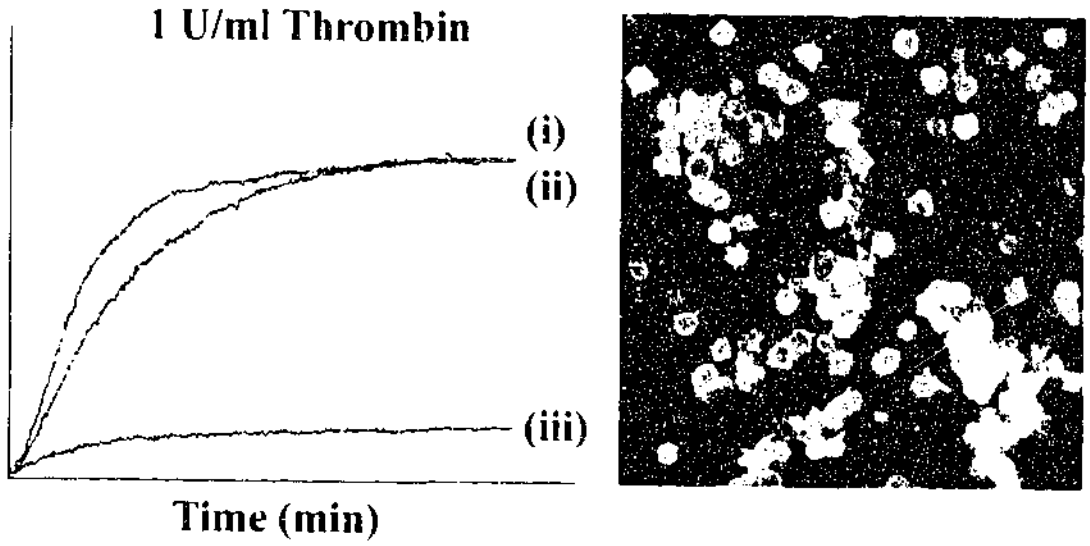
(a)



(b)



(c)



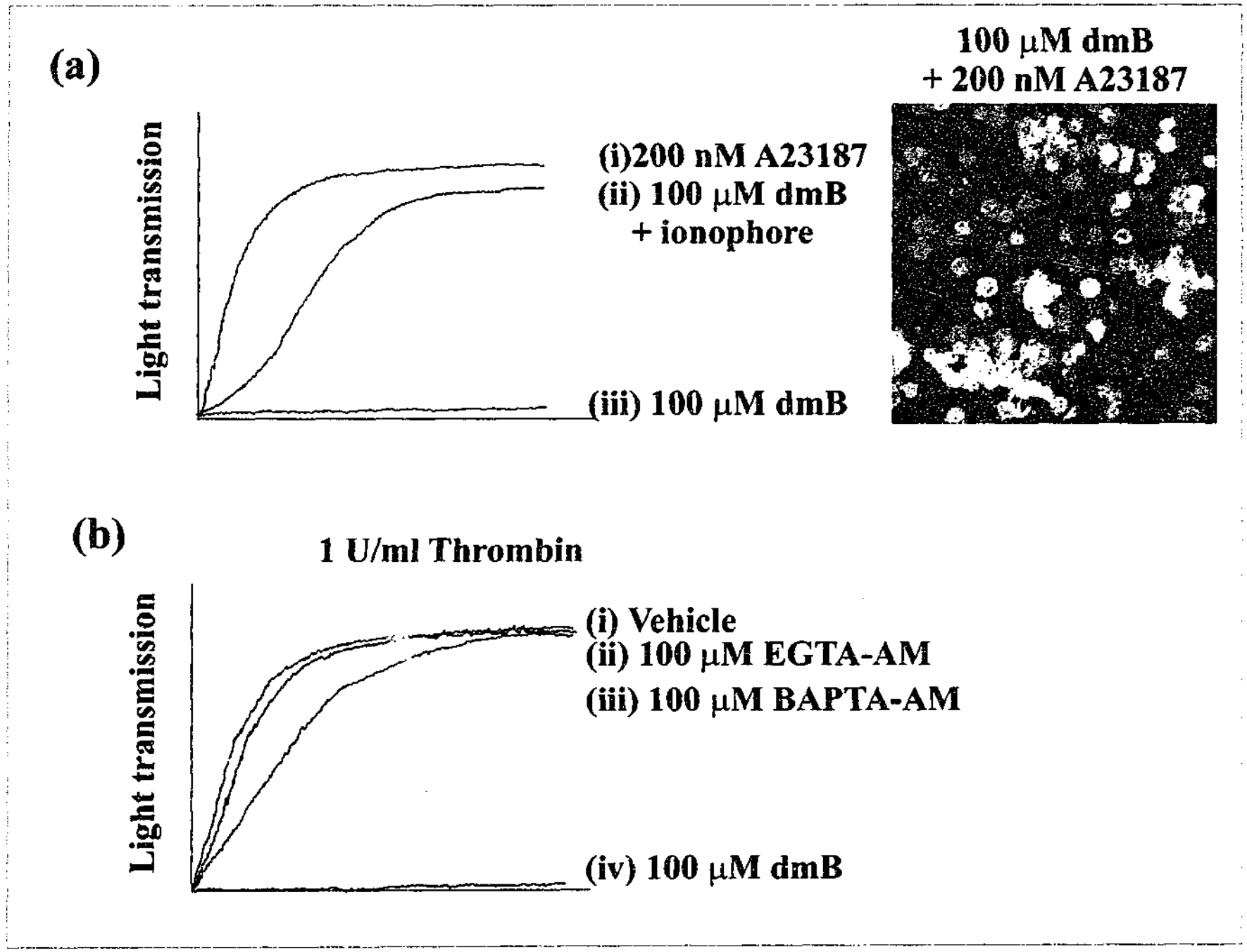
(i) Vehicle (ii) 30μM dm-B (iii) 100μM dm-B

(fig. 3.1a & b aggregation tracings), and abolished PAC-1 binding to individual cells (fig 3.1a & b fluorescence images). DM-BAPTA,AM (100μM) was also found to effectively inhibited large aggregate formation in response to high dose thrombin stimulation, however, small aggregates (micro aggregates of typically 10-20 cells) still formed and a high proportion of cells in these aggregates stained strongly with PAC-1 (fig 3.1c fluorescence images). These studies suggest that cytosolic calcium is important for platelet aggregation in response to soluble agonist stimulation and are consistent with previous studies demonstrating that DM-BAPTA,AM is a potent inhibitor of integrin $\alpha_{IIb}\beta_3$ activation in suspension-activated platelets (Jen et al., 1996; Paul et al., 1999). However, the persistence of PAC-1 binding and microaggregate formation in DM-BAPTA,AM (100μM) treated platelets in response to high dose thrombin (1 U/ml) (fig. 3.2a), raises the possibility that $\alpha_{IIb}\beta_3$ activation in some platelets in response to thrombin occurs through a calcium independent mechanism. Alternatively, calcium in these cells may not have been completely chelated with DM-BAPTA,AM.

A potential concern associated with loading platelets with high concentrations of DM-BAPTA,AM is that hydrolysis of the AM-ester by cytosolic esterases results in the release of acetic acid and formaldehyde in the cell cytosol. This may result in cell toxicity and consequently to non-specific inhibition of platelet activation. However, two lines of evidence suggest that the effects of DM-BAPTA,AM (100 μM) are likely to be due to calcium chelation, rather than a non-specific inhibitory effect of these metabolites. First, stimulating DM-BAPTA,AM-treated platelets with calcium ionophore A23187 (200 nM), induced platelet aggregation and PAC-1 binding to the surface of platelets treated with 100 μM DM-BAPTA,AM (fig. 3.2a). Second, the same concentration (100 μM) of lower affinity membrane-permeable calcium chelators, EGTA-AM (Kd 268 versus Kd 40 nM for DM-BAPTA,AM) did not inhibit platelet aggregation induced by high dose (1 U/ml)

Figure 3.2 Reversibility of the cytosolic calcium chelators DM-BAPTA,AM, EGTA-AM and BAPTA-AM by platelet stimulation with calcium ionophore or high dose thrombin.

(a) Washed platelets were treated with vehicle (0.25% Me_2SO) or 100 μM DM-BAPTA,AM (dm-B), prior to stimulation with 200 nM calcium ionophore (A23187). In (b) platelets were treated with (0.25% Me_2SO) or with 100 μM of either EGTA-AM or BAPTA-AM prior to stimulation with 1 U/ml thrombin. Platelets were stirred continuously for 10 minutes as described in figure 3.1. The aggregation tracings are from one experiment representative of four individual experiments. At the end of 10 minutes stimulation, platelets treated with 100μM DM-BAPTA,AM were fixed in 1% paraformaldehyde and imaged by fluorescence confocal microscopy. Red staining indicates the binding of WM23 mAb to GPIb and green staining indicates binding of PAC-1 mAb to activated $\alpha_{IIb}\beta_3$ (a-fluorescence image).


(a)
100 μM dmB
+ 200 nM A23187
Light transmission
(i)200 nM A23187
(ii) 100 μM dmB
+ ionophore
(iii) 100 μM dmB
(b)
1 U/ml Thrombin
Light transmission
(i) Vehicle
(ii) 100 μM EGTA-AM
(iii) 100 μM BAPTA-AM
(iv) 100 μM dmB

thrombin (fig. 3.2b). Identical results were observed using high concentrations (100 μM) of another cytosolic calcium chelator BAPTA-AM (Kd = 160 nM) (data not shown). These studies demonstrate a key role for cytosolic calcium during integrin $\alpha_{IIb}\beta_3$ activation in response to soluble agonist stimulation.

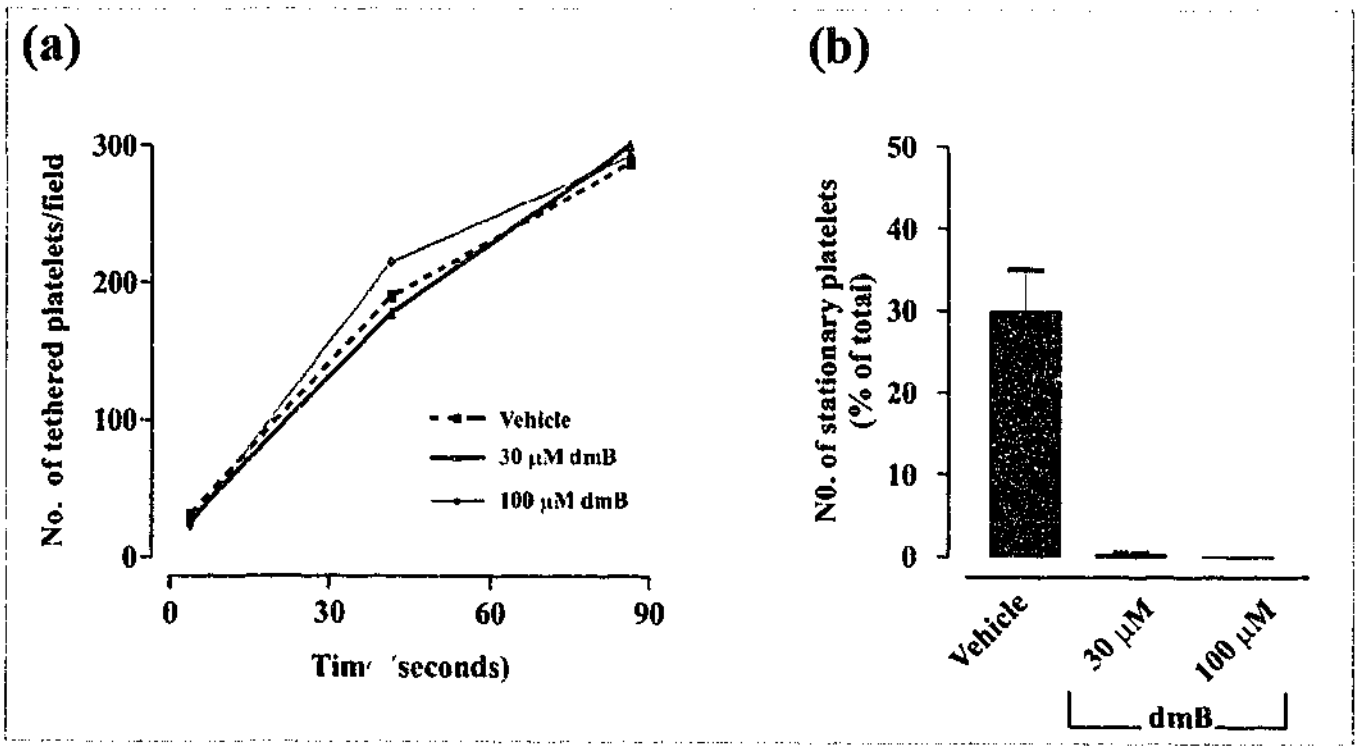
3.3 Effect of DM-BAPTA,AM on Platelet Adhesion and Integrin $\alpha_{IIb}\beta_3$ Activation Induced by vWf and Collagen.

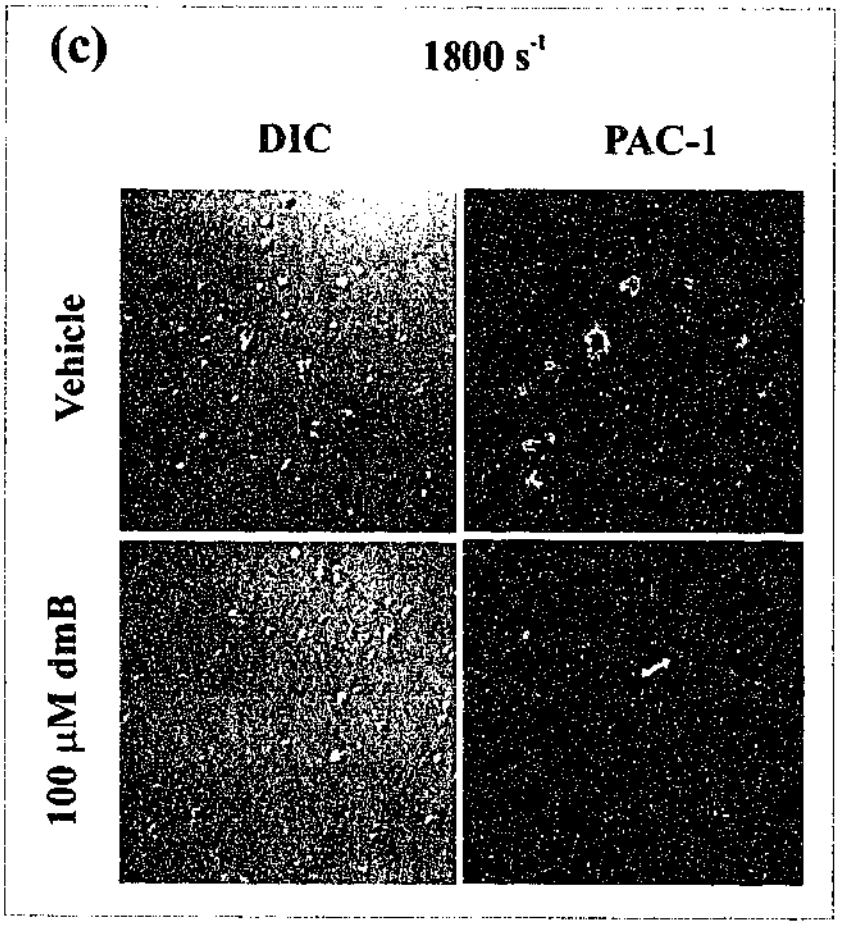
Next the requirement for cytosolic calcium flux for integrin $\alpha_{IIb}\beta_3$ activation in response to stimulation by adhesive matrices was examined. In these studies, flow based adhesion assays were performed on vWf and collagen matrices, using DM-BAPTA,AM-treated platelets. Integrin $\alpha_{IIb}\beta_3$ activation in these studies was assessed in by the ability of platelets to form stationary adhesion contacts with immobilised vWf and more directly, by the ability of platelets to bind PAC-1 following exposure to vWf or collagen.

To examine the requirement for cytosolic calcium for platelet adhesion to vWf, washed platelets were treated with vehicle (0.25% Me_2SO) or DM-BAPTA,AM (30 or 100 μM), then reconstituted with washed red blood cells (50% haematocrit) and perfused at 1800s^{-1} over purified vWf (100 μg/ml). Platelet adhesion was quantified by counting the mean number of tethered platelets in five random fields, and stationary adhesion was defined as platelets moving less than one cell diameter for a period of at least 10 seconds. Figure 3.3a shows the level of platelet tethering and figure 3.3b shows the level of stationary adhesion formation of vehicle and DM-BAPTA,AM-treated platelets perfused in the presence of PAC-1 antibody (1mg/ml), over an immobilised vWf matrix. Perfusion of vehicle treated platelets over a vWf matrix at 1800 s^{-1} demonstrated that 30 % of the tethered platelet population was able to form stationary adhesion contacts with the vWf matrix and to bind PAC-1 (fig. 3.3c), indicating that integrin $\alpha_{IIb}\beta_3$ is activated in

Figure 3.3 Effect of chelating cytosolic calcium on platelet adhesion and integrin $\alpha_{IIb}\beta_3$ activation on vWf under flow.

Washed platelets (1×10^7 cells/ml) were treated with vehicle (0.25% Me_2SO), 30 μM or 100 μM DM-BAPTA,AM (dm-B) and then perfused at 1800 s^{-1} through vWf-coated (100 μg/ml) microcapillary tubes in the presence of PAC-1 antibody (1 μg/ml) for 2 minutes. The number of platelets tethering over the first 90 seconds of flow was determined (a). Individual tethered platelets were monitored for a further 30 seconds and the percentage of platelets forming stationary adhesion contacts for a period of at least 10 seconds was determined in five random fields (b). The results represent the mean ± SEM from three separate experiments, and are expressed as a percentage relative to the results obtained from control platelets. Non-adherent cells were washed from the microcapillary tubes and irreversibly adherent platelets were fixed, labelled with a FITC-conjugated secondary antibody to PAC-1 and imaged by differential interference contrast (DIC) and fluorescence microscopy. (c) DIC images of vWf adherent platelets and fluorescence images showing PAC-1 binding to single vWf-adherent platelets.


(a)
No. of tethered platelets/field
300
200
100
0
0
30
60
90
Time (seconds)
Vehicle
30 μM dmB
100 μM dmB
(b)
NO. of stationary platelets (% of total)
50
40
30
20
10
0
Vehicle
30 μM
100 μM
dmB


(c)
1800 s-1
DIC
PAC-1
Vehicle
100 μM dmB

stationary platelets. Both 30 and 100 μM DM-BAPTA,AM, totally abolished the ability of platelets to form stationary adhesion contacts with vWf and to bind PAC-1 (fig. 3.3b & c), but did not affect GP Ib mediated tethering of platelets to the matrix (fig. 3.3c). This data demonstrates an important role for cytosolic calcium in vWf-mediated integrin $\alpha_{IIb}\beta_3$ activation and stationary contact formation on a vWf matrix.

To investigate the requirement for cytosolic calcium for integrin $\alpha_{IIb}\beta_3$ activation by collagen, flow-based adhesion assays were performed on a collagen matrix in the presence of the PAC-1 antibody in order to directly assess integrin $\alpha_{IIb}\beta_3$ activation under these conditions. As shown in figure 3.4a, vehicle treated platelets were able to adhere to collagen fibres under high shear conditions (1800 s^{-1}) and to bind PAC-1, indicating that integrin $\alpha_{IIb}\beta_3$ is activated in response to collagen binding. Loading platelets with 100 μM DM-BAPTA,AM inhibited irreversible platelet adhesion to the collagen matrix by >95% (fig. 3.4b). Unlike platelets perfused over a vWf matrix which remain tethered to the matrix even in the absence of stationary adhesion formation, the majority of DM-BAPTA,AM treated platelets interacted only briefly with the collagen matrix before detaching and returning to the bulk flow. Of the remaining 5% of DM-BAPTA,AM-treated platelets that retained the ability to adhere irreversibly to collagen fibres, all cells bound PAC-1 (fig. 3.4a). Furthermore, the importance of intracellular calcium flux for platelet adhesion and thrombus formation on a collagen matrix was assessed by perfusing DM-BAPTA,AM (100 μM) treated platelets, at high density over type I collagen at 1800 s^{-1}. Figure 3.5 (a and b) demonstrates a profound inhibitory effect of DM-BAPTA,AM on thrombus growth, suggesting an important role of cytosolic calcium during platelet primary adhesion and thrombus formation on collagen. These results highlight the critical importance for cytosolic calcium in promoting platelet adhesion to collagen, however, the existence of a residual population of collagen adherent DM-BAPTA,AM treated platelet

Figure 3.4 Effect of DM-BAPTA,AM on platelet adhesion and integrin $\alpha_{IIb}\beta_3$ activation in response to collagen.

Washed platelets (1 x 10^7 cells/ml) were treated with vehicle (0.25% Me_2SO) or 100 μM DM-BAPTA,AM (dm-B) and then perfused at 1800 s^{-1} through type I fibrillar collagen-coated (2.5 mg/ml) microcapillary tubes in the presence of PAC-1 antibody (1 μg/ml). Non-adherent cells were washed from the microcapillary tubes and irreversibly adherent platelets were fixed, labelled with a FITC-conjugated secondary antibody and imaged by differential interference contrast and fluorescence microscopy. (a) DIC images of platelets adherent to collagen fibres under flow and fluorescence images showing PAC-1 binding to single collagen-adherent platelets. (b) The percentage of platelets forming stationary adhesion contacts was determined in five random fields. The results represent the mean ± SEM from three separate experiments, and are expressed as a percentage relative to the results obtained from control platelets.

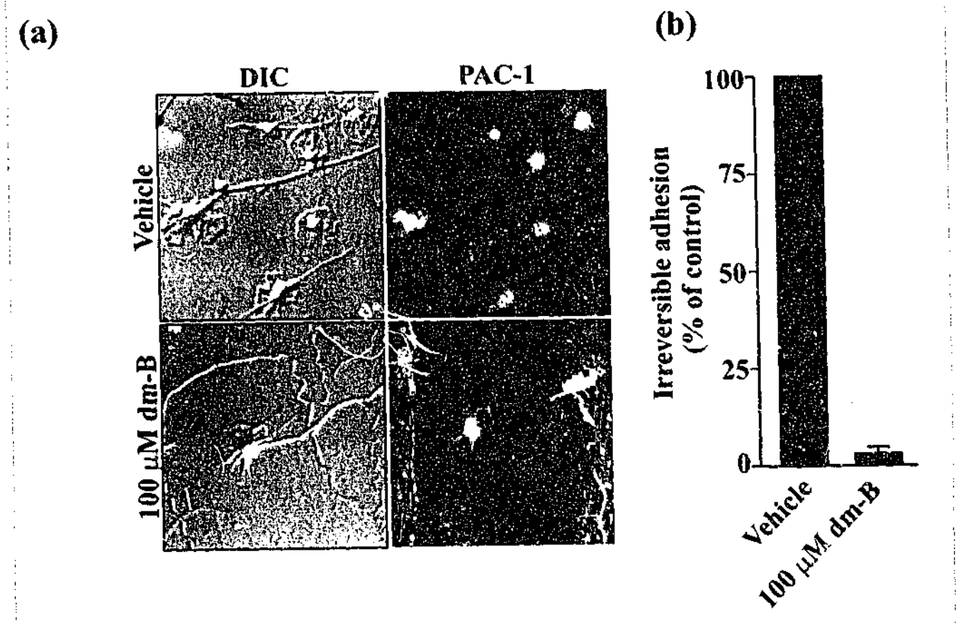
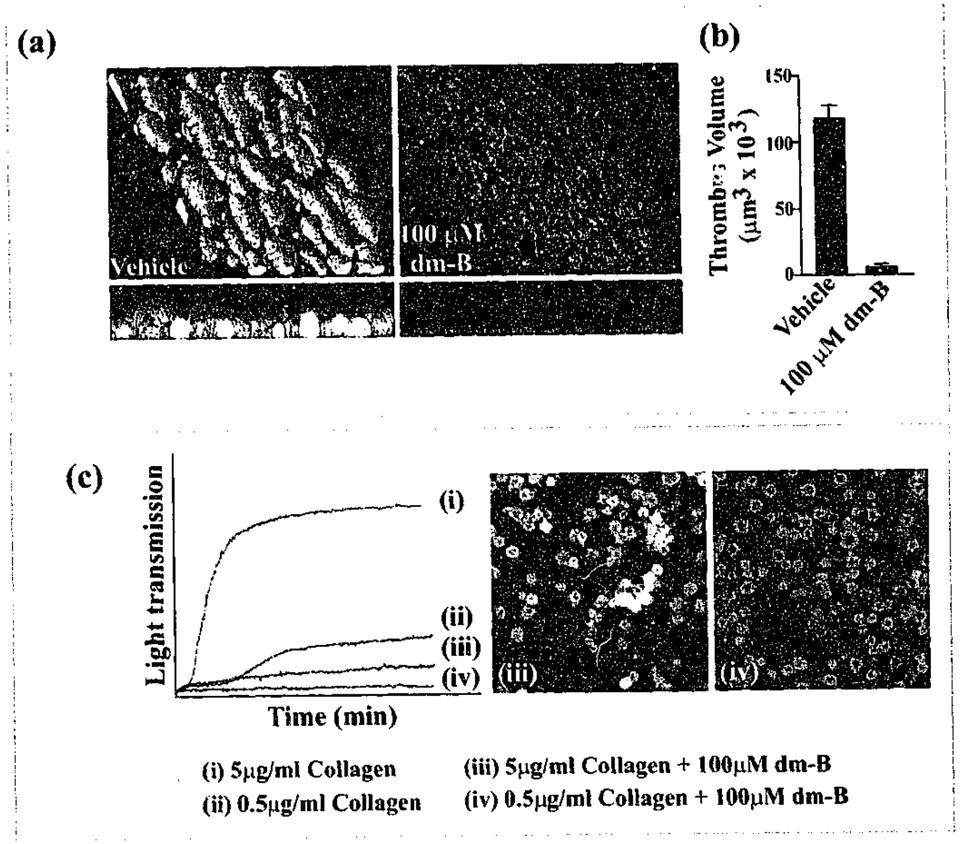


Figure 3.5 Effect of DM-BAPTA,AM on platelet thrombus formation on immobilised collagen under flow.

Washed platelets (1 x 10^7 cells/ml) were treated with vehicle (0.25% Me_2SO) or 100 μM DM-BAPTA,AM (dm-B) as indicated prior to perfusion through collagen-coated microcapillary tubes at 1800 s^{-1} for 5 minutes as described in section 2.6.4. Platelet thrombi formed after 5 minutes were subsequently imaged by confocal microscopy and reconstructed using image analysis software. (a) The upper panels show an oblique view of the imaged area to demonstrate surface coverage, while the lower panel shows a side view to demonstrate differences in thrombus height. (b) Total thrombus volume in the field of interest was calculated using image tool software. The results represent the mean ± SEM from four separate experiments. In (c), washed platelets were treated with either 0.25% Me_2SO (i and ii) or 100 μM DM-B (iii and iv). Platelet aggregation assays were then performed using collagen at 5 μg/ml (i and iii) or 0.5 μg/ml (ii and iv). Platelet aggregates were fixed in suspension and imaged by fluorescence confocal microscopy. Red fluorescence indicates binding of WM23 to GPIb and green fluorescence indicates binding of PAC-1 to activated $\alpha_{IIb}\beta_3$. The aggregation tracings are from one experiment representative of four individual experiments.



that express active $\alpha_{IIb}\beta_3$, suggest that cytosolic calcium may not be completely chelated in all platelets, or that adhesion of this platelet population to collagen occurs through the activation of calcium-independent mechanisms.

Given the profound inhibition of irreversible platelet adhesion to collagen upon DM-BAPTA,AM treatment, it was not possible to directly examine integrin $\alpha_{IIb}\beta_3$ activation on the majority of cells that tethered only transiently to the collagen surface. To assess $\alpha_{IIb}\beta_3$ activation in response to collagen stimulation in non-adherent platelets, suspension-based platelet aggregation studies were performed using acid-soluble type I collagen. As demonstrated in figure 3.5c, DM-BAPTA,AM (100 μM) inhibited macroscopic and microscopic aggregation induced by low dose collagen (0.5 μg/ml) and completely eliminated PAC-1 binding to individual cells. In contrast, stimulation of DM-BAPTA,AM-loaded platelets with high dose collagen (5 μg/ml) resulted in the formation of small platelets aggregates (~10-20 cells) that readily bound PAC-1 (Fig 3.5c fluorescence images). Together, these results suggest that cytosolic calcium is important to support activation of integrin $\alpha_{IIb}\beta_3$ in response to collagen binding.

3.4 Measurement of Cytosolic Calcium Transients during Platelet Stimulation with Soluble Agonists.

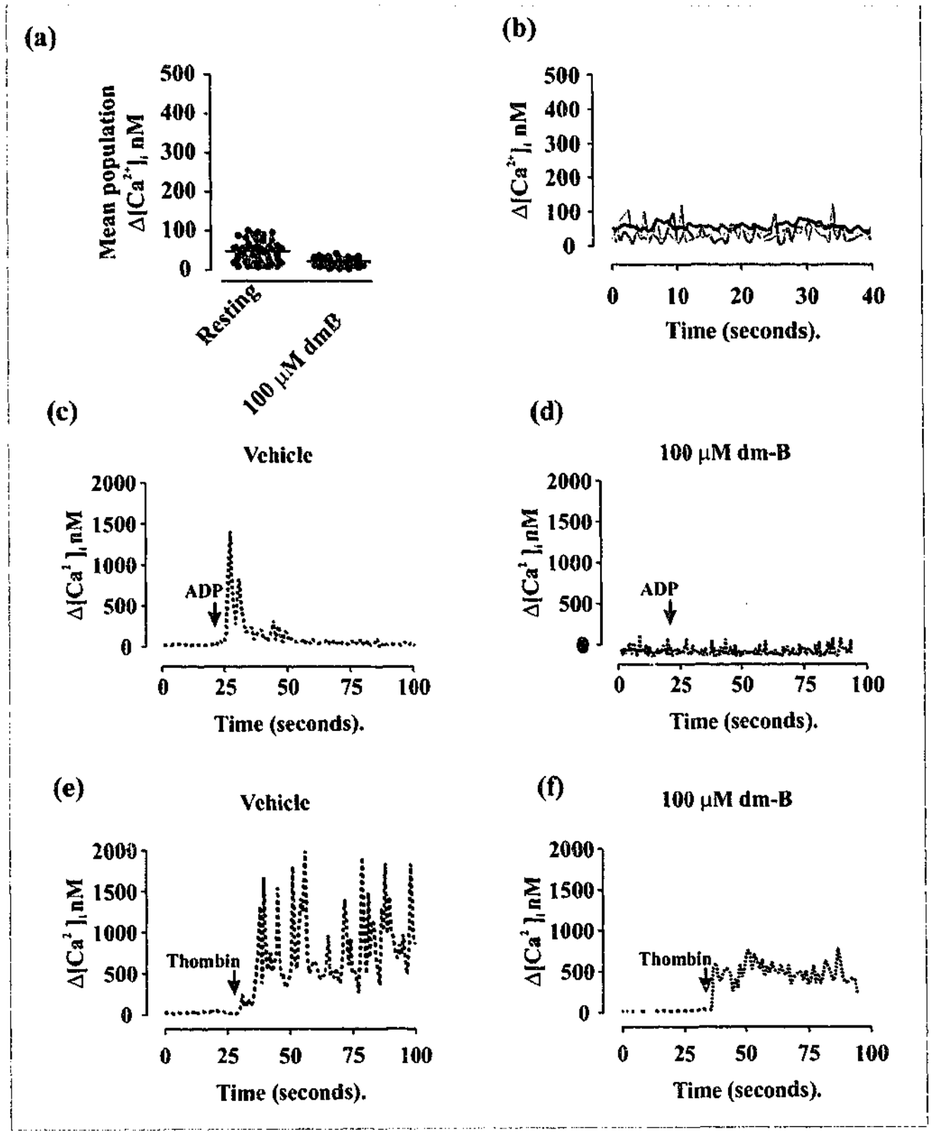
The observation that high dose (1 U/ml) thrombin (fig. 3.1c) induced small aggregate formation in DM-BAPTA,AM treated cells, and that a small population of DM-BAPTA,AM treated cells retained the ability to adhere irreversibly to collagen fibres under flow (fig. 3.4a), raised the possibility that DM-BAPTA,AM, even at high concentrations (100 μM), did not completely chelate calcium in all cells. To test this possibility, intracellular calcium levels were measured directly in suspension-based and adhesion-based platelet activation assays.

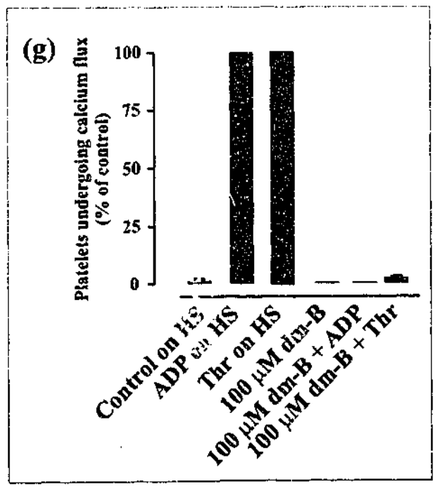
To measure cytosolic calcium changes induced by soluble agonists, a confocal based ratiometric calcium assay was used in non-stirred platelet suspensions. This assay allows direct cytosolic calcium measurements to be made both at a population level and at a single platelet level. In these studies, washed platelets were loaded with the calcium indicator dyes Oregon-green BAPTA-AM and Fura Red-AM as described in section 2.7, and allowed to settle onto a non-reactive surface (human serum blocked glass coverslides) prior to stimulation with ADP (12.5 μM) or thrombin (1 U/ml). Population analysis of cytosolic calcium levels in resting platelets demonstrated a mean basal calcium level of $\Delta[Ca^{2+}]_i$ = 50 ± 35 nM (fig. 3.6a). Figure 3.6b shows the typical non-oscillatory calcium profile of three single representative resting platelets. Addition of ADP (12.5 μM) resulted in 100 % of platelets undergoing a transient calcium response (fig. 3.6c & g) peaking at $\Delta[Ca^{2+}]_i$ = 1100nM ± 300 nM, and returning back to basal levels within 8.5 ± 3.5 seconds post ADP addition (fig. 3.6c). Pretreatment of washed platelets with 100 μM DM-BAPTA.AM reduced the mean cytosolic calcium levels of unstimulated platelets to $\Delta[Ca^{2+}]_i$ < 25 nM (fig. 3.6a), and totally abolished ADP induced calcium transients (fig. 3.6d & g). This data demonstrates that 100 μM DM-BAPTA,AM completely abolishes calcium transients induced by ADP.

Addition of 1 U/ml thrombin resulted in 100% of platelets undergoing an elevation in cytosolic calcium (fig. 3.6g). As shown in figure 3.6e, the calcium profiles of platelets stimulated with thrombin were very different from that induced by ADP. Cytosolic calcium levels in thrombin-stimulated platelets rose rapidly to levels approaching $\Delta[Ca^{2+}]_i$ = 2000 nM and remained elevated for the duration of the 2 minute monitoring period. DM-BAPTA.AM-pretreatment of thrombin stimulated platelets abolished calcium mobilisation in 98% of the platelet population (fig. 3.6g). Single cell calcium analysis of the remaining 2% of platelets that did undergo a calcium response, revealed that these platelets also

Figure 3.6 Effect of DM-BAPTA,AM on thrombin and ADP induced calcium flux in platelets.

Calcium dye loaded washed platelets were allowed to settle under static conditions onto the surface of 10% (v/v) human serum blocked cover slides. Changes in cytosolic calcium concentrations in vehicle (0.25% Me_2SO) or 100 μM DM-BAPTA,AM (dm-B) treated platelets were monitored by fluorescence confocal microscopy. The scatter-plots in (a) show the distribution of cytosolic calcium concentrations expressed in a platelet population in contact with the non-reactive surface. The data presented is from one experiment representative of five individual experiments. (Note: the mean cytosolic calcium concentration around which calcium levels oscillate is demonstrated by the thick solid line in the scatter plots). (b) Typical cytosolic calcium profiles of single resting platelets on serum blocked cover slides. Typical calcium response of single platelets treated with (c and e) vehicle or (d and f) dm-B (100 μM) upon addition of ADP (12.5 μM) (c and d) or thrombin (1 U/ml) (e and f). The arrows mark the time of agonist addition. (g) The proportion of vehicle and dm-B treated platelets undergoing defined calcium spikes was quantitated in the presence or absence of ADP (12.5 μM) or thrombin (1 U/ml). The results represent the mean ± SEM from three separate experiments, and are expressed as a percentage relative to the results obtained from control platelets.





expressed sustained cytosolic calcium levels, although the amplitude of the calcium response in DM-BAPTA,AM treated platelets was much lower (peak $\Delta[Ca^{2+}]_i$ = 500 nM) (fig. 3.6f) than observed in control platelets. This data highlights the difference in the cytosolic calcium responses induced by weak (ADP) and strong (thrombin) platelet agonists. Moreover, these results suggest that DM-BAPTA,AM does not load equally throughout the platelet population and that while it is present at saturating levels capable of abolishing calcium flux in most platelets, a subset of platelets (~2%) loads inefficiently with DM-BAPTA,AM and are able to express sufficient levels of calcium (~500nM) to support integrin $\alpha_{IIb}\beta_3$ activation and aggregation. This data supports the hypothesis that residual platelet aggregation, and integrin $\alpha_{IIb}\beta_3$ activation, in thrombin stimulated platelets treated with DM-BAPTA,AM (100 μM), is due to incomplete chelation of cytosolic calcium rather than the activation of alternative, calcium independent activation pathways.

3.5 Effect of DM-BAPTA,AM on Cytosolic Calcium Transients During Platelet Adhesion to vWf and Collagen.

To investigate more closely the relationship between cytosolic calcium levels and platelet adhesion to vWf and collagen under shear conditions, washed platelets were loaded with calcium indicator dyes and calcium levels were determined during platelet adhesion to vWf and collagen under flow conditions (see section 2.7). The advantage of this system over suspension-based calcium assays is that it allows simultaneous measurement of platelet translocation and cytosolic calcium in individual platelets. To investigate the effects of DM-BAPTA,AM on cytosolic calcium levels during platelet adhesion to immobilised vWf or collagen, calcium dye loaded platelets were perfused at a density of 5 x 10^9/L through vWf or collagen-coated microcapillary tubes at 1800 s^{-1}. To facilitate accurate correlation of platelet translocation with cytosolic calcium

measurements in individual platelets, platelets were classified as stationary if they did not move more than one platelet diameter for at least 10 seconds.

Consistent with data reported by (Nesbitt et al., 2002), perfusion of platelets over vWf revealed three sub-populations of platelets that could be divided according to their calcium profiles and translocation behaviour. A small proportion (~5%) of platelets tethering to the vWf surface were found to form prolonged and firm stationary adhesion contacts (10 seconds) with the vWf matrix and these platelets displayed sustained high-range $\Delta[Ca^{2+}]_i$ approaching 1200nM (mean = 100 nM) (fig. 3.7a). A second population of platelets tethered to the vWf matrix translocated rapidly and continuously over the vWf surface with $\Delta[Ca^{2+}]_i < 20$ nM (fig. 3.7b), and a third population consisted of platelets that translocated on the vWf matrix with a stop-start pattern in which periods of stationary adhesion coincided with the generation of transient calcium flux ranging between $\Delta[Ca^{2+}]_i$ = 20 and 65 nM (fig. 3.7c). Pretreatment of platelets with DM-BAPTA,AM (100 μM) prior to perfusion over immobilised vWf, reduced mean cytosolic calcium concentrations of the adherent population to below basal levels ($\Delta[Ca^{2+}]_i < 20$ nM) and totally abolished stationary adhesion formation on vWf (fig. 3.7d), demonstrating that stationary adhesion formation on this matrix is absolutely dependent on cytosolic calcium.

Similar perfusion studies were conducted on type I fibrillar collagen. In these experiments, 100% of vehicle treated platelets tethering to the matrix adhered essentially instantaneously and irreversibly, with all adherent cells displaying high oscillatory calcium responses approaching $\Delta[Ca^{2+}]_i$ = 2000 nM (mean calcium level of 550±50 nM; n = 5) (fig 3.8a, b & c). As shown in figure 3.8b, 94 % of platelets loaded with 100 μM DM-BAPTA,AM failed to form stationary adhesion contacts with the collagen matrix. Analysis of cytosolic calcium in DM-BAPTA,AM treated platelets showed a major reduction in the mean calcium level in the platelet population down to $\Delta[Ca^{2+}]_i$ = 45 ± 7 nM. Moreover,

Figure 3.7 Cytosolic calcium flux during platelet adhesion and translocation over vWf.

Dye loaded platelets (5 x 10^9 cells/L) were treated with vehicle (0.25% Me_2SO) or 100 μM DM-BAPTA,AM (dm-B) and then perfused at 1800 s^{-1} through vWf-coated (100 μg/ml) microcapillary tubes. Changes in cytosolic calcium levels ($\Delta[Ca^{2+}]_i$) in individual platelets were measured by confocal microscopy and correlated with translocation behaviour on vWf under flow. Single cell calcium profiles and concomitant displacement over time graphs of representative platelets are shown. (a) Typical high-range, sustained oscillatory calcium profile of platelets forming stationary adhesion contacts with the vWf matrix. (b) Represents the low level calcium profile of platelets translocating rapidly and continuously over the vWf matrix. (c) Shows the typical intermediate $\Delta[Ca^{2+}]_i$ expressed in platelets translocating on vWf with a stop-start translocation behaviour. (d) Demonstrates the inhibitory effect of cytosolic calcium chelation with dm-B (100 μM) on calcium flux and the translocation behaviour of vWf adherent platelets.

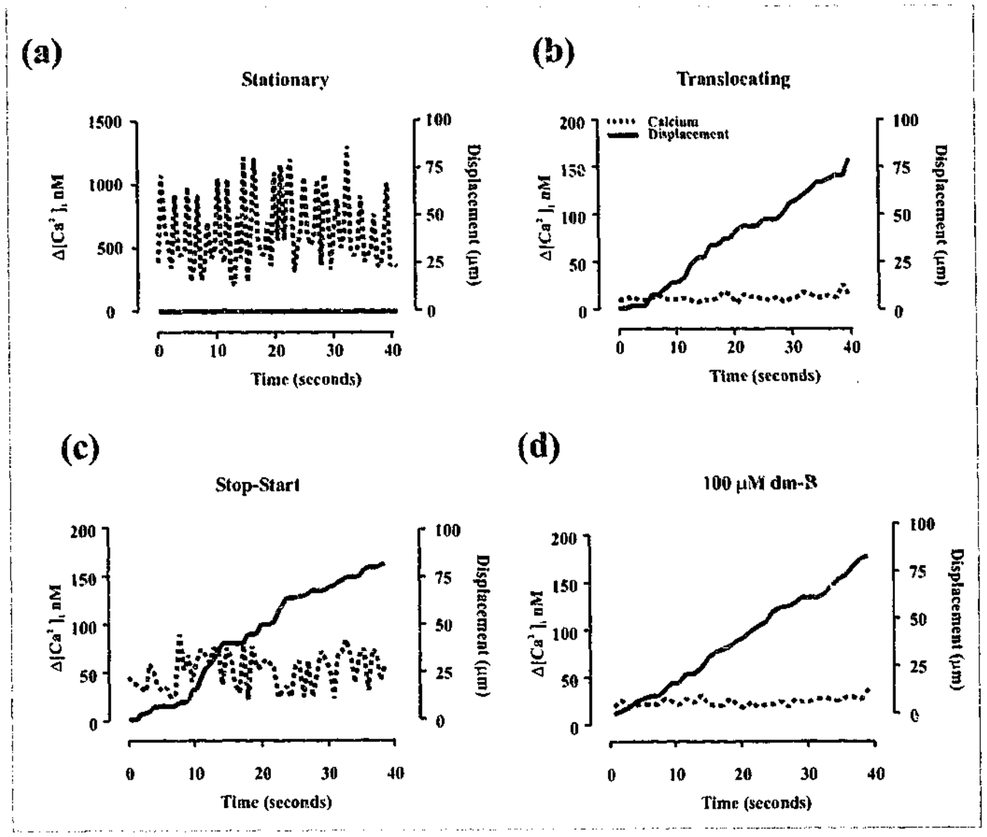
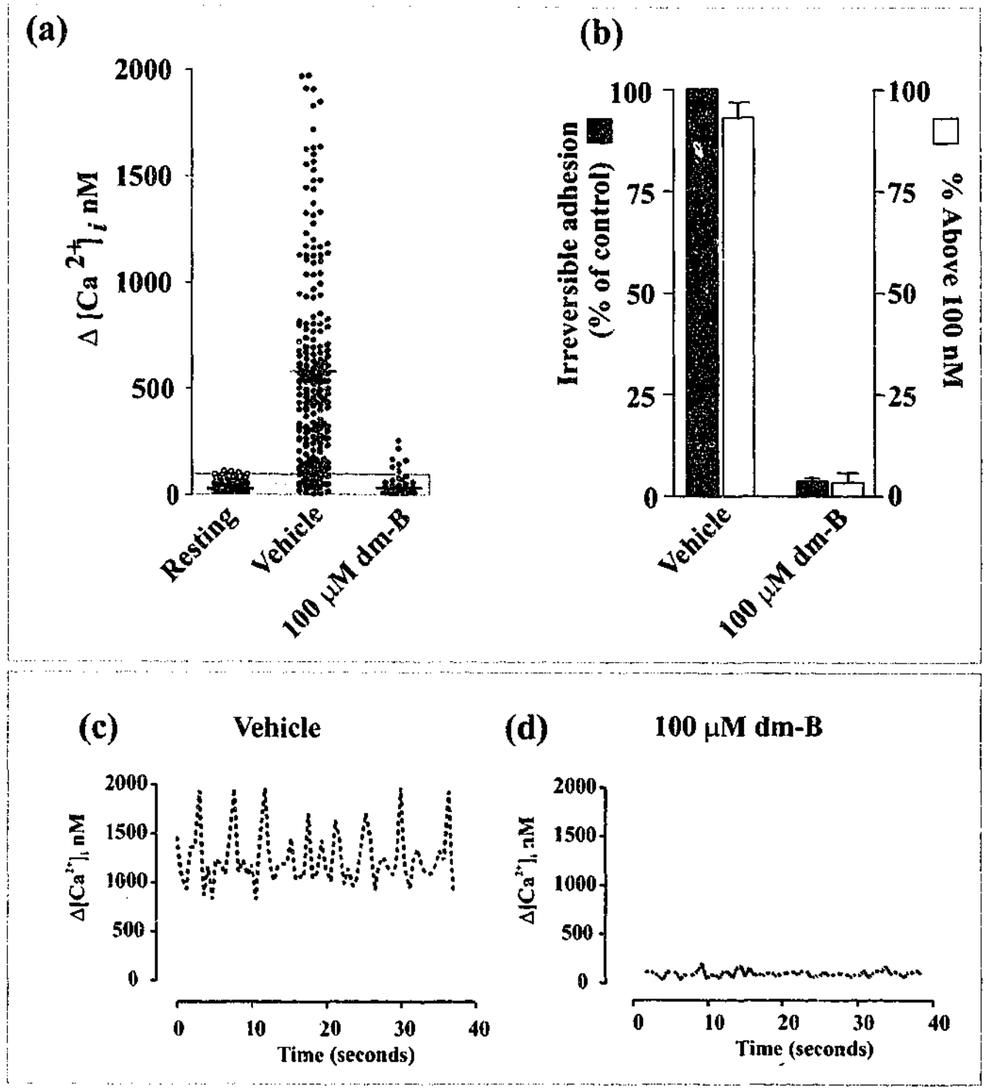


Figure 3.8 Effect of DM-BAPTA,AM on cytosolic calcium flux and platelet adhesion on immobilised collagen under flow.

Washed platelets (1 x 10^7 cells/ml) were treated with vehicle (0.25% Me_2SO), 30 μM or 100 μM DM-BAPTA,AM (dm-B) as indicated, then perfused at 1800 s^{-1} through collagen-coated (2.5 mg/ml) microcapillary tubes for 2 minutes. Changes in cytosolic calcium concentrations during platelet adhesion to collagen fibres were monitored by confocal microscopy. (a) The distribution of cytosolic calcium concentrations expressed in a resting platelet population, and in platelets adhering to collagen fibres under flow. The data presented is from one experiment representative of five individual experiments. (Note: the mean cytosolic calcium concentration around which calcium levels oscillate is demonstrated by the thick solid line in the scatter plots, while the shaded box marks the 100 nM calcium level). The proportion of recorded calcium events lying above 100 nM (grey bars, n=5) was determined and expressed as a percentage of all calcium events recorded in the adherent platelet population over a 30 second time frame (b, right axis). The level of irreversible adhesion (black bars) was determined and expressed as a percentage relative to the results obtained from control platelets. The results presented represent the mean ± SEM from five separate experiments (b, left axis). Representative single platelet oscillatory calcium flux recorded in vehicle (0.25% Me_2SO) treated (c) dm-B treated platelets (d) forming irreversible adhesions on type I fibrillar collagen.

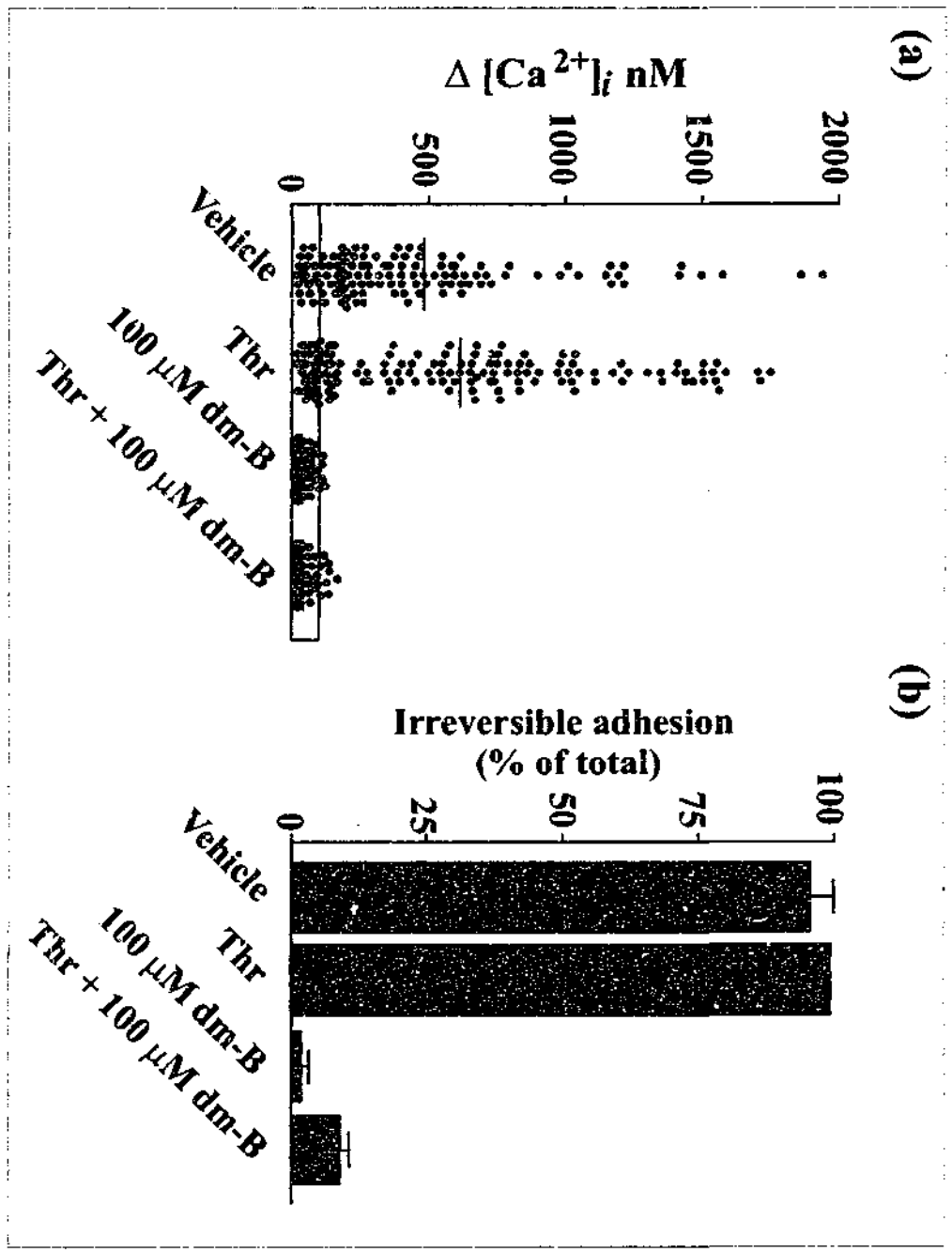


analysis of cytosolic calcium levels in the collagen adherent platelet population showed a close correlation between the percentage of platelets that retain the ability to form stationary adhesion contacts on type I collagen, and the proportion of calcium events lying above $\Delta[Ca^{2+}]_i$ = 100nM (fig. 3.8b). Subsequent single cell analysis of the remaining 6 % of cells forming stationary adhesion contacts with the collagen matrix confirmed that all cells exhibited $\Delta[Ca^{2+}]_i \geq 100$ nM (fig. 3.8d).

To investigate whether cytosolic calcium is essential for stationary platelet adhesion when these cells are exposed to multiple platelet activating stimuli, flow experiments were performed on a type I collagen matrix in the presence of thrombin (1 U/ml). In these experiments, platelets were perfused over collagen for 90 seconds and then exposed to 1 U/ml of thrombin for a further 30 seconds. As shown in figure 3.9a, addition of thrombin induced only a slight enhancement in the mean cytosolic calcium level in platelets adherent to immobilised collagen, suggesting near maximal physiological calcium release induced by collagen stimulation alone. Pretreating the cells with DM-BAPTA,AM (100μM) reduced the mean cytosolic calcium level to $\Delta[Ca^{2+}]_i$ = 52 nM and 63 nM in the absence and presence of thrombin respectively, and inhibited stationary platelet adhesion in 91 % of cells (fig. 3.9b). In the remaining 9 % of stationary cells, all cells had a residual calcium level above $\Delta[Ca^{2+}]_i$ = 100 nM. Combined with the results presented in figure 3.8, these studies provide further evidence to suggest that the inability of DM-BAPTA,AM to completely eliminate irreversible platelet adhesion (fig. 3.5a - c) and aggregation (fig. 3.1a - c) is not due to the existence of a calcium-independent pathway responsible for integrin $\alpha_{IIb}\beta_3$ activation, but due to incomplete chelation of calcium in a subset of cells.

Figure 3.9 Effect of thrombin and collagen co-stimulation on platelet adhesion and cytosolic calcium flux in platelets loaded with DM-BAPTA,AM.

Calcium dye-loaded platelets were treated with vehicle (0.25% Me_2SO) or 100 μM DM-BAPTA,AM (dm-B) prior to perfusion through collagen-coated microcapillary tubes at 1800 s^{-1}. Where indicated, adherent platelets were exposed to 1 U/ml thrombin (Thr). The scatter plots in (a) demonstrate the distribution of cytosolic calcium concentrations in the platelet population adherent to collagen fibres. The data presented is from one experiment representative of three individual experiments. (b) The number of irreversibly adherent platelets were quantitated in five random fields and the data expressed as a percentage of total adherent (reversible and irreversible) platelets. The data represents the mean ± SEM of three individual experiments.


(a)
Δ [Ca 2+]i nM
0
500
1000
1500
2000
Vehicle
Thr
100 μM dm-B
Thr + 100 μM dm-B
(b)
Irreversible adhesion
(% of total)
0
25
50
75
100
Vehicle
Thr
100 μM dm-B
Thr + 100 μM dm-B

3.6 Discussion.

The results presented in this chapter support previous findings for a central role of calcium in regulating the affinity status of integrin $\alpha_{IIb}\beta_3$ (Lapetina et al., 1985; Quinton et al., 2002a; Saitoh et al., 1989; Walker and Watson, 1993). Whether calcium is indispensable for integrin $\alpha_{IIb}\beta_3$ activation has remained less clearly defined, with several recent reports suggesting the possible existence of calcium-independent pathways involving phosphoinositide 3-kinases, calcium-independent forms of PKC, non-receptor tyrosine kinases and small molecular weight GTPases (Haimovich et al., 1996; Jen et al., 1996; Kuwahara et al., 1999; Quinton et al., 2002a; Rotondo et al., 1997; Watson and Hambleton, 1989). The nature and importance of these alternative pathways, remains unclear. While the studies in this chapter do not exclude a potentially important role for these enzymes in regulating integrin $\alpha_{IIb}\beta_3$ activation, they nonetheless suggest an indispensable role for calcium in this process. The apparent discrepancy between the conclusions drawn in this chapter and those derived from previous reports may primarily reflect methodological differences in the experimental approaches used. For example, many studies have been performed using lower doses of DM-BAPTA,AM or using lower affinity calcium chelators such as EGTA-AM and BAPTA-AM. The present studies have demonstrated that high concentrations of DM-BAPTA,AM (100 μM) are required to effectively prevent increases in cytosolic calcium in response to strong agonist stimulation. Loading platelets with 30 μM DM-BAPTA,AM or high concentrations (100 μM) of lower affinity calcium chelators, such as EGTA-AM or BAPTA-AM, does not completely inhibit activation induced by strong platelet agonists, and thus, may lead to erroneous interpretation of the role of calcium in platelet functional responses. The studies presented in this chapter also highlight the importance of analysing calcium levels at the single cell level when assessing the efficiency of calcium chelators. For example, at a population level

high concentrations of DM-BAPTA,AM (100 μM) appear to completely eliminate agonist-induced increases in cytosolic calcium. However, single cell analysis detected a sub-population of platelets in which calcium was incompletely chelated. By monitoring cytosolic calcium changes and platelet adhesion concomitantly, it was demonstrated that these incompletely chelated cells were able to maintain stable platelet adhesion on type I collagen under flow. These observations provide the simplest and most likely explanation for why a residual level of platelet aggregation occurs when platelets are exposed to potent activating stimuli, such as thrombin and collagen. The reason why the cytosolic calcium in this small proportion of platelets is not completely chelated is unknown, but may reflect the fact that DM-BAPTA,AM does not load equally well in all platelets, or that it is actively extruded from the cytosol of these platelets at a greater rate.

In addition to its central role in promoting integrin $\alpha_{IIb}\beta_3$ activation, the above studies also suggest a critical role for cytosolic calcium in stable platelet adhesion on collagen. This conclusion is based on the observation that DM-BAPTA,AM-treated platelets that did not maintain a relative cytosolic calcium level greater than 100 nM, were unable to sustain stable adhesion contacts and withstand the detaching effects of high shear. Sustained platelet adhesion to collagen under flow requires the synergistic contribution of integrins $\alpha_2\beta_1$ and the GPVI/FcRγ complex (Nieswandt et al., 2001a; Savage et al., 1998), with recent evidence supporting a major role for the GPVI/FcRγ complex in this process (Tsuji et al., 1997). Integrin $\alpha_2\beta_1$, like $\alpha_{IIb}\beta_3$, is subject to affinity regulation by signals generated from within the cell (Jung and Moroi, 1998; Moroi et al., 1996), however to date, there is no evidence for a similar mode of regulation for the GPVI/FcRγ complex. However, there is data supporting an important role for GPVI/FcRγ-derived activating signals, based on studies from FcRγ-deficient mice (Poole et al., 1997; Tsuji et al., 1997), in enabling platelets to remain firmly adherent to collagen. These

observations, combined with the studies reported in this chapter, suggest that GPVI/FcRγ-mediated PLCγ activation and subsequent intracellular calcium mobilisation are critical signalling events required to support the normal adhesive function of integrins $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$ and the GPVI/FcRγ complex.

While the precise mechanism by which calcium regulates the affinity status of integrins has not been clearly defined, it is of interest that a number of calcium-binding proteins, including calreticulin, calcium and integrin-binding protein (CIB) and calpain, have been demonstrated to be physically or functionally linked to integrins in a number of cell types. Future studies will be required to delineate the relative roles of these calcium-binding proteins in regulating integrin $\alpha_{IIb}\beta_3$ affinity.

CHAPTER 4:

COMPLEX SIGNALLING RELATIONSHIP BETWEEN CYTOSOLIC CALCIUM AND PROTEIN KINASE C IN REGULATING INTEGRIN $\alpha_{IIb}\beta_3$ ACTIVATION AND PLATELET ADHESION TO vWf.

Chapter 4.

Complex Signalling Relationship between Cytosolic Calcium and Protein Kinase C in Regulating Integrin $\alpha_{IIb}\beta_3$ Activation and Platelet Adhesion to vWf.

4.1 Introduction.

The studies presented thus far have established a critical requirement for cytosolic calcium flux for integrin $\alpha_{IIb}\beta_3$ activation. Previous studies have demonstrated that PKC activation is a key calcium-dependent signalling event required for integrin $\alpha_{IIb}\beta_3$ activation in response to stimulation with soluble agonists (Shattil, 1999). However, there is conflicting evidence as to the absolute requirement for PKC in inducing integrin $\alpha_{IIb}\beta_3$ activation. While several studies have demonstrated a central role for PKC in platelet integrin $\alpha_{IIb}\beta_3$ activation (Kaibuchi et al., 1983; Rink et al., 1983; Rotondo et al., 1997; Saitoh et al., 1989; Walker and Watson, 1993), others have shown that platelet secretion (Sloan and Haslam, 1997) and integrin $\alpha_{IIb}\beta_3$ activation (Iorio et al., 1996; Quinton et al., 2002a) can occur independent of PKC activity. Another area of controversy involves the signalling mechanisms utilised by PKC to promote platelet activation. For instance, while many studies have suggested that PKC and cytosolic calcium operate synergystically to promote integrin $\alpha_{IIb}\beta_3$ activation (Kaibuchi et al., 1983; Rink et al., 1983; Rotondo et al., 1997; Saitoh et al., 1989; Walker and Watson, 1993), others have demonstrated a that PKC can induce integrin $\alpha_{IIb}\beta_3$ activation in the absence of a measurable calcium response (Rink et al., 1983; Watanabe et al., 2001). Therefore, while the important role for PKC in platelet signal transduction and activation is well established, several issues including the absolute

requirement for PKC, and the exact signalling relationship between cytosolic calcium and PKC for integrin $\alpha_{IIb}\beta_3$ activation remain unclear.

Another important unresolved issue is the mechanism by which PKC becomes activated in response to vWf stimulation. It has become increasingly clear that upon binding of vWf, GP Ib/V/IX is able to mediate intracellular signalling events leading to integrin $\alpha_{IIb}\beta_3$ activation. Much of the current understanding of vWf-induced platelet aggregation and PKC activation is derived from suspension-based activation studies. In these studies GP Ib-vWf binding was induced in two ways, (i) by the application of high shear stresses, **S**hear-**I**nduced **P**latelet **A**ggregation (SIPA), or (ii) by the use of artificial modulators (such as ristocetin) (Ikeda et al., 1993; Kroll et al., 1991; Kroll et al., 1993; Moake et al., 1988; Oda et al., 1995; Peterson et al., 1987). These studies have demonstrated that GP Ib binding of vWf at high shear promotes calcium influx (Chow et al., 1992), ADP release (Moake et al., 1988; (Chow et al., 1992) and PKC activation (Kroll et al., 1993). The results of these studies have lead to the establishment of a model of PKC activation whereby vWf binding to GPIb/V/IX induces an initial transmembrane calcium influx, which promotes the release of endogenous soluble agonists such as TXA_2 and ADP. These agonists then bind to their receptors on the platelet surface and induce intracellular signalling mechanisms leading to PKC activation and subsequently to integrin $\alpha_{IIb}\beta_3$ activation (Dopheide et al., 2001). This model suggests that release of endogenous agonists is important for vWf-mediated PKC and integrin $\alpha_{IIb}\beta_3$ activation. Recent adhesion based platelet activation studies have challenged the importance of TXA_2 and ADP release in GP Ib-induced integrin $\alpha_{IIb}\beta_3$ activation, demonstrating that integrin $\alpha_{IIb}\beta_3$ activation on a vWf surface under both static and high shear conditions is not abolished in the presence of ADP or TXA_2 inhibitors (Nesbitt et al., 2002; Yap et al., 2000). However, it is not known under these conditions if inhibitors of ADP and TXA_2 affect PKC activation.

The specific roles of GP Ib/V/IX and integrin $\alpha_{IIb}\beta_3$ play in regulating PKC activity in platelets are also unclear. Studies in CHO cells, He La cells and vascular smooth muscle cells have demonstrated a role for integrin mediated outside-in signalling in PKC activation and cell spreading (Chun et al., 1996; Disatnik and Rando, 1999; Haller et al., 1998; Vuori and Ruoslahti, 1993). While early suspension based studies have suggested that in platelets, PKC can be activated downstream of GP Ib/V/IX engagement, little is known about the role of integrin $\alpha_{IIb}\beta_3$ outside-in signalling in promoting PKC activation in platelets.

The studies presented in this chapter aim to provide further insight into the role of GP Ib/V/IX, integrin $\alpha_{IIb}\beta_3$ and endogenous agonist release in the regulation of PKC in platelets, and to investigate the mechanism by which PKC and calcium co-operate to promote integrin $\alpha_{IIb}\beta_3$ activation and stable platelet adhesion on vWf.

4.2 GP Ib and Integrin $\alpha_{IIb}\beta_3$ Regulation of Protein Kinase C.

Previous studies have demonstrated that PKC activation in vWf stimulated platelets primarily occurs downstream of GPIb. These studies demonstrated that PKC activation was sensitive to ADP and TXA_2 inhibition, but was not significantly inhibited by integrin $\alpha_{IIb}\beta_3$ antagonists (Kroll et al., 1991; Kroll et al., 1993). These studies were performed in suspension-based assays in which soluble vWf was induced to bind GPIb by the application of high shear, or alternatively by the use of an artificial modulator (Kroll et al., 1991; Kroll et al., 1993).

In initial studies, the relative roles of GPIb and integrin $\alpha_{IIb}\beta_3$ in promoting PKC activation on vWf under static conditions were investigated. In recent adhesion based studies on immobilised vWf, it was demonstrated that platelet spreading under static conditions is also dependent on PKC activation. Under these conditions spreading could occur independent of endogenous agonist secretion, but the rate of platelet spreading on vWf could be reduced by the presence of the ADP scavenging enzyme, apyrase (Yap et al., 2000). To determine the time-point at which platelet spreading on vWf proceeds independent of endogenous agonists, platelet spreading was monitored over 60 minutes in the presence or absence of aspirin and apyrase. Consistent the studies of (Yap et al., 2000), figure 4.1 demonstrates that aspirin and apyrase treatment had no significant effect on the extent of spreading observed at 60 minutes, suggesting that integrin $\alpha_{IIb}\beta_3$ activation and platelet spreading at this time point is not dependent on ADP or TXA_2 secretion.

In subsequent studies, ^{32}P-HPO_4-loaded platelets were applied to a human vWf matrix for 60 minutes in the presence or absence of the integrin $\alpha_{IIb}\beta_3$ antagonist, c7E3 Fab, and PKC activation was monitored indirectly by examining phosphorylation of the PKC-specific substrate, pleckstrin. As demonstrated in figures 4.2a and c, in the absence of

Figure 4.1. Ro'e of the Endogenous Agonists ADP and Thromboxane A_2 in Promoting Platelet Spreading on vWf.

Washed platelets were allowed to spread under static conditions on vWf-coated coverslips for 60 minutes in the presence or absence of 0.25% Me_2SO (vehicle), 1.5 mM aspirin (ASA), 2 U/ml apyrase (APY) or c7E3 mAb (c7E3) as indicated. Mean surface area measurements of platelets spreading on purified vWf were quantitated at the indicated time points by analysing five random fields of platelets using MCID™ software. The data presented shows mean ± S.E.M of three independent experiments performed in duplicate.

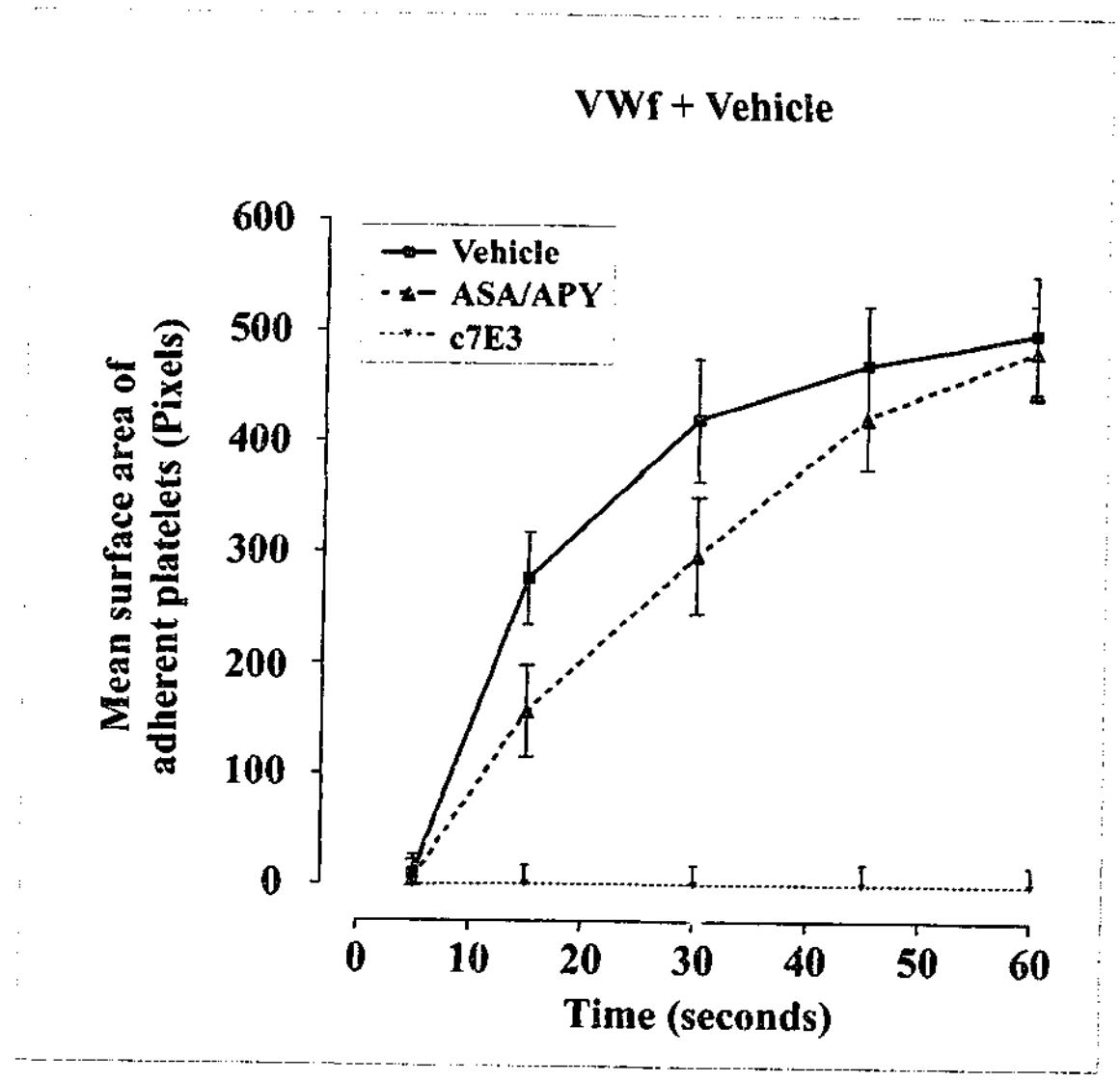
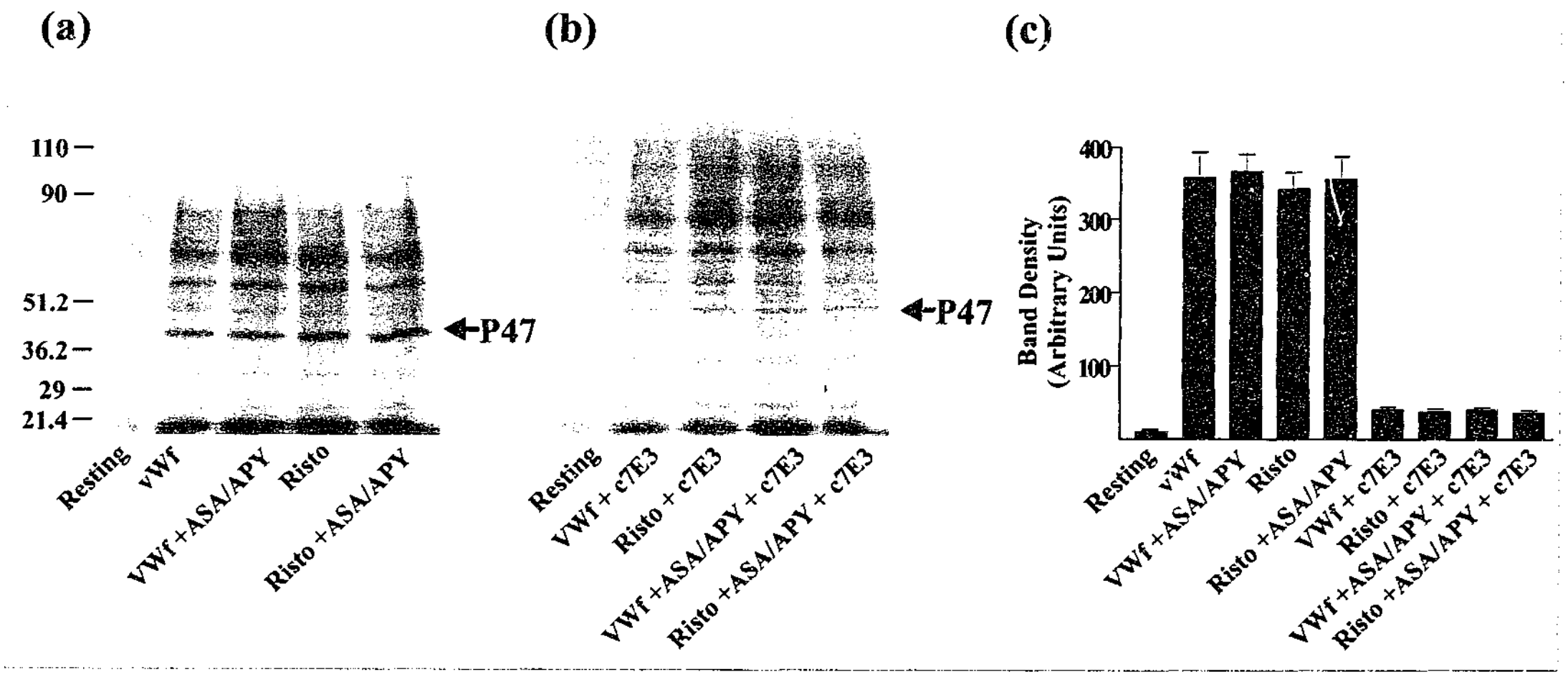


Figure 4.2. PKC Activation and Pleckstrin Phosphorylation during Platelet Adhesion to vWf.

Washed platelets loaded with [^{32}P]-P_i were allowed to spread under static conditions on vWf-coated coverslips for 60 minutes, in the presence of Ristocetin (Risto) and/or c7E3 as indicated. (a) and (b) Platelet lysates were then harvested and analysed by autoradiography according to the procedure outlined in section 2.4.2 and 2.5. (a) Treatment of platelets with (ASA) aspirin or (APY) apyrase does not inhibit pleckstrin phosphorylation (labelled p47) on vWf. (b) Demonstrates a reduction in level of pleckstrin phosphorylation in the presence of c7E3 Fab. (c) Densitometric analysis was performed on p47 bands to quantitated the level of pleckstrin phosphorylation, the histogram represents mean ± S.E.M of three independent experiments (n = 3), the results of which are presented as band density in arbitrary units.



c7E3 Fab, pleckstrin phosphorylation was readily observed in platelets spreading on vWf (mean ± S.E.M = 369.4 ± 22.74 arbitrary units). Increasing the affinity of vWf for GPIb by adding ristocetin to the adhesion assays, did not show any significant effect on pleckstrin phosphorylation (mean ± S.E.M= 376.8 ± 7.09 arbitrary units), whereas pretreating platelets with c7E3 Fab, inhibited pleckstrin phosphorylation by ~87% (fig. 4.2b and c) (mean ± S.E.M = 46.89 ± 12.47 arbitrary units), even when ristocetin was present in the assay (mean ± S.E.M = 47.44 ± 18.67 arbitrary units). The ability of integrin $\alpha_{IIb}\beta_3$ to promote PKC activation was not dependent on the release of endogenous agonists, such as ADP or TXA_2, as similar levels of pleckstrin phosphorylation were also observed in aspirin and apyrase-treated platelets (fig. 4.2a – c, vWf + ASA/APY). These studies suggest a major role for integrin $\alpha_{IIb}\beta_3$ in regulating PKC activation during platelet adhesion on vWf.

4.3 PKC-Dependent and Independent Signalling Mechanisms Regulating Platelet Adhesion Under Flow.

A key unresolved issue relates to the role of PKC inside-out and outside-in signalling in regulating integrin $\alpha_{IIb}\beta_3$ activation. The studies described above demonstrate that PKC activation is primarily mediated through integrin $\alpha_{IIb}\beta_3$ engagement, suggesting an important role for PKC in signalling events occurring post integrin $\alpha_{IIb}\beta_3$-binding of vWf. On the other hand, it has previously been established that PKC activation is essential for the inside-out signalling pathway(s) leading to the initial integrin $\alpha_{IIb}\beta_3$ activation (Shattil, 1999; Yap et al., 2000).

To determine if PKC activation is absolutely necessary for initial integrin $\alpha_{IIb}\beta_3$ activation leading to platelet spreading and stationary adhesion formation on immobilised vWf, static and flow based platelet adhesion studies were performed in the presence of GF109203X (IC_{50} = 10 nM), a competitive antagonist of the ATP-binding site on all PKC

isoforms (Gekeler et al., 1996). Consistent with recent studies by (Shattil, 1999; Yap et al., 2000), platelet treatment with 500 nM GF109203X completely eliminated PAC-1 binding and platelet spreading on vWf under static conditions (fig. 4.3a), suggesting that under these conditions, PKC is critical for inside-out signalling mechanisms that lead to $\alpha_{IIb}\beta_3$ activation.

To investigate the importance of PKC in promoting integrin $\alpha_{IIb}\beta_3$ activation and stationary adhesion formation under flow conditions, platelet were perfused over vWf at $1800s^{-1}$ in the presence of GF109203X and PAC-1. As demonstrated in figure 4.3b, GF109203X treatment dose-dependently reduced the level of stationary adhesion formation on vWf. In contrast to the static adhesion assays, where platelet spreading was completely abolished by PKC inhibition, under shear conditions, up to 20-30% of platelets treated with the PKC inhibitor retained their capacity to form stationary adhesion contacts with the vWf matrix and bind the activation-specific antibody against integrin $\alpha_{IIb}\beta_3$, PAC-1 (fig. 4.3b & c) (Shattil, 1999; Yap et al., 2000). This data raises the possibility that under high shear conditions, GF109203X only partially inhibits PKC activation in platelets.

To investigate the latter hypothesis, PKC activity was measured in the presence or absence of GF109203X. Pretreating platelets with GF109203X at concentrations as low as 100 nM, totally abolished pleckstrin phosphorylation induced by immobilised vWf (fig. 4.3d). Furthermore, 500 nM GF109203X was able to completely inhibit pleckstrin phosphorylation induced by potent PKC activators, including the phorbol ester, PMA, and thrombin (1 U/ml) (fig. 4.3e & f). This evidence suggests that incomplete PKC inhibition does not account for the incomplete inhibition of stationary adhesion formation by GF109203X. An alternative explanation for this phenomenon may be that a subset of platelets utilises PKC-independent signalling mechanisms to regulate integrin $\alpha_{IIb}\beta_3$ activation under flow.

Figure 4.3. Effect of the PKC inhibitor GF109203X on pleckstrin phosphorylation and platelet adhesion.

Washed platelets treated with vehicle (0.25% Me_2SO) or GF109203X (200 or 500 nM as indicated) were allowed to spread on a vWf matrix under static conditions (a), or under shear conditions at $1800s^{-1}$ (b and c), in the presence of PAC-1 mAb. Platelets were subsequently fixed with 3.8 % paraformaldehyde and imaged by DIC and fluorescence microscopy. (a) DIC images of platelet morphology, and fluorescence images of PAC-1 binding to vWf adherent platelets. (b) Demonstrates the reduction in the level of irreversible adhesion on vWf in response to 200 nM and 500 nM GF109203X (mean ± SEM, n = 4). (c) DIC images of platelets forming stationary adhesion contacts on the vWf matrix under flow, and fluorescence images demonstrating PAC-1 binding to the adherent platelets. Washed platelets loaded with $[^{32}P]$-P_i were incubated with the indicated concentrations of GF109203X prior to exposure for 60 minutes to (d) a purified vWf matrix, or for 5 minutes to (e) 200 nM PMA or (f) 1 U/ml of thrombin (Thr) as indicated. Whole platelet lysates were prepared as described in section 2.4.2 and 2.5, and subjected to SDS-PAGE and autoradiography. The results in (d) and (e) demonstrate the dose-dependent effects of GF109203X on pleckstrin phosphorylation induced by vWf or PMA. (f) Demonstrates the inhibition of PMA and thrombin-induced pleckstrin phosphorylation by 500 nM GF109203X.

(a)

Static

Vehicle GF109203X

DIC

PAC-1

(b)

1800s^{-1}

Irreversible adhesion (% of control)

100 80 60 40 20 0

Vehicle 200 nM 500 nM

GF109203X

(c)

1800s^{-1}

Vehicle GF109203X

DIC

PAC-1

(d)

←p47

Vehicle 100 200 500

GF109203X (nM)

(e)

←p47

Resting PMA 100 200 500

GF109203X (nM)

(f)

←p47

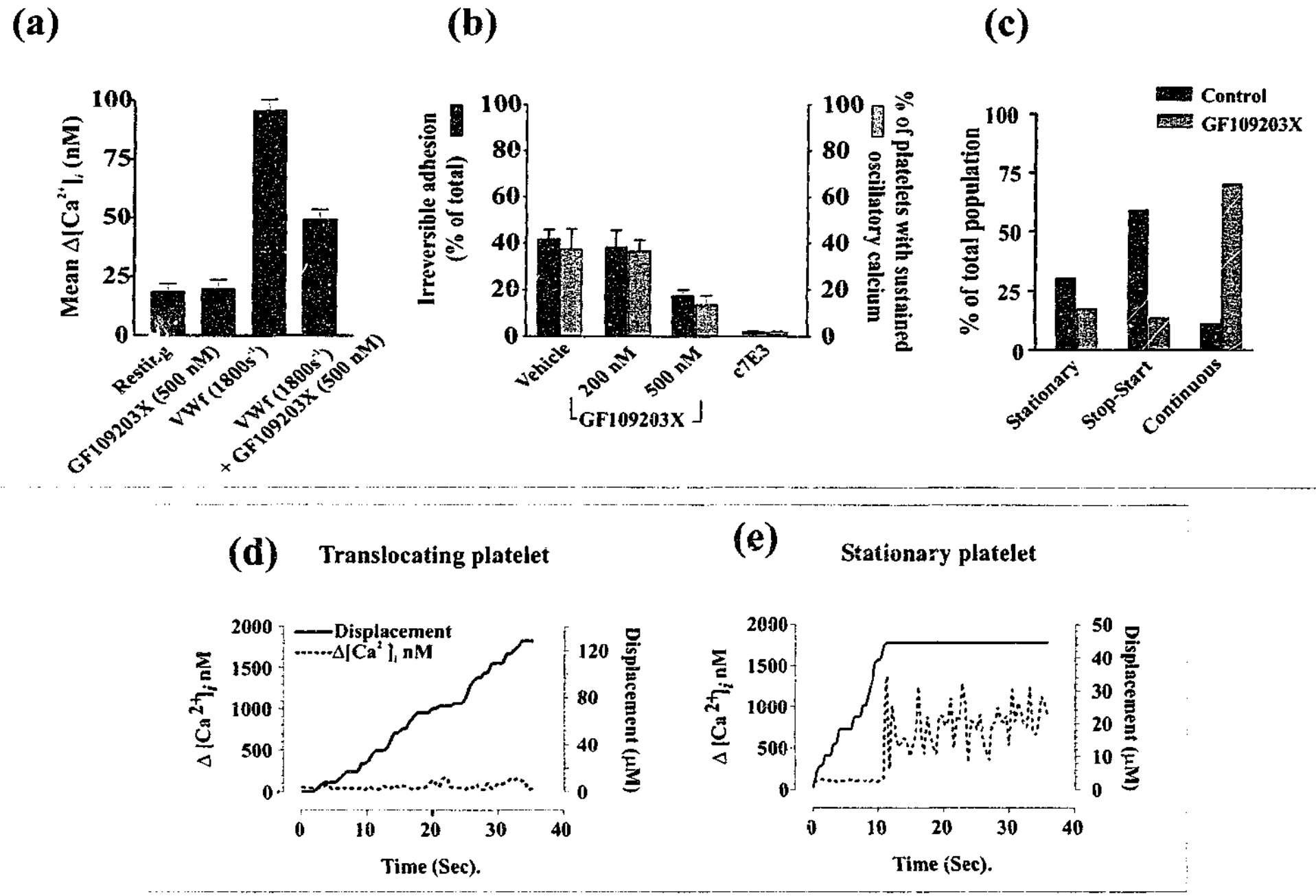
Resting Thr PMA Thr + GF109203X PMA + GF109203X

4.4 Relationship Between Protein Kinase C Activation, Calcium Mobilisation and Integrin $\alpha_{IIb}\beta_3$-dependent Platelet Adhesion.

Studies presented in section 3.5 and in previous studies (Nesbitt et al., 2002), demonstrated a strong correlation between cytosolic calcium levels and the translocation behaviour of platelets under flow conditions (fig. 3.7). To gain further insight into the role of PKC in promoting integrin $\alpha_{IIb}\beta_3$ activation and platelet adhesion under flow conditions on vWf, the effect of PKC inhibition on cytosolic calcium flux was examined in individual platelets during surface translocation on vWf. In these studies, real-time analysis of platelet translocation behaviour was performed and correlated with temporal changes in cytosolic calcium responses. Pretreating platelets with GF109203X (500 nM) had no effect on the basal cytosolic calcium levels in resting platelets (fig. 4.4a). Consistent with data presented in figure 4.3, GF109203X (500 nM) treatment significantly reduced the proportion of platelets forming irreversible stationary adhesions (fig. 4.4b), and hence undergoing a sustained oscillatory cytosolic calcium response (fig. 4.4a & b). This resulted in a reduction in the mean calcium level (from ~100 to 60 nM) in the entire population of translocating platelets (fig. 4.4a). Detailed analysis of the calcium response in individual platelets revealed that inhibiting PKC significantly increased the proportion of rapidly translocating platelets (11.4 to 70%) exhibiting low cytosolic calcium levels ($\Delta[Ca^{2+}]_i$ <20 nM). This increase in rapidly translocating platelets was associated with a proportional reduction in the percentage of platelets showing stop-start translocation behaviour (58.7 to 12.6%), and expressing intermediate calcium levels ($\Delta[Ca^{2+}]_i$ = 20-65 nM), and in platelets expressing high range calcium levels ($\Delta[Ca^{2+}]_i$ approaching 1200 nM) forming stationary adhesions (29.9 to 17.4%) (fig. 4.4c). While most GF109203X-treated platelets failed to form stationary adhesion contacts and showed very low cytosolic calcium levels (fig. 4.4d), all of the platelets that retained their ability to form stationary adhesion contacts with

Figure 4.4. Effect of the PKC inhibitor GF109203X on platelet adhesion and calcium mobilisation on immobilised vWf.

Cytosolic calcium flux was measured in platelets as described in section 2.7. (a) Shows the mean cytosolic calcium concentrations in a population of platelets in suspension undergoing non-synchronised calcium flux in the presence or absence of 500 nM GF109203X (1st and 2nd bars), or during adhesion to vWf under flow (3rd and 4th bars) (mean ± S.E.M, n=5). Washed platelets (1 x 10^7 cells/ml) were treated with vehicle (0.25% Me_2SO) or GF109203X (200 nM or 500 nM) prior to perfusion through vWf-coated microcapillary tubes at 1800 s^{-1}. (b) Dose dependent reduction in the number of platelets undergoing sustained calcium oscillations (right axis) and in the level of irreversible adhesion (left axis) on vWf in the presence of GF109203X (mean ± SEM, n = 4). Figure (c) shows the proportion of vehicle (Me_2SO) and (500 nM) GF109203X-treated platelets displaying stationary adhesion, continuous or stop-start translocation behaviours. Typical calcium profiles of single vWf adherent platelets either translocating rapidly across the vWf matrix (d), or forming stationary adhesion contacts (e), in the presence of GF109203X. The solid line shows platelet displacement over time while the dotted line demonstrates cytosolic calcium flux profiles.


(a)
Mean Δ[Ca2+]i (nM)
100
75
50
25
0
Resting
GF109203X (500 nM)
VWf (1800s-1)
VWf (1800s-1) + GF109203X (500 nM)
(b)
Irreversible adhesion (% of total)
% of platelets with sustained oscillatory calcium
100
80
60
40
20
0
Vehicle
200 nM
500 nM
c7E3
GF109203X
(c)
Control
GF109203X
% of total population
Stationary
Stop-Start
Continuous
(d) Translocating platelet
Displacement
Δ[Ca2+]i nM
Δ [Ca2+]i, nM
Displacement (μM)
2000
1500
1000
500
0
120
80
40
0
10
20
30
40
Time (Sec).
(e) Stationary platelet
50
40
30
20
10

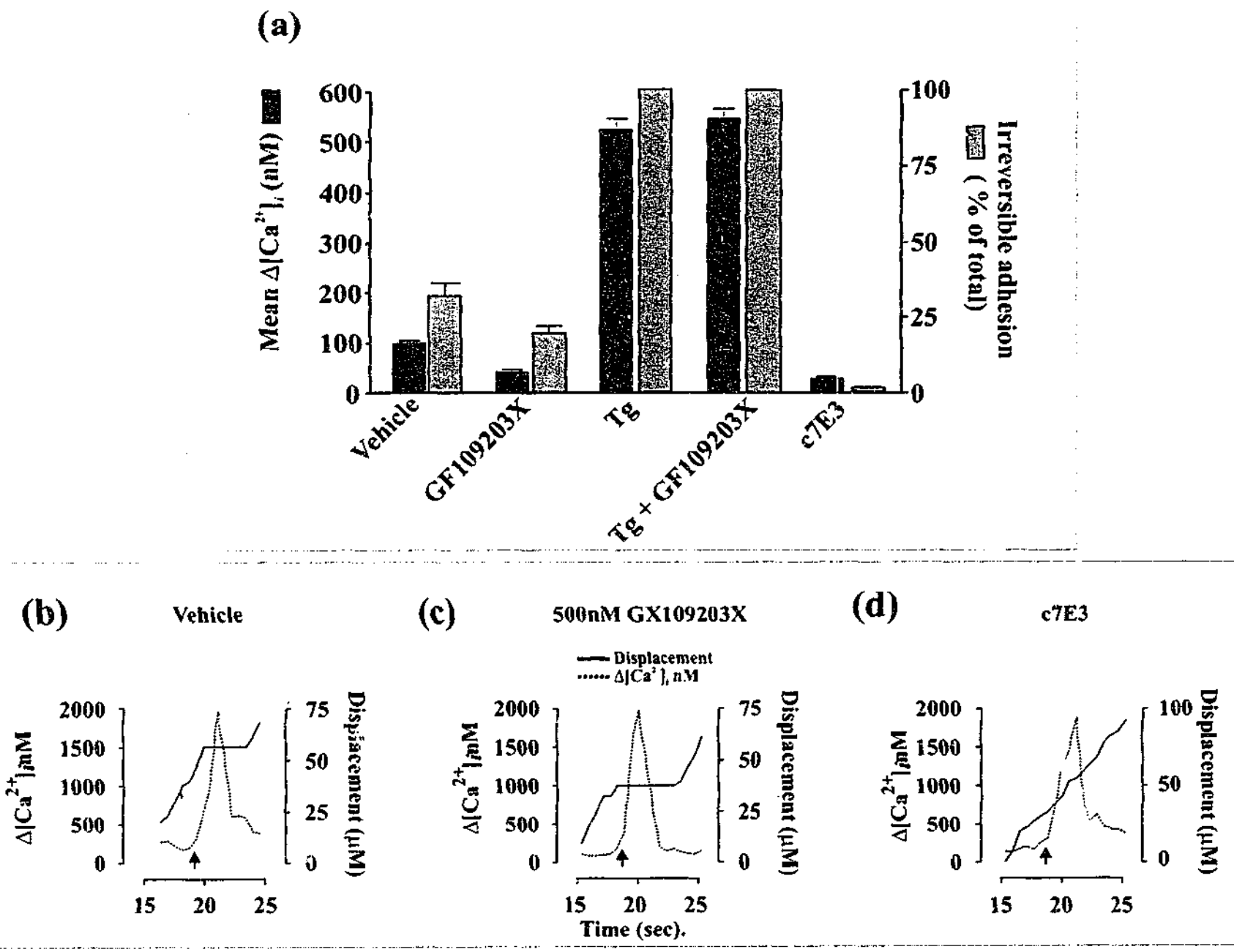
the vWf matrix, displayed high oscillatory calcium levels (peak levels > 800 nM) (fig. 4.4e). These results raise the possibility that PKC regulation of platelet adhesion under flow is at least in part due to an effect on cytosolic calcium levels. These studies also suggest that when cytosolic calcium levels are elevated beyond a certain threshold (100 nM), PKC activation is no longer absolutely necessary for integrin $\alpha_{IIb}\beta_3$ activation and stationary adhesion formation on vWf. In control experiments, c7E3 was added to GF109203X-pretreated platelets prior to perfusion over vWf. Integrin $\alpha_{IIb}\beta_3$ blockade was found to completely abolish stationary adhesion formation and sustained calcium oscillations (fig. 4.4b), suggesting that the calcium flux displayed in stationary GF109203X-treated platelets was integrin $\alpha_{IIb}\beta_3$-dependent.

4.5 Threshold Cytosolic Calcium Concentration Required for Protein Kinase C-dependent Integrin $\alpha_{IIb}\beta_3$ Activation and Platelet Adhesion.

To investigate the possibility that high levels of cytosolic calcium can induce integrin $\alpha_{IIb}\beta_3$ activation independent of PKC, the effects of artificially elevating cytosolic calcium levels on the ability of GF109203X to inhibit stable platelet adhesion on vWf were investigated. In initial studies, cytosolic calcium levels were elevated by treating platelets with thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase. This compound inhibits active re-uptake of cytosolic calcium into the platelet dense tubular system, thereby allowing accumulation of calcium within the cytosol. Platelets were pretreated with 100 nM thapsigargin prior to perfusion over immobilised vWf at 1800 s^{-1}. As shown in figure 4.5a, treatment with 100 nM thapsigargin dramatically elevated the mean cytosolic calcium levels in vWf adherent platelets from $\Delta[Ca^{2+}]_i$ = 100 nM, to $\Delta[Ca^{2+}]_i = 550 \pm 15$ nM. This increase in cytosolic calcium was associated with 100% of translocating cells forming stationary adhesion contacts with the vWf matrix (fig. 4.5a).

Figure 4.5. Effect of artificially elevating cytosolic calcium on platelet translocation and integrin $\alpha_{IIb}\beta_3$ activation, and the ability of GF109203X to inhibit platelet adhesion on vWf.

Washed platelets (1 x 10^7 cells/ml) were treated with vehicle (0.25% Me_2SO) or 500 nM GF109203X, prior to the addition of 100 nM thapsigargin (Tg). Platelets were then perfused through vWf-coated microcapillary tubes at 1800 s^{-1}. (a) Demonstrates the mean cytosolic calcium concentrations expressed by the adherent platelet population (left axis), and the increase in the number of irreversibly adherent platelets upon thapsigargin treatment (right axis) both in the presence or absence of GF109203X (mean ± SEM, n = 4). (b – d) Washed platelets were loaded with 10μM of the caged calcium compound NP-EGTA and perfused through vWf coated microcapillary slides at 1800s^{-1}. The platelets were allowed to translocate for approximately 18 seconds before being exposed to a near UV light source for 0.6 seconds (marked by the arrow). (b) Demonstrates the displacement vs. time graph of a representative control NP-EGTA loaded platelet exposed to UV light. (c) and (d) show displacement vs. time graphs of a representative NP-EGTA loaded platelet treated with 500nM GF109203X or c7E3 Fab respectively, prior to exposure to UV light.


(a)
Mean Δ[Ca2+]i (nM)
Irreversible adhesion
(% of total)
600
500
400
300
200
100
0
100
75
50
25
0
Vehicle
GF109203X
Tg
Tg + GF109203X
c7E3
(b)
Vehicle
(c)
500nM GX109203X
(d)
c7E3
Displacement
Δ[Ca2+]i nM
Δ[Ca2+]inM
Displacement (μM)
2000
1500
1000
500
0
75
50
25
0
100
15
20
25
Time (sec).

Pretreating platelets with GF109203X (500 nM) had minimal inhibitory effect on the platelet calcium response or on the ability of thapsigargin-treated platelets to form stationary adhesion contacts with the vWf surface (fig. 4.5a). In control studies, stationary adhesion formation was inhibited by treated platelets with the integrin $\alpha_{IIb}\beta_3$ antagonist, c7E3 Fab, confirming that the increase in stationary adhesion under these experimental conditions was due to activation of integrin $\alpha_{IIb}\beta_3$ (fig. 4.5a). These studies support the hypothesis that PKC activity is not an absolute requirement for integrin $\alpha_{IIb}\beta_3$ activation and that accumulation of high cytosolic calcium levels can trigger $\alpha_{IIb}\beta_3$ activation through signalling mechanisms not requiring PKC activation.

Thapsigargin treatment of translocating platelets induced high, sustained cytosolic calcium levels, and as such, acted as a very potent stimulus of platelet activation. Recent studies by (Nesbitt et al., 2002) have demonstrated that a subset of platelets translocating on a vWf matrix may experience high range but transient elevations in cytosolic calcium which correspond to temporary arrest of platelet translocation. To investigate whether transient calcium elevation could also induce integrin $\alpha_{IIb}\beta_3$ activation independent of PKC, a caged calcium platelet activation assay was adopted. In these studies, platelets were loaded with a membrane permeable caged calcium chelator called nitrophenyl EGTA (NP-EGTA). This is a UV sensitive calcium chelator, which exhibits a marked increase in Kd for calcium following exposure to UV light. Briefly, NP-EGTA entering the platelet cytosol binds free cytosolic calcium. Exposure of NP-EGTA-loaded platelets to UV or near UV light (wavelengths of 300 - 400 nm), leads to an irreversible change in the chemical structure of the NP-EGTA that results in a rapid release of relatively large quantities of calcium within the cytosol.

As demonstrated in figure 4.5b, initiation of high-range transient calcium spikes (peak calcium levels approaching 2000 nM), in NP-EGTA-treated platelets translocating

on a vWf matrix under flow conditions, resulted in all cells forming transient stationary adhesion contacts with the vWf matrix, which returned to translocation as cytosolic calcium concentrations returned to basal levels (~100 nM). Pretreating NP-EGTA-loaded platelets with GF109203X resulted in a reduction in the percentage of cells forming stationary adhesion contacts prior to uncaging of calcium, however, similar to vehicle treated platelets, upon exposure to UV light, all platelets formed transient stationary adhesion contacts with the vWf matrix (fig. 4.5c). In control studies, it was confirmed that stationary adhesion under these conditions was mediated by integrin $\alpha_{IIb}\beta_3$, as it was completely abolished by pretreating platelets with the $\alpha_{IIb}\beta_3$ antagonists, c7E3 Fab (fig. 4.5d). Together, these studies suggest that the induction of high cytosolic calcium levels (mean cytosolic calcium levels >500 nM) promotes integrin $\alpha_{IIb}\beta_3$ activation independent of PKC, whereas integrin $\alpha_{IIb}\beta_3$ activation at lower cytosolic calcium levels are dependent on PKC activation.

4.6 Role of PKC in GP Ib and Integrin $\alpha_{IIb}\beta_3$ Derived Calcium Signals.

Recent studies have shown that during adhesion to vWf, GP Ib and integrin $\alpha_{IIb}\beta_3$ elicit distinct calcium signals that serve to co-operatively regulate platelet adhesion and activation on immobilised vWf (Nesbitt et al., 2002). Moreover, there is evidence that both PKC activation and intracellular calcium elevation are critical signalling events required to regulate integrin $\alpha_{IIb}\beta_3$ on an immobilised vWf matrix under both static and high shear conditions (Shattil, 1999; Yap et al., 2000). However, to date the precise signalling relationship between the GPIb and integrin $\alpha_{IIb}\beta_3$-derived calcium signals and PKC activation has not been defined.

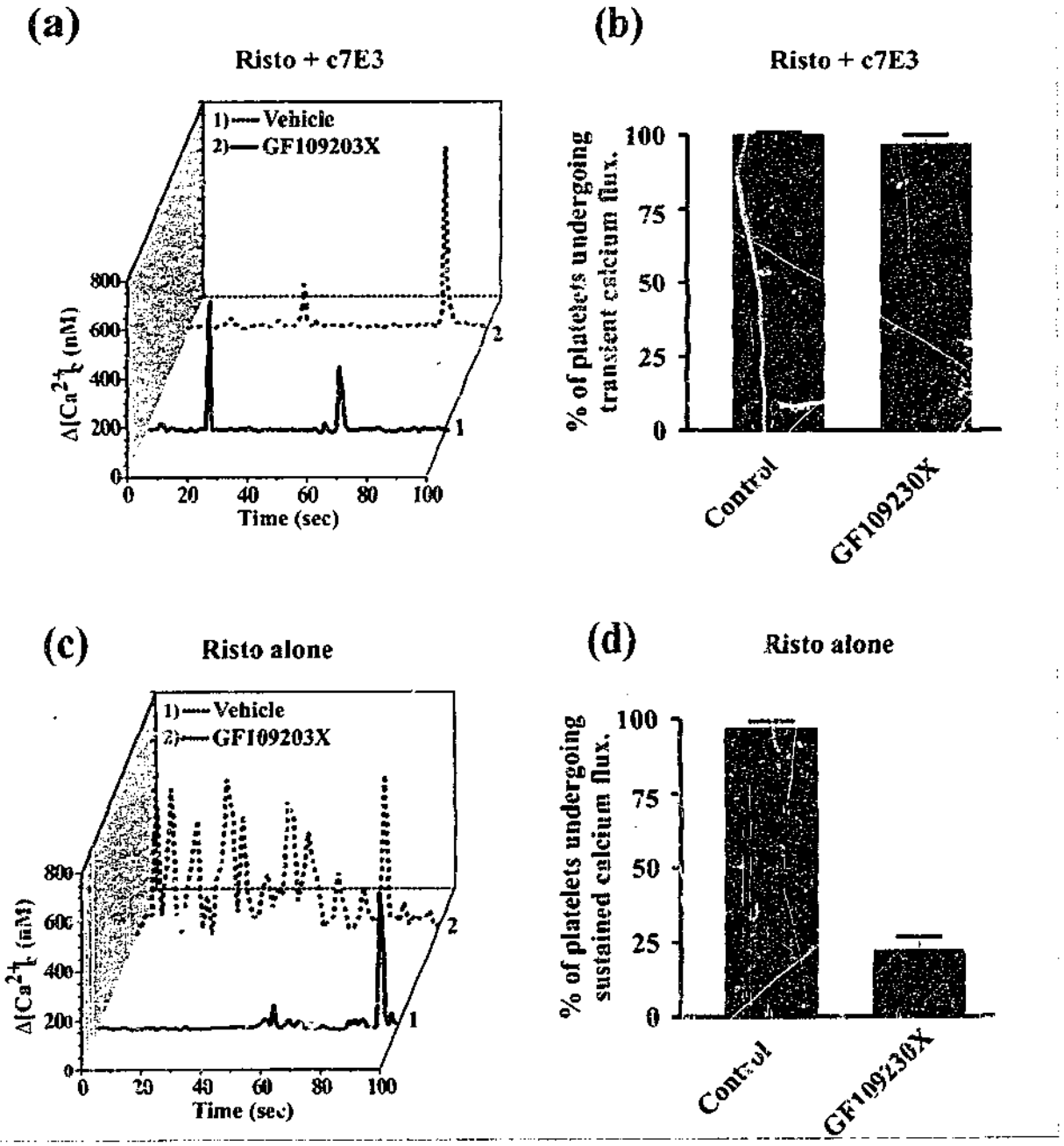
To examine the relationship between PKC and the GPIb and $\alpha_{IIb}\beta_3$-derived calcium signals during platelet adhesion to vWf calcium dye-loaded washed platelets were

reconstituted with washed red blood cells (50 % haematocrit) and perfused over a vWf matrix at 1800 s^{-1} in the presence or absence of GF109203X. Initially to investigate the GPIb derived calcium signals, c7E3 Fab-treated platelets were perfused over an immobilised vWf matrix in the presence of the artificial modulator ristocetin, and real-time changes in cytosolic calcium flux were monitored. Ristocetin increases the binding affinity between GP Ib and vWf, such that, even in the absence of integrin $\alpha_{IIb}\beta_3$ engagement, platelets remain stationary through the GP Ib-vWf interaction in a shear field. Figure 4.6a, shows the typical GP Ib-derived calcium profile of individual platelets adhering to vWf in the presence of ristocetin and c7E3 Fab under flow conditions. This figure illustrates the transient and infrequent nature of the cytosolic calcium flux induced by GPIb signalling. PKC inhibition with a dose of GF109203X (500 nM), which has been previously shown to specifically inhibit PKC activation and prevent platelet spreading and stationary adhesion on vWf (Davis et al., 1992a; Davis et al., 1992b; Toullec et al., 1991; Yap et al., 2000), had no effect on GPIb-derived calcium signals. All platelets exhibited transient GPIb-induced calcium flux both on the presence or absence of GF109203X (fig. 4.6a & b), demonstrating that GPIb induced calcium signalling is not PKC sensitive.

Studies presented in section 3.5 of this thesis demonstrated that the formation of integrin $\alpha_{IIb}\beta_3$-dependent stationary adhesion contacts on vWf under high shear ($1800s^{-1}$) conditions, coincides with the generation of a sustained oscillatory cytosolic calcium response (fig. 3.7). Figure 4.6c, demonstrates that in the absence of the integrin $\alpha_{IIb}\beta_3$ antagonist c7E3 Fab, vWf adherent platelets display a similar sustained oscillatory calcium flux in the presence of ristocetin. GF109203X (500 nM) treatment reduced the proportion of platelets undergoing a sustained, $\alpha_{IIb}\beta_3$-derived oscillatory calcium response by 75 % and converted the calcium profiles in these cells to GPIb-like transient spikes (fig. 4.6c &

Figure 4.6. Effect of GF109203X on the GP Ib/V/IX and $\alpha_{IIb}\beta_3$ derived calcium mobilisation on vWf under flow.

Washed platelets (1 x 10^7 cells/ml) were treated with vehicle (0.25% Me_2SO) or 500 nM GF109203X, prior to perfusion through vWf coated microcapillary tubes at 1800 s^{-1} in the presence of ristocetin (Risto) and/or c7E3 Fab as indicated. (a) Demonstrates that GP Ib-derived cytosolic calcium transients in single adherent platelet are not effected by 500 nM GF109203X. (b) Demonstrates that GF109203X treatment has no effect on the percentage of the platelet population undergoing transient calcium spiking. (c) Demonstrates that sustained oscillatory calcium flux induced by $\alpha_{IIb}\beta_3$ are blocked by GF109203X treatment. (c) Indicates the reduction in the percentage of platelets undergoing a sustained calcium oscillation on vWf in the presence of GF109203X. Figure (a) and (c) show the calcium responses of single platelets representative of 25 independent platelets per experiment.


(a)
Risto + c7E3
1)—Vehicle
2)—GF109203X
Δ[Ca2+]t (nM)
Time (sec)
(b)
Risto + c7E3
% of platelets undergoing transient calcium flux.
Control
GF109230X
(c)
Risto alone
1)—Vehicle
2)—GF109203X
Δ[Ca2+]t (nM)
Time (sec)
(d)
Risto alone
% of platelets undergoing sustained calcium flux.
Control
GF109230X

d). These studies demonstrate a potentially important role for PKC in regulating cytosolic calcium flux in platelets by regulating integrin $\alpha_{IIb}\beta_3$-dependent calcium signals.

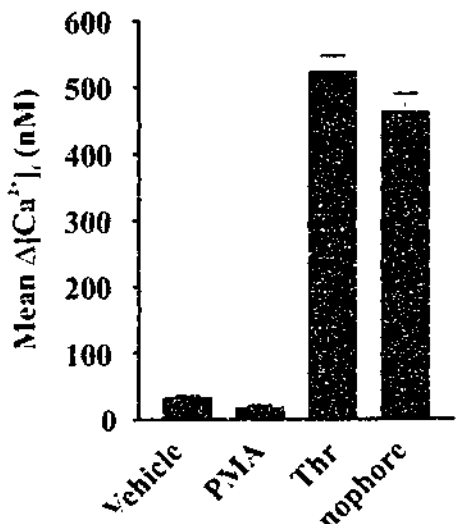
4.7 Protein Kinase C Potentiates Calcium Mobilisation via an Integrin $\alpha_{IIb}\beta_3$-Dependent Mechanism.

The studies presented in this chapter so far have established that PKC can modulate calcium flux induced by integrin $\alpha_{IIb}\beta_3$ signalling. The exact mechanism through which this occurs remains unclear. Previous studies have demonstrated that PKC can directly phosphorylate the IP_3 receptor in reconstituted lipid vesicles (Ferris et al., 1991), suggesting that a potential means for PKC to regulate calcium flux may be through modulating the IP_3 receptor. To investigate further the mechanism through which PKC induces calcium signalling, the effect of PMA stimulation on platelet integrin $\alpha_{IIb}\beta_3$ activation and calcium transients was investigated in suspension and adhesion-based assays on vWf. As demonstrated in figure 4.7a, stimulating platelets in suspension with 200 nM PMA did not result in a significant increase in the mean cytosolic calcium level, suggesting that PKC is not able to directly mobilise calcium from intracellular stores. Interestingly, it induced an approximate 3 fold increase in the level of PAC-1 binding to the surface of these cells (fig. 4.7b), suggesting that direct PKC stimulation can induce a low level of integrin $\alpha_{IIb}\beta_3$ activation in the absence of a detectable calcium rise. This raises the possibility that PKC acts through an indirect $\alpha_{IIb}\beta_3$ dependent mechanism to potentiate calcium flux. It should be noted that the extent of calcium elevation and magnitude of PAC-1 binding induced by PMA under these experimental conditions was low relative to that induced by ionophore A23187 or thrombin (fig. 4.7a & b) confirming a dominant role for calcium in integrin $\alpha_{IIb}\beta_3$ activation.

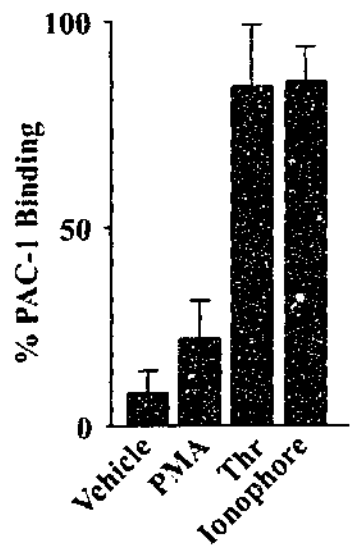
Figure 4.7. Relationship between PKC and integrin $\alpha_{IIb}\beta_3$ activation in regulating cytosolic calcium flux.

Calcium dye-loaded platelets were stimulated in suspension under non-stirred conditions with 200 nM PMA, 1 U/ml thrombin (Thr) or 100 nM ionophore A23187. (a) Demonstrates that PMA stimulation of platelets in suspension does not induce a significant rise in the mean cytosolic calcium level in the platelet population while a dramatic calcium rise is induced by thrombin and ionophore stimulation (mean ± SEM, n = 4). (b) Demonstrates the level of PAC-1 binding to platelets stimulated with 200 nM PMA, 1 U/ml thrombin or 100 nM ionophore A23187 under non-stirred conditions. The level of PAC-1 binding is expressed as a percentage relative to the results obtained from thrombin-stimulated platelets (mean ± SEM, n = 6). (c and d) Calcium dye-loaded platelets treated with vehicle (0.25% Me_2SO) or 200 nM PMA were allowed to settle on a vWf matrix under static conditions for 60 minutes at 37°C, in the presence or absence of the anti-$\alpha_{IIb}\beta_3$ antagonist, aggrastat (AS). (c) Demonstrates the percentage of cells spread on the vWf matrix over a 60 minute time period. (d) Demonstrates real-time changes in mean cytosolic calcium concentrations over 60 minutes. These results are expressed as mean ± SEM from four individual experiments.

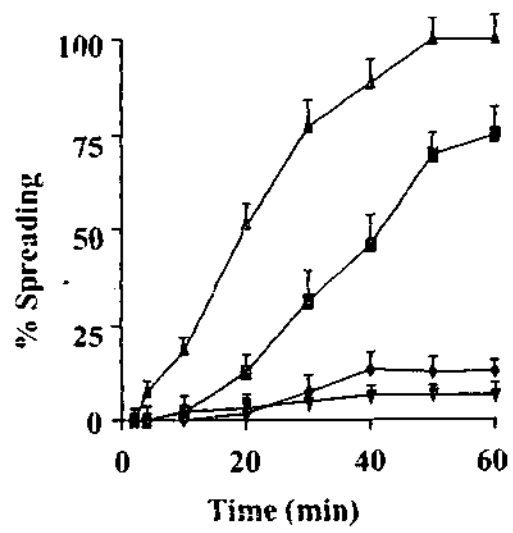
(a)


600
500
400
300
200
100
0
Mean Δ[Ca2+]i (nM)
Vehicle
PMA
Thr
Ionophore

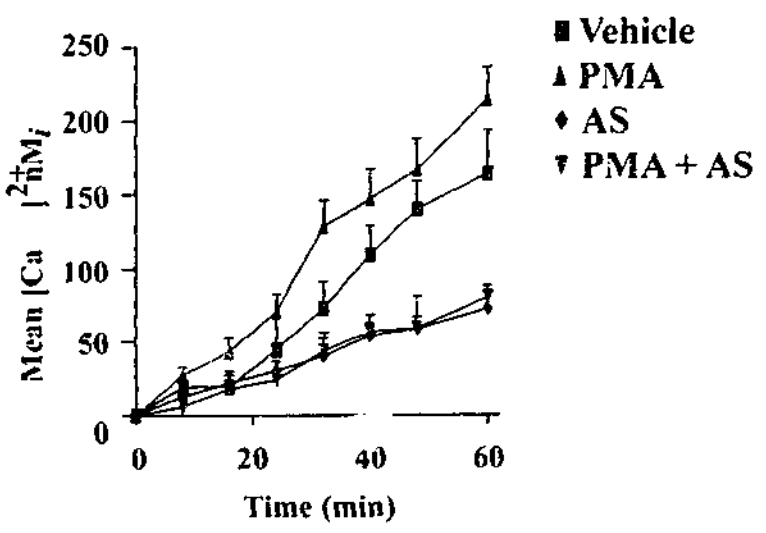
(b)


100
50
0
% PAC-1 Binding
Vehicle
PMA
Thr
Ionophore

(c)


100
75
50
25
0
% Spreading
0
20
40
60
Time (min)

(d)

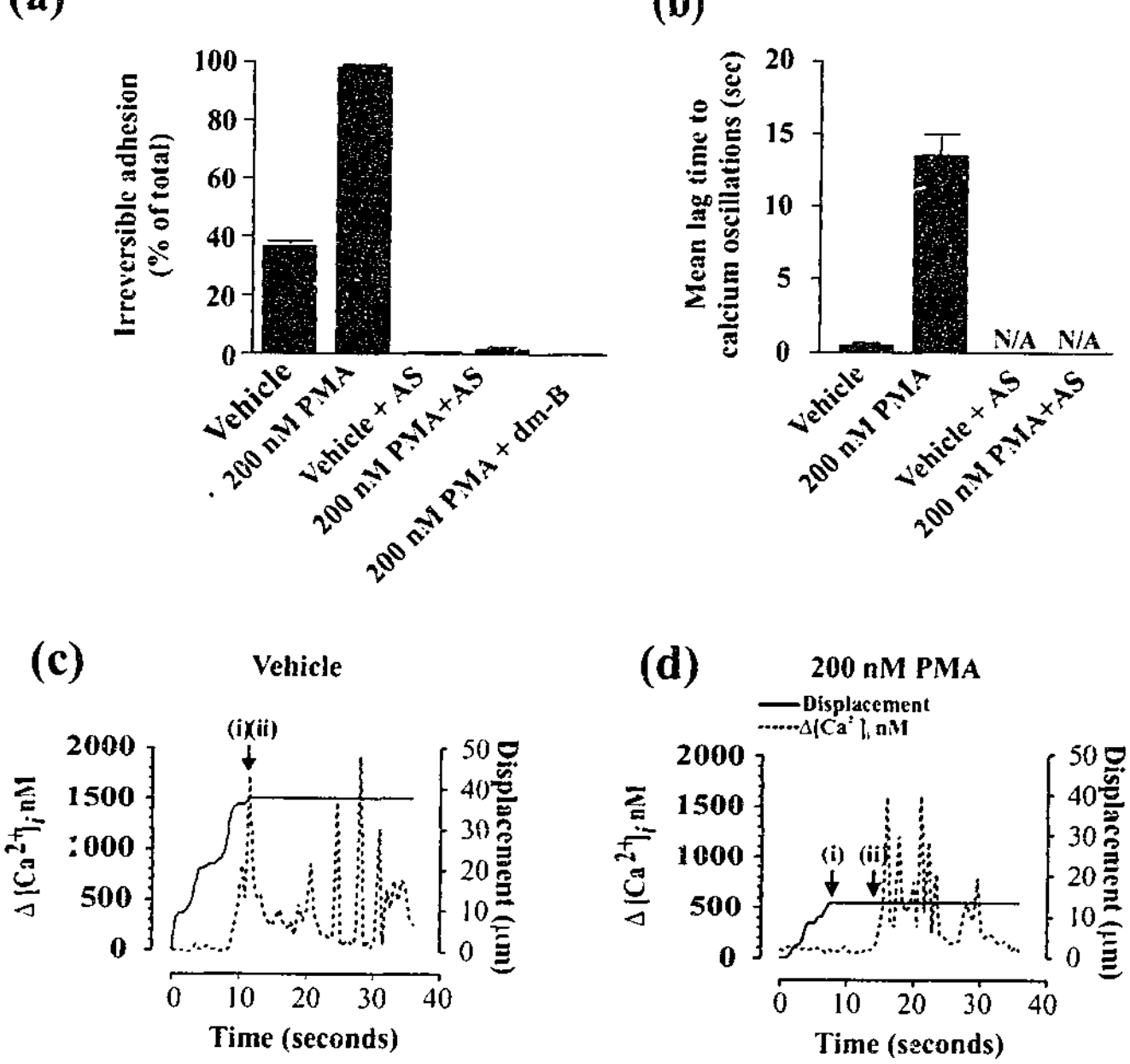

250
200
150
100
50
0
Mean [Ca2+]i nM
0
20
40
60
Time (min)
Vehicle
PMA
AS
PMA + AS

In contrast to suspension-based assays, stimulating vWf adherent platelets under static conditions with PMA, resulted in an increase in the overall level of platelet adhesion and spreading on this matrix, and this coincided with an enhanced cytosolic calcium response throughout the entire platelet population adherent to vWf (fig. 4.7c and d). Pretreating platelets with aggrastat abolished stable adhesion and calcium flux in control and PMA-stimulated platelets, suggesting that the potentiating effects of PKC on cytosolic calcium flux occurs indirectly, as a result of the upregulation of the adhesive and signalling function of integrin $\alpha_{IIb}\beta_3$ (fig. 4.7c & d).

To investigate further the relationship between PKC activation, calcium mobilisation and $\alpha_{IIb}\beta_3$ engagement, platelets were perfused through vWf-coated microcapillary tubes in the presence or absence of PMA. In control studies, 37% of washed platelets tethering to the vWf matrix formed stationary adhesion contacts. Analysis of the effects of PMA on platelet adhesion to vWf under flow conditions demonstrated a dramatic increase in the proportion of cells forming firm adhesion contacts with the vWf matrix (>98%) (fig. 4.8a). Detailed analysis of the translocation behaviour of individual platelets and the corresponding cytosolic calcium levels in these cells revealed that vehicle treated platelets translocating over the vWf surface typically exhibiting a relatively low basal calcium level (below 100nM), prior to forming stationary adhesion contacts. In all cells analysed (>100 individual platelets), the onset of sustained calcium oscillations coincided with the moment of firm adhesion contact formation. Similar single cell analysis of PMA-treated platelets revealed that unlike vehicle treated platelets, there was a considerable lag time (~15 seconds) between the onset of stationary adhesion and the subsequent oscillatory calcium response in platelets stimulated with PMA (fig. 4.8b, c & d). The initiation of stationary adhesion contacts, as well as the sustained oscillatory calcium response, in both vehicle and PMA-treated platelets was dependent on integrin $\alpha_{IIb}\beta_3$ engagement of vWf as

Figure 4.8. Relationship between PKC and calcium in regulating integrin $\alpha_{IIb}\beta_3$ activation under flow conditions.

Calcium dye-loaded platelets (1×10^7 cells/ml) were perfused through vWf-coated microcapillary slides at $1800s^{-1}$ in the presence of vehicle (0.25% Me_2SO) or 200 nM PMA. Where indicated, platelets where also pre-incubated with aggrastat (AS) or 100 µM dimethyl BAPTA.AM (dm-B) prior to perfusion. (a) Demonstrates the level of stationary platelet adhesion on immobilised vWf in response to 200 nM PMA stimulation in the presence of aggrastat (AS) or dm-B (mean ± SEM, n = 4). (b) Demonstrates the lag time between stationary adhesion contact formation and the onset of calcium oscillations in adherent platelets. (c and d) indicate typical translocation behaviours and calcium profiles of single platelets adherent to vWf in the presence (d) or absence of PMA (c). The arrows in (c and d) indicate the onset of stationary adhesion (i) and the subsequent onset of calcium mobilisation (ii).


(a)
Irreversible adhesion (% of total)
100
80
60
40
20
0
Vehicle
200 nM PMA
Vehicle + AS
200 nM PMA+AS
200 nM PMA + dm-B
(b)
Mean lag time to calcium oscillations (sec)
20
15
10
5
0
N/A
N/A
Vehicle
200 nM PMA
Vehicle + AS
200 nM PMA+AS
(c)
Vehicle
(i)(ii)
Δ[Ca2+]i, nM
2000
1500
1000
500
0
Displacement (μm)
50
40
30
20
10
0
0
10
20
30
40
Time (seconds)
(d)
200 nM PMA
Displacement
Δ[Ca2+]i, nM
(i)
(ii)
Δ[Ca2+]i, nM
2000
1500
1000
500
0
Displacement (μm)
50
40
30
20
10
0
0
10
20
30
40
Time (seconds)

both were abolished by pretreating platelets with Aggrastat (AS) (fig. 4.8a & b). The demonstration of a temporal dissociation between $\alpha_{IIb}\beta_3$ dependent stationary adhesion and the onset of an oscillatory calcium response further supports the hypothesis that PKC primes integrin $\alpha_{IIb}\beta_3$ outside-in signalling, and has an indirect effect on calcium signalling in vWf adherent platelets.

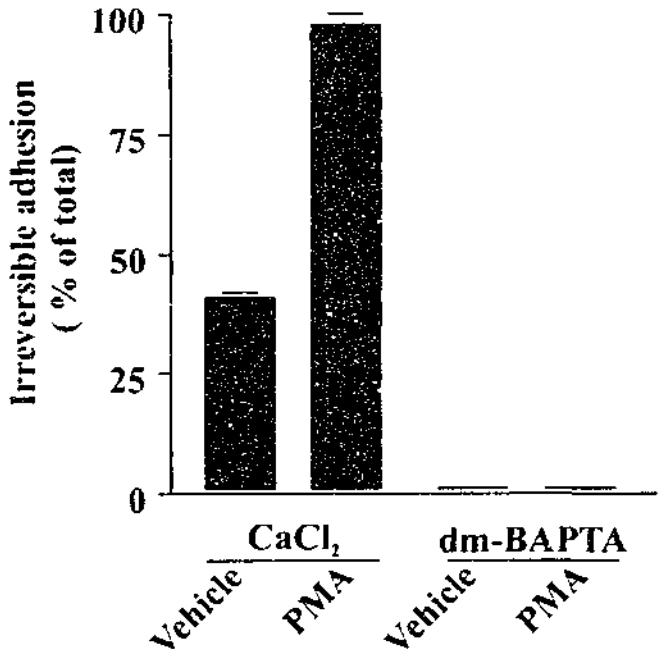
4.8 Protein Kinase C Potentiates Integrin $\alpha_{IIb}\beta_3$- activation Through a Calcium Dependent Mechanism.

Finally, to investigate whether PKC regulation of integrin $\alpha_{IIb}\beta_3$ activation, is absolutely dependent on intracellular calcium mobilisation, flow studies were performed on platelets pretreated with the membrane permeable calcium chelator DM-BAPTA,AM, to chelate cytosolic calcium. As demonstrated in figure 4.9a, PMA was able to induce stationary adhesion contact formation in 100% of vehicle treated platelets but was unable to induce stationary adhesion of DM-BAPTA,AM-treated platelets, with all platelets translocating in a rapid continuous manner. Furthermore, PMA-induced PAC-1 binding to the surface of platelets was completely eliminated by pretreating platelets with DM-BAPTA,AM (fig. 4.9b). The inability of PMA to induce integrin $\alpha_{IIb}\beta_3$ activation under these experimental conditions was not due to the inhibition of pleckstrin phosphorylation (fig. 4.9c), as PMA induced robust pleckstrin phosphorylation in DM-BAPTA,AM-treated platelets, presumably through the activation of one or more calcium-independent (atypical and/or novel) isoforms of PKC. Moreover, DM-BAPTA,AM also inhibited PAC-1 binding to thrombin stimulated platelets (fig. 4.9b), even though pleckstrin phosphorylation was unaffected in these platelets (fig. 4.9c). These studies support a complex co-operative relationship between cytosolic calcium and PKC during integrin $\alpha_{IIb}\beta_3$ activation.

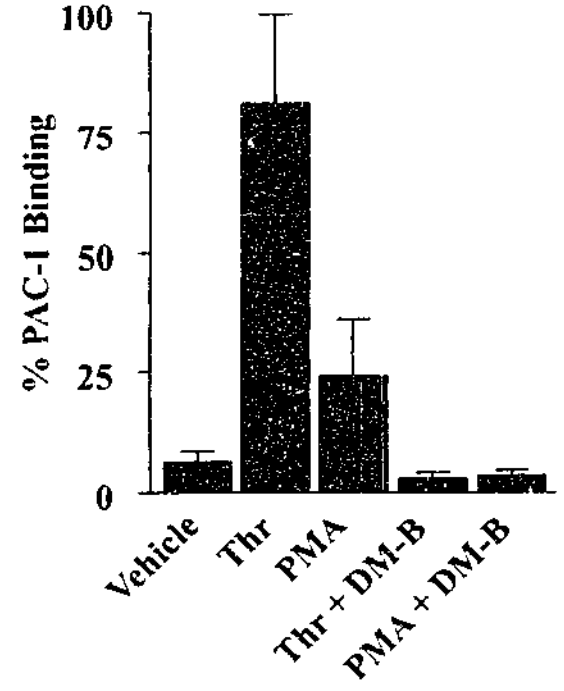
Figure 4.9. Effect of extracellular and intracellular calcium chelation on integrin $\alpha_{IIb}\beta_3$ activation and pleckstrin phosphorylation induced by PMA.

Calcium dye-loaded platelets treated with vehicle (0.25% Me_2SO) or 100 μM dimethyl-BAPTA,AM (dm-B), were perfused through vWf-coated microcapillary slides at $1800s^{-1}$, in the presence or absence of 200 nM PMA. (a) Demonstrates a significant increase in the level of stationary adhesion on immobilised vWf in response to 200nM PMA stimulation. The data also shows PMA induced stationary adhesion formation can be abolished by pre-treatment of platelets with 100 μM dm-B (mean ± SEM, n = 4). Washed platelets treated with vehicle (0.25% Me_2SO) or 100 μM dm-B, were stimulated in suspension under non-stirred conditions with 200 nM PMA or 1 U/ml thrombin (Thr) in the presence of PAC-1 mAb. (b) Demonstrates the level of PAC-1 binding to PMA or thrombin-stimulated platelets in suspension. The level of PAC-1 binding is expressed as a percentage relative to the results obtained from thrombin-stimulated platelets (mean ± SEM, n = 6). Washed platelets loaded with $[^{32}P]$-P_i were incubated with or without 100 mM DM-B prior to exposure for 5 minutes to 200 nM PMA or 1 U/ml of thrombin (Thr) as indicated. (c) Demonstrates that 100 μM dm-B treatment does not inhibit thrombin or PMA induced pleckstrin phosphorylation (p47).

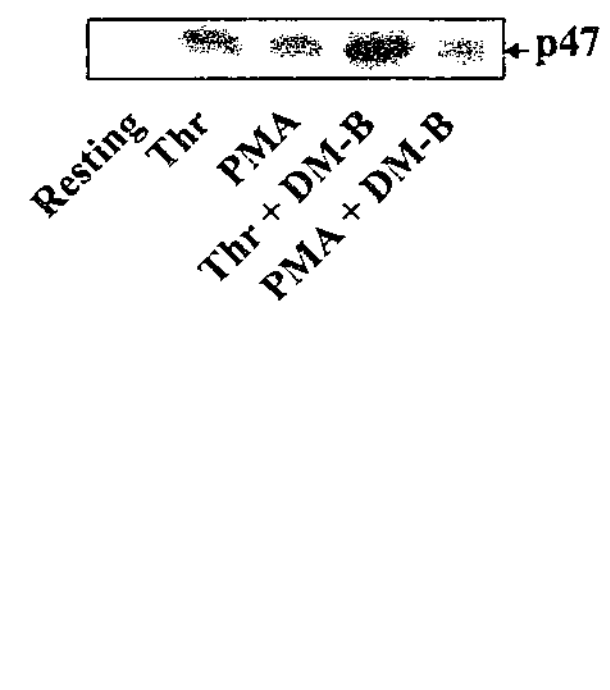
(a)


100
75
50
25
0
Irreversible adhesion
(% of total)
CaCl2
dm-BAPTA
Vehicle
PMA
Vehicle
PMA

(b)


100
75
50
25
0
% PAC-1 Binding
Vehicle
Thr
PMA
Thr + DM-B
PMA + DM-B

(c)


p47
Resting
Thr
PMA
Thr + DM-B
PMA + DM-B

4.9 Discussion.

Unravelling the signalling mechanisms regulating integrin $\alpha_{IIb}\beta_3$ activation during platelet adhesion under shear conditions is of fundamental importance for haemostasis and thrombosis. To date, the signalling processes regulating integrin $\alpha_{IIb}\beta_3$ activation during platelet adhesion on vWf have been relatively poorly defined, although recent studies have suggested a potentially important role for integrin $\alpha_{IIb}\beta_3$-derived calcium signals in this process (Nesbitt et al., 2002). The studies presented in this chapter have demonstrated the existence of a complex signalling relationship operating between PKC, calcium and integrin $\alpha_{IIb}\beta_3$ that serves to regulate the transition from surface translocation to irreversible platelet adhesion. More specifically, they demonstrate that bi-directional integrin $\alpha_{IIb}\beta_3$ signalling through PKC is important for initiating and maintaining integrin $\alpha_{IIb}\beta_3$ activation and stationary platelet adhesion on vWf. Moreover, the present studies have established that the level of the cytosolic calcium response in the platelet cytosol dictates the requirement for PKC during the process of integrin $\alpha_{IIb}\beta_3$ activation.

An unexpected finding from the current study was the demonstration that PKC activation during platelet adhesion on vWf primarily occurred as a consequence of vWf binding to integrin $\alpha_{IIb}\beta_3$. This contradicts previous findings demonstrating that PKC activation primarily occurs downstream of GPIb (Kroll et al., 1991; Kroll et al., 1993). The difference between the present studies and the data reported by Kroll et al., may reflect technical differences in the experimental approaches of these two studies. For example, our studies were performed on immobilised vWf matrices to which platelets bind under physiological flow conditions. Under these conditions, GPIb plays a minor role in PKC activation and most PKC activity occurs primarily via direct outside-in signal transduction downstream integrin $\alpha_{IIb}\beta_3$. On the other-hand, earlier studies of vWf induced integrin $\alpha_{IIb}\beta_3$ activation were performed using artificial modulators of vWf binding or under

pathological shear conditions (Kroll et al., 1991; Kroll et al., 1993) where PKC activation occurs primarily through inside-out signalling as a results of released soluble agonists binding to their rèspective surface receptors following GPIb engagement. These findings indicate that a level of caution should be exercised when attempting to directly extrapolate findings based on aggregation studies from those obtained from adhesion experiments.

Defining the relative contribution of GPIb and integrin $\alpha_{IIb}\beta_3$ in regulating PKC activation and calcium flux is a critical issue with respect to the mechanism by vWf induces integrin $\alpha_{IIb}\beta_3$ activation. For example, if PKC and calcium are primarily regulated downstream of GPIb, with little contribution of integrin $\alpha_{IIb}\beta_3$ outside-in signalling, this would support a model in which these molecules participate in a direct linear signalling pathway linking GP Ib to integrin $\alpha_{IIb}\beta_3$ activation. However, this long-held view of GPIb signalling is not consistent with the current findings that PKC is primarily regulated downstream of integrin $\alpha_{IIb}\beta_3$, and that integrin $\alpha_{IIb}\beta_3$-dependent calcium flux is required for sustained integrin $\alpha_{IIb}\beta_3$ activation and firm platelet adhesion on vWf (Nesbitt et al., 2002). Moreover, the studies presented in this chapter have demonstrated that while direct PKC activation with PMA results in a low level of integrin $\alpha_{IIb}\beta_3$ activation, sustained integrin $\alpha_{IIb}\beta_3$-dependent stationary adhesion formation on vWf does not require ongoing PKC activity if cytosolic calcium levels are sufficiently elevated. These studies suggest that PKC may be important for priming integrin $\alpha_{IIb}\beta_3$-dependent outside-in signalling processes leading to the establishment of a positive feedback loop that promotes the mobilisation of intracellular calcium and hence further integrin $\alpha_{IIb}\beta_3$ activation and stable adhesion formation. This also suggests that the current linear model of GPIb-dependent integrin $\alpha_{IIb}\beta_3$ activation does not adequately explain the signalling relationship between calcium, PKC and integrin $\alpha_{IIb}\beta_3$ activation. The present studies support an alternative

model in which PKC inside-out signalling primarily serves to potentiate integrin $\alpha_{IIb}\beta_3$ activation and transient stable adhesion, whereas subsequent outside-in signalling establishes a positive feedback loop that promotes intracellular calcium mobilisation, and further integrin $\alpha_{IIb}\beta_3$ activation.

Studies examining the effects of PKC inhibitors and phorbol esters on calcium flux during platelet adhesion on vWf revealed that, despite its ability to promote integrin $\alpha_{IIb}\beta_3$ activation, direct PKC activation does not induce a cytosolic calcium response. Furthermore, enhanced calcium flux was only observed following platelet integrin $\alpha_{IIb}\beta_3$ engagement of the vWf substrate and was specifically abolished by blocking ligand binding to $\alpha_{IIb}\beta_3$, supporting an indirect role for PKC in promoting calcium release from internal stores. These results were unexpected in light of the fact that PKC has previously been shown to promote an increase in calcium decay rates in activated platelet, rather than promoting calcium flux. The signalling mechanism by which PKC potentiates $\alpha_{IIb}\beta_3$-induced calcium mobilisation is unclear. It has previously been established that PKC can phosphorylate the IP_3 receptor in reconstituted lipid vesicles presenting as a potential means of regulating intracellular release (Ferris et al., 1991). However, the studies in this chapter do not support a direct effect of PKC on the IP_3 receptor, rather, they demonstrate that the effects of PKC on calcium flux are mediated indirectly, through enhancement of integrin $\alpha_{IIb}\beta_3$-dependent calcium mobilisation. One possible mechanism by which PKC promotes cytosolic calcium flux is by promoting integrin $\alpha_{IIb}\beta_3$ activation, thereby enhancing platelet adhesion contact formation with the vWf substrate and indirectly potentiating outside-in signalling events linked to intracellular calcium mobilisation.

The present studies also demonstrate that the requirement for PKC during integrin $\alpha_{IIb}\beta_3$ activation is dictated by the cytosolic calcium concentration. Figure 4.8 demonstrates that in the presence of intracellular calcium chelators, direct activation of

PKC is unable to induce integrin $\alpha_{IIb}\beta_3$ activation, despite robust pleckstrin phosphorylation under these experimental conditions. These findings suggest that the calcium-activated (conventional) isoforms of PKC may be required for integrin $\alpha_{IIb}\beta_3$ activation. Additionally, these studies have demonstrated that when the mean cytosolic calcium levels are sufficiently elevated, PKC is no longer required for integrin $\alpha_{IIb}\beta_3$ activation. These findings suggest that PKC regulation of integrin $\alpha_{IIb}\beta_3$ occurs within a limited calcium concentration range, a finding that may explain previous observations that PKC inhibitors do not prevent platelet aggregation induced by potent platelet activating stimuli, including thrombin (Iorio et al., 1996; Watson and Hambleton, 1989; Watson et al., 1988).

Numerous studies have established an important synergistic role for PKC and calcium in promoting integrin $\alpha_{IIb}\beta_3$ activation (Kaibuchi et al., 1983; Rotondo et al., 1997; Saitoh et al., 1989; Walker and Watson, 1993). While this synergy undoubtedly plays an important role in potentiating platelet activation, these studies raise the interesting possibility that it may also be important for limiting the irreversible platelet activating effects of calcium. For example, previous studies have demonstrated that a low, transient cytosolic signal induces a sufficient level of integrin $\alpha_{IIb}\beta_3$ activation to reduce platelet translocation velocity on the vWf surface, however adhesion contact formation under these conditions always remains reversible. In contrast, the induction of a high, sustained cytosolic calcium response induces irreversible platelet activation, leading to stable platelet adhesion, spreading and granule release. Thus, the modest effects of PKC on integrin $\alpha_{IIb}\beta_3$ affinity at basal calcium levels (see Fig. 4.6a &b), provides a potential mechanism for 'fine-tuning' the transition from surface translocation to stationary platelet adhesion. Finally our studies demonstrate that the low level of $\alpha_{IIb}\beta_3$ activation induced by PMA in platelet suspensions, was sufficient to promote stationary contact formation in almost all

platelets tethered to an immobilised vWf matrix. However, the onset of the sustained oscillatory calcium response in stationary PMA-treated platelets was delayed significantly compared to vehicle treated platelets, where stationary adhesion coincides exactly with the onset of the sustained calcium flux. The reason for such a delay is unclear. Recent studies have suggested that low levels of calcium in the cytosol are able to sensitise the IP_3 receptor responsible for intracellular calcium mobilisation, such that lower levels of the IP_3 metabolite can mediate calcium release from intracellular stores (Bootman et al., 1997; Merchant, 2001; Merchant, 1999; Tovey et al., 2001). One could speculate that under normal conditions, GP Ib/V/IX engagement of vWf results in signalling events that promote both a low level of PKC activation and also in transient GP Ib/V/IX-derived calcium spikes. This low level calcium signalling may prime the IP_3 receptor in platelets such that, when integrin $\alpha_{IIb}\beta_3$ is engaged, the resulting outside in signalling results in rapid calcium mobilisation. Platelet activation with excess phorbol ester treatment bypasses the initial calcium signalling events required to prime the IP_3 receptor which might account for the delay in calcium flux downstream of integrin $\alpha_{IIb}\beta_3$ activation. However further studies are required to clarify this issue. Regardless of the exact mechanism, these studies support an important role for PKC in promoting integrin $\alpha_{IIb}\beta_3$ bi-directional signalling relevant to platelet adhesion under flow.

CHAPTER 5:

INTERCELLULAR CALCIUM SIGNALLING REGULATES PLATELET AGGREGATION AND THROMBUS GROWTH

Chapter 5.

Intercellular Calcium Signalling Regulates Platelet Aggregation and Thrombus Growth.

5.1 Introduction.

The extent of platelet activation at sites of vascular injury is regulated by several factors. These include, the generation of soluble agonists such as thrombin, ADP and TXA_2 at the site of vessel injury (Hourani and Cusack, 1991; Shattil et al., 1998), the exposure of highly reactive subendothelial matrix proteins such as collagen type I and III (Baumgartner, 1977a; Baumgartner, 1977b; Fuster and Chesebro, 1986; van Zanten et al., 1994; Weiss et al., 1989), and on the shear forces generated at areas of stenosis (Goto et al., 1998; Ruggeri, 1993a). While collagen appears to be the most potent thrombogenic protein expressed in the subendothelial matrix (Baumgartner et al., 1977), recent *in vitro* and *in vivo* studies have demonstrated that platelet adhesion to collagen at high shear rates requires a second adhesive protein, vWf. In fact, it is well established that vWf itself is able to mediate both primary platelet adhesion and subsequent thrombus growth under high shear conditions (Badimon et al., 1989a; Badimon et al., 1989b; Denis et al., 1998; Goto et al., 1998; Kulkarni et al., 2000; Mazzucato et al., 1999; Ni et al., 2000; Ruggeri et al., 1999; Savage et al., 1998; Savage et al., 1996; Tsuji et al., 1999). The molecular mechanism(s) underlying the differences in the thrombogenic potential of these two matrices is unknown.

Platelet adhesion to vWf occurs through a multi-step mechanism involving the sequential binding of platelet GPIb, which mediates the initial (reversible) tethering of platelets to the vWf surface, and the subsequent binding of integrin $\alpha_{IIb}\beta_3$, which promotes

firm (irreversible) adhesion to the vWf matrix (Ruggeri, 2000; Savage et al., 1996). This multi-step adhesion mechanism appears to be equally relevant for platelet adhesion and thrombus growth under high shear conditions. Recent intravital studies have highlighted the dynamic nature of platelet thrombus growth *in vivo*, and have confirmed the importance of the sequential interaction between vWf expressed on the platelet surface with GPIb and integrin $\alpha_{IIb}\beta_3$ on flowing platelets in this process (Denis et al., 1998; Kulkarni et al., 2000; Ni et al., 2000). Intravital microscopy studies have demonstrated that platelets tethering to the luminal surface of thrombi tend to translocate for a variable period of time before either forming stationary adhesion contacts or detaching from the thrombus surface altogether (Kulkarni et al., 2000).

The rate of conversion from reversible to irreversible platelet adhesion on a thrombus surface may ultimately determine the extent of thrombus growth in blood vessels. While it is well known that stationary platelet adhesion to the surface of developing thrombi requires active integrin $\alpha_{IIb}\beta_3$, to date, the mechanism(s) regulating the conversion from surface translocation to firm platelet adhesion on vWf have been poorly defined. It has been shown in leukocytes that the conversion from surface translocation, to stationary adhesion is mediated by inside-out signalling events induced by soluble cytokines which increase the binding strength of β_2-integrins (von Andrian et al., 1991). The studies presented in chapter 3 and 4 of this thesis and by (Nesbitt et al., 2002), have demonstrated that calcium signalling events generated by GPIb and integrin $\alpha_{IIb}\beta_3$ engagement are sufficient to promote stationary platelet adhesion to vWf independent of soluble platelet agonists Recent studies by (Kuwahara et al., 1999) have also demonstrated a potentially important role for integrin $\alpha_{IIb}\beta_3$-dependent calcium flux in promoting platelet aggregation and thrombus growth on vWf.

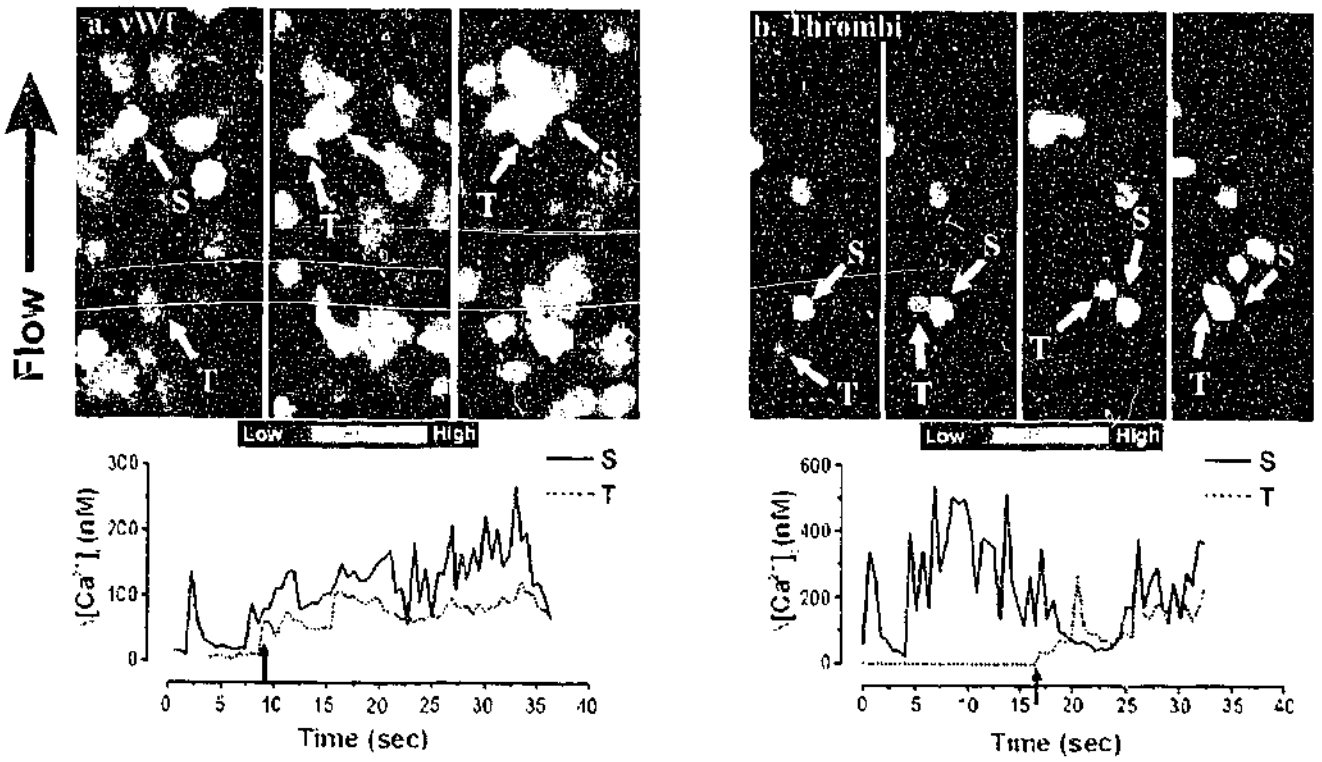
Given the importance of the GPIb and integrin $\alpha_{IIb}\beta_3$ derived calcium signals in promoting stationary platelet adhesion on vWf under high shear conditions, it is conceivable that the calcium signals generated downstream of GPIb and $\alpha_{IIb}\beta_3$ engagement may also play an important role in regulating platelet aggregation. In this chapter a confocal based imaging technique that enables real-time analysis of platelet calcium flux was used to assess the role of calcium during thrombus formation.

5.2 Inter-cellular Calcium Communication (ICC) Promotes Platelet aggregate formation on the Surface of Thrombi.

To assess the role of cytosolic calcium during platelet cohesion and thrombus formation on a vWf matrix, calcium dye loaded platelets were reconstituted with washed red blood cells (50% haematocrit) at 150 x 10^9/L in order to maximise platelet-platelet interactions, and then perfused over purified vWf (100 µg/ml) at 1800 s^{-1}. Platelets that formed irreversible stationary adhesion contacts on the vWf matrix were found to exhibit high range sustained oscillatory calcium flux as described previously in this thesis (fig 3.7a, section 3.5). Once firm adhesion contacts were established, the 'primary' stationary platelets appeared to act as reactive surfaces able to recruit other neighbouring platelets and initiate the formation of small aggregates. As demonstrated in figure 5.1a, Translocating platelets (T) that came into close proximity to 'primary' stationary platelets (S) that expressed high oscillatory calcium levels, tethered to these cells and formed stationary contacts themselves. Moreover, the 'primary' stationary platelets appeared to transfer or communicate their 'calcium activation status' to the tethering platelets, such that, the newly adherent cells also displayed similar calcium oscillations to those seen in primary stationary platelets. The lower panel in figure 5.1a shows an initially stationary cell (S) undergoing high range sustained calcium oscillations on a vWf surface, inducing a

Figure 5.1 Inter-cellular Calcium Communication (ICC)

Platelets (150 x10^9/L) were perfused at and a shear rate of 1800 s^{-1} over immobilised vWf or over the surface of pre-formed thrombi *in vitro*. Single channel Oregon Green fluorescence images of platelets undergoing ICC during aggregate formation on immobilised vWf (a-top panel) or during platelet interactions on the surface of preformed thrombi (b- top panel). **S**, marks a primary stationary adherent cell; and **T**, a tethering cell. Representative single-cell calcium flux recordings demonstrating the duration and amplitude of the cytosolic calcium signal in primary adherent cells (S-solid line) and tethering cells (T-dotted line) are shown below the fluorescence images. The arrow bold arrow (↑) indicates the point at which the tethering platelet physically interacts with the stationary platelet and propagation of the calcium signal first occurs. $\Delta[Ca^{2+}]_c$ recordings are representative of 25 platelets from 3 independent experiments. (c) Single channel Oregon Green fluorescence images demonstrating a lack of sustained adhesion, aggregation and ICC propagation when the interaction of tethering platelets (dotted marques) is not timed within 0.6-s of moment when the calcium flux in a primary stationary cell (→) is at its peak. As shown, lack of ICC results in both the primary adherent and tethering cells returning to surface translocation. (d) The percentage of platelets tethering with a primary adherent platelet and undergoing ICC, was quantified at time points where the primary adherent cell was expressing high $\Delta[Ca^{2+}]_c$, or low $\Delta[Ca^{2+}]_c$. ICC occurs in 53 % of cells when tethering coincides with a period where the primary adherent platelet expresses high calcium levels, but does not occur at all when the primary adherent platelet expresses minimal calcium levels (n = 25 platelets).


a. vWf
b. Thrombi
Flow
S
T
Low
High
S
T
[Ca2+] (nM)
Time (sec)
c. Timing
2μm
Flow
0-s
1.2-s
8.5-s
10.8-s
21.0-s
23.9-s
High
Low
d.
Tethering Platelets Undergoing Calcium Oscillations (% Total)
75
50
25
0
High Δ[Ca2+]c
Low Δ[Ca2+]c

rapid increase in calcium flux in a translocating platelet (T), at the point at which the two platelets come into contact with each other (marked by the arrow). This resulted in the formation of small but relatively stable platelet aggregates (6-10 cells) that underwent sustained oscillatory calcium flux throughout the 3 minute observation period (fig. 5.1a). This data demonstrates for the first time, that platelets within a developing aggregate are capable of transmitting calcium activation signals to adjacent cells. This process will be referred to as Inter-platelet Calcium Communication (ICC).

Detailed single cell analysis of the calcium responses in developing platelet aggregates revealed that efficient propagation of the calcium signal from a stationary platelet (S) to its tethered counterparts (T), required that contact between the cells be made within a narrow temporal window (≤ 0.6 seconds) at the point at which the $\Delta[Ca^{2+}]_i$ of the primary adherent cell was at its peak (fig. 5.1c). Of the platelets that tethered to a primary stationary cell over the observation period (3 minutes), only 53 % were found to form stationary adhesions and display elevated calcium oscillations (fig. 5.1d).

To determine whether ICC also plays a role in the subsequent recruitment of platelets to the surface of growing thrombi, similar perfusion studies were conducted on preformed thrombi. In these studies, thrombi were generated by perfusing citrated whole blood over type I fibrillar collagen for 5 minutes as described in section 2.7.3. Subsequently, calcium dye loaded platelets (150 x 10^9/L) were perfused over the preformed thrombi and calcium ratio measurements were taken of platelets interacting with the thrombus surface. A similar 'transfer' of calcium activation status was also observed on the surface of thrombi (fig. 5.1b), where stationary platelets expressing elevated calcium levels (S) were found to propagate their calcium status to other platelets within the local population of tethered cells (T). These studies suggest a potentially important role for ICC in promoting thrombus growth by facilitating the arrest of translocating platelets.

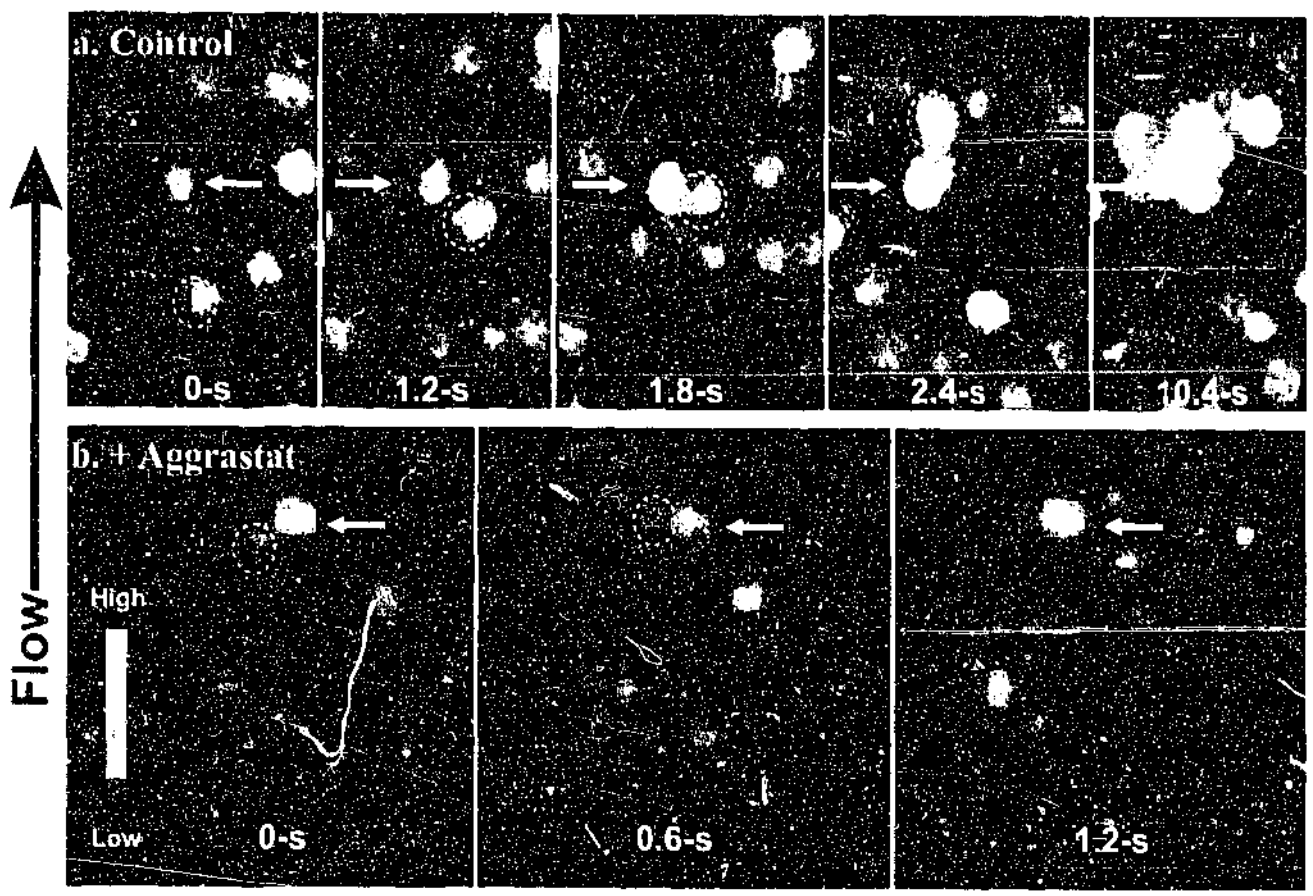
5.3 Integrin $\alpha_{IIb}\beta_3$ Requirement for Inter-platelet Calcium Communication.

The studies presented in chapter 3 and 4 of this thesis highlight the central role for integrin $\alpha_{IIb}\beta_3$ in promoting cytosolic calcium flux and sustained platelet adhesion and aggregation on immobilised vWf. To determine whether integrin $\alpha_{IIb}\beta_3$ is required for promoting calcium communication, the effect of integrin $\alpha_{IIb}\beta_3$ inhibition on ICC was examined. In these studies an initial population of 'firmly adherent' platelets undergoing sustained calcium oscillations was established by perfusing calcium dye-loaded platelets over an immobilised vWf matrix and then washing away loosely adherent or translocating platelets. A second population of platelets treated with either control buffer or with the integrin $\alpha_{IIb}\beta_3$ antagonist, aggrastat, was then perfused over these primary adherent platelets at a shear rate of 1800 s^{-1}. As demonstrated in figure 5.2a, rolling platelets that tethered to pre-adherent platelets expressing sustained calcium oscillations, were able to form stationary adhesion contacts themselves and the calcium activation status of the primary spread cell was propagated to its tethering partners, demonstrating that ICC could be observed on vWf under these assay conditions.

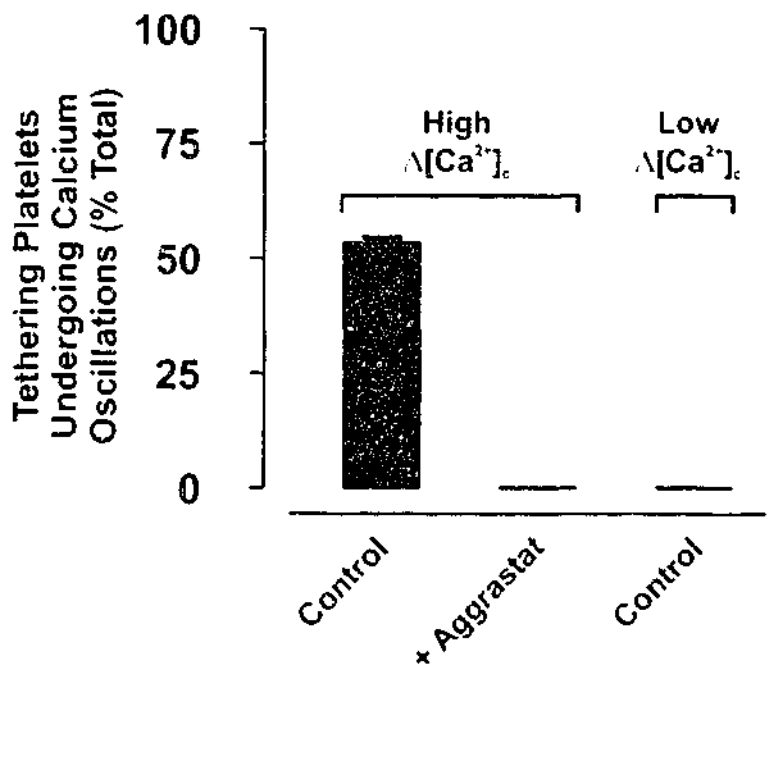
In subsequent experiments, platelets were pre-incubated with aggrastat to block ligand binding to integrin $\alpha_{IIb}\beta_3$ on the surface of flowing platelets prior to perfusion over pre-adherent platelets. This was found to completely inhibit inter-platelet calcium communication suggesting that integrin $\alpha_{IIb}\beta_3$ plays a key role in promoting ICC (fig. 5.2b and c). Interestingly, aggrastat did not inhibit the ability of stationary 'pre-adherent' platelets to undergo sustained oscillatory calcium flux despite its inhibitory effects on integrin $\alpha_{IIb}\beta_3$ activity at the luminal surface of these platelets. Presumably, this is because integrin $\alpha_{IIb}\beta_3$-mediated signal transduction in the pre-adherent platelets can still occur through ligated integrins on their basal surface.

Figure 5.2 ICC Requires Integrin $\alpha_{IIb}\beta_3$ Engagement

Platelets (150 x10^9/L) were perfused over immobilised vWF at 1800 s^{-1} to establish an initial population of stationary adherent cells undergoing sustained calcium oscillations (→). A second population of platelets treated with or without 200 nM Aggrastat (dotted marques) were subsequently perfused over this reactive population and platelet-platelet interactions monitored in real-time. (a) Control flows demonstrating efficient ICC and aggregate formation between primary stationary (→) and tethering platelets (dotted marques). (b) Aggrastat treatment effectively inhibited the propagation of the initial calcium signal and prevented aggregate formation at the surface of the vWF matrix. (c) The percentage of platelets coming into contact with an initially adherent platelet and undergoing concomitant calcium oscillations (ICC), was quantified at time points where the primary adherent cell was expressing high $\Delta[Ca^{2+}]_c$ or low $\Delta[Ca^{2+}]_c$. ICC is dependent on integrin-$\alpha_{IIb}\beta_3$ engagement and is completely abolished by aggrastat (n = 25 platelets).


a. Control
0-s
1.2-s
1.8-s
2.4-s
10.4-s
b. + Aggrastat
Flow
High
Low
0-s
0.6-s
1.2-s

c.

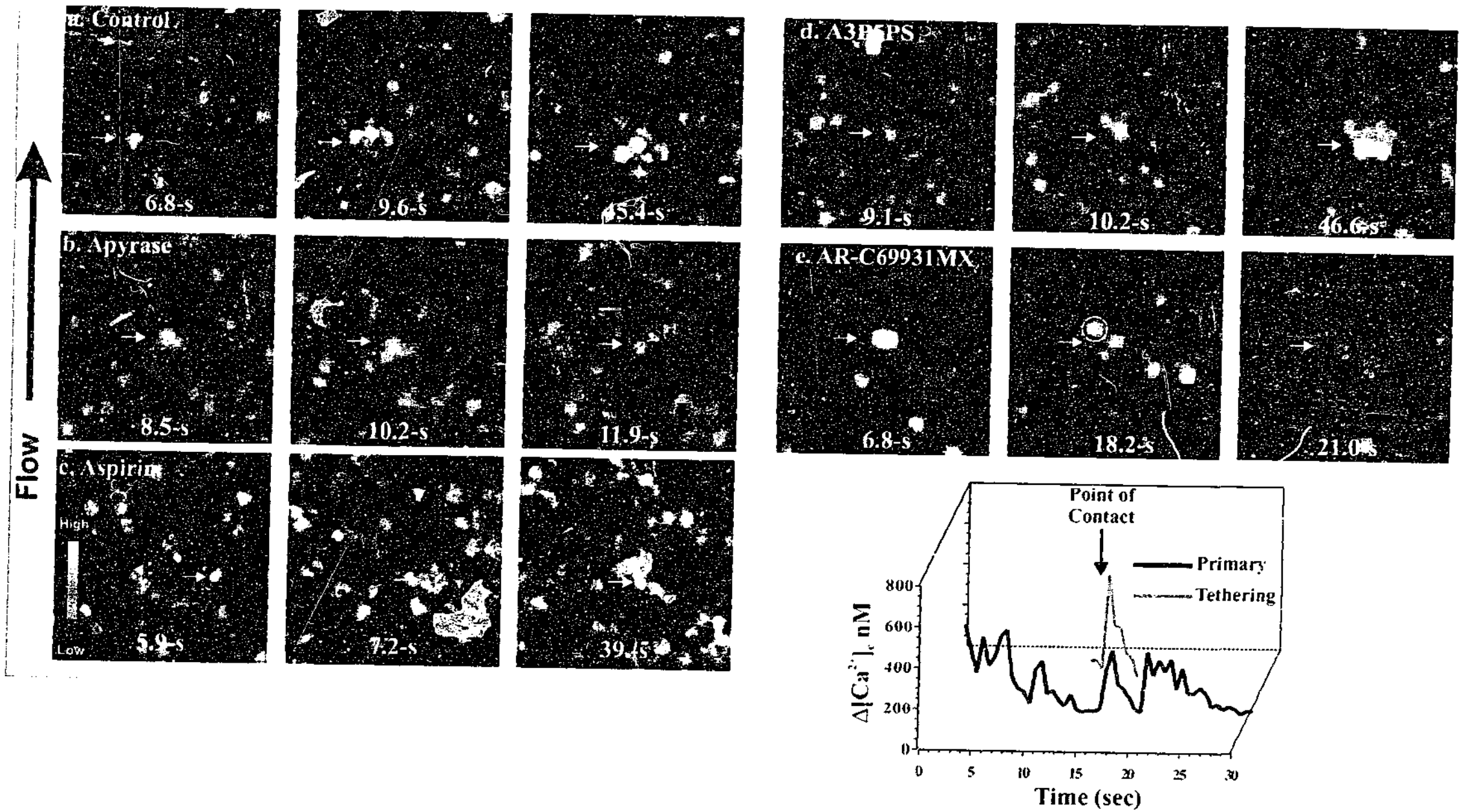

Tethering Platelets Undergoing Calcium Oscillations (% Total)
100
75
50
25
0
High Δ[Ca2+]c
Low Δ[Ca2+]c
Control
+ Aggrastat
Control

5.4 Role of Secreted ADP for Inter-platelet Calcium Communication.

Conflicting evidence exists regarding the role of secreted platelet agonists such as ADP and TXA_2 during platelet adhesion and activation on reactive matrix proteins. TXA_2 generation and the release of endogenous ADP from platelet dense granules is known to be important for stable thrombus formation both *in vivo* and *in vitro* (Cattaneo and Gachet, 1999; Fabre et al., 1999; Hourani and Cusack, 1991; Huang et al., 2000; Leon et al., 1999a; Leon et al., 1999b; Nieswandt et al., 2001b). ADP in particular, has also been shown to play a key role in promoting and stabilising platelet aggregates during stimulation with many physiological agonists, including vWf (Cattaneo et al., 1990; Chow et al., 1992; Gachet, 2001a; Ikeda et al., 1991; Moake et al., 1988; Moritz et al., 1983; Oda et al., 1995; Peterson et al., 1987; Turner et al., 2001). To investigate the possibility that released ADP is also required to promote platelet aggregation and inter-cellular calcium communication on vWf, calcium dye loaded platelets (150 x 10^9/L) were perfused through vWf coated microcapillary slides in the presence of the ADP scavenging enzyme apyrase (1.5 U/ml). In agreement with previous studies (Nesbitt et al., 2002), a primary adherent population of platelets expressing sustained oscillatory calcium flux could still be established in the presence of apyrase (fig. 5.3a and b). However, as shown in figure 5.3b, the ability of these primary adherent cells to induce calcium signals in their tethering counterparts was completely inhibited, as was the ability of tethering platelets to form stable adhesion contacts with primary adherent platelets and to form platelet aggregates. Identical results were observed when these experiments were performed in the combined presence of two specific purinergic receptor antagonists, A3P5PS and AR-C69931MX, to selectively inhibit ADP signalling through the platelet $P2Y_1$ and $P2Y_{12}$ receptors respectively (Boyer et al., 1996; Humphries, 2000; Ingall et al., 1999) (data not shown). This data supports a key role for released ADP for ICC and aggregate formation on a vWf matrix. To

Figure 5.3 Secreted ADP Drives ICC and Platelet Aggregation on a vWf matrix.

Calcium dye-loaded platelets (150 x10^9/L) were perfused over immobilised vWf at 1800 $s^{-1}$and Oregon Green fluorescence images were recorded in real time. (a) Single channel Oregon Green fluorescence images of vehicle (Me_2SO) treated platelets on immobilised vWf. (→) Indicates a primary adherent platelet undergoing vWf dependent calcium flux. The cluster of platelets formed at the 9.6-s and 45.4-s time points, demonstrate that ICC and aggregate formation takes place at sites of primary platelet-vWf adhesion. (b) Single channel Oregon Green fluorescence images of apyrase (1.5 U/ml) treated platelets on immobilised vWf. (-→) Indicates a single platelet undergoing transient calcium flux that correlates with short duration stationary adhesion formation. Images taken at 10.2-s and 11.9-s demonstrate that although platelet-platelet contacts occur in the shear field, apyrase treatment effectively abolishes ICC. Treating platelets with (c) aspirin (1.5 μM) or (d) A3P5PS (100 μM) prior to perfusion over immobilised vWf does not inhibit ICC at sites of primary platelet-vWf adhesion demonstrating that ICC does not require $TX^{\wedge}_2$ or $P2Y_1$ signalling. However treating platelets with (e) AR-C69931MX (200nM) prior to perfusion over immobilised vWf abolishes ICC demonstrating that $P2Y_{12}$ signalling is required for ICC. (e-lower panel) representative calcium flux profile of a primary stationary platelet (black line) and a tethering platelet (red line) showing that platelet-platelet contact in the presence of AR-C69931MX results in a transient intra-platelet calcium spike that is not sustained resulting in only transient platelet-platelet contact and lack of ICC and aggregate formation.


a. Control
6.8-s
9.6-s
15.4-s
b. Apyrase
8.5-s
10.2-s
11.9-s
c. Aspirin
High
Low
5.9-s
7.2-s
39.4-s
Flow
d. A3P5PS
9.1-s
10.2-s
46.6-s
e. AR-C69931MX
6.8-s
18.2-s
21.0-s
Point of Contact
Primary
Tethering
800
600
400
200
0
0
5
10
15
20
25
30
Time (sec)

investigate the role of TXA_2 in ICC and platelet aggregation on vWf, calcium dye loaded platelets (150 x 10^9/L) were pre-treated with 1.5mM aspirin prior perfusion over the surface of immobilised vWf. Consistent with earlier studies (Nesbitt et al., 2002), aspirin pre-treatment did not inhibit the ability of platelets to form primary stationary adhesions with the vWf matrix, nor did it inhibit ICC at the surface of vWf under flow conditions (fig. 5.3c). In control studies 1.5mM aspirin was found to completely inhibit the platelet aggregation response to arachidonic acid (data not shown).

To gain further insight into the mechanism by which ADP promotes ICC and aggregate formation on vWf under shear conditions, the individual role of platelet ADP receptors $P2Y_1$ or $P2Y_{12}$ was examined. In these experiments, calcium dye loaded washed platelets were perfused at high shear (1800 s^{-1}) over immobilised vWf, in the presence of either A3P5PS or AR-C69931MX, to selectively inhibit ADP signalling through $P2Y_1$, or $P2Y_{12}$, respectively. Treatment of platelets with A3P5PS (200 μM) did not inhibit ICC or effect the ability of platelets to form aggregates on the vWf matrix (fig. 5.3d). Conversely, blockade of the $P2Y_{12}$ receptor using AR-C69931MX (200 nM), resulted in inhibition of ICC and sustained aggregate. As shown in figure 5.3e, while initial primary stationary adhesion formation and calcium spiking associated with platelet-vWf contact was not inhibited by AR-C69931MX treatment, the propagation of ICC and the generation of a sustained oscillatory calcium response in platelets tethering to primary adherent platelet was totally abolished. These results suggest that the $P2Y_{12}$–linked G_i signalling pathway plays is critical for supporting ICC and platelet aggregation under shear conditions.

5.5 Inter-platelet Calcium Communication Drives Thrombus Growth.

Earlier studies presented in this thesis demonstrated that a type I collagen matrix elicited stronger calcium responses in adherent platelets than vWf. Given the importance of

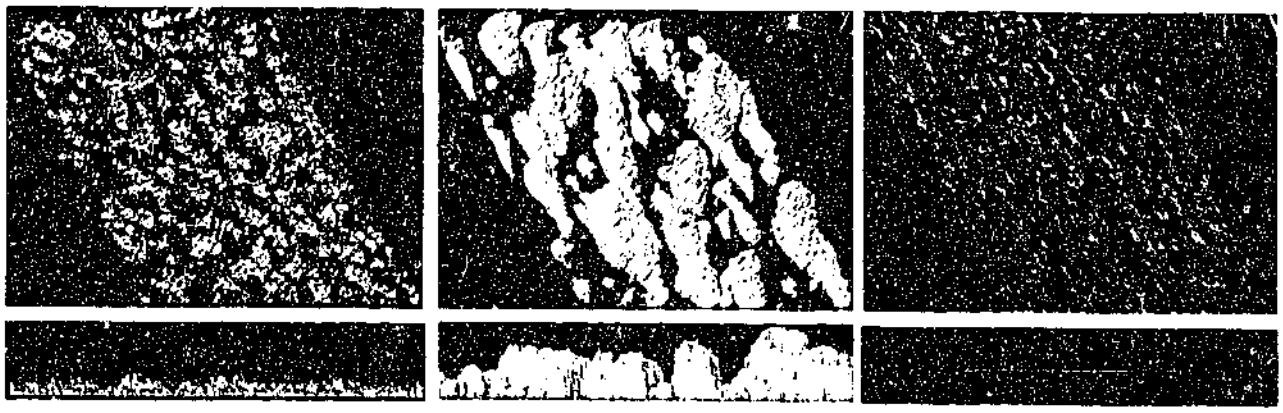
inter-cellular calcium communication during the process of platelet aggregation on vWf under high shear, it is possible that the rate and extent of thrombus growth under flow conditions may be regulated by the extent of calcium mobilisation elicited by the adhesive matrix, and subsequently, the efficiency by which calcium signals are propagated throughout an aggregating platelet population. To test this hypothesis, platelets (150 x 10^9/L) were reconstituted with red blood cells (50 % haematocrit) and citrated plasma, and perfused at 1800 s^{-1} through vWf or collagen coated microcapillary slides to compare the level of thrombus formation on these matrices. Generally, the thrombi generated on a vWf matrix were relatively small, typically measuring <20, 000 μm^3 in volume, and formed in discrete clusters over the vWf surface (fig. 5.4a and b). On the other hand, platelet aggregates formed on collagen fibrils were extremely large compared to vWf and measured ≥ 110, 000 μm^3 in volume (fig. 5.4a and b). Detailed analysis of primary platelet adhesion on the vWf matrix showed that only 6.5 ± 1.7% of tethered cells maintained stationary adhesion contacts for longer than 30 seconds (fig. 5.4c), indicating that the majority of adhesion contacts formed under these experimental conditions remain reversible. In contrast, 100% of platelets interacting with collagen fibrils formed immediate and irreversible stationary adhesion contacts with this matrix (fig. 5.4c) and they provided highly efficient nuclei for the recruitment of additional platelets, resulting in the rapid formation of very large platelet aggregates.

To investigate the hypothesis that ICC propagation regulates the rate and extent of thrombus formation, real-time changes in cytosolic calcium levels were monitored during platelet thrombus formation on a type I fibrillar collagen or vWf matrix. Single cell analysis of platelet calcium flux and adhesive behaviour under flow revealed that only a small percentage of tethered platelets (6.5 ± 1.7%) formed prolonged stationary adhesion contacts and exhibited an observable calcium response on a vWf matrix. However all

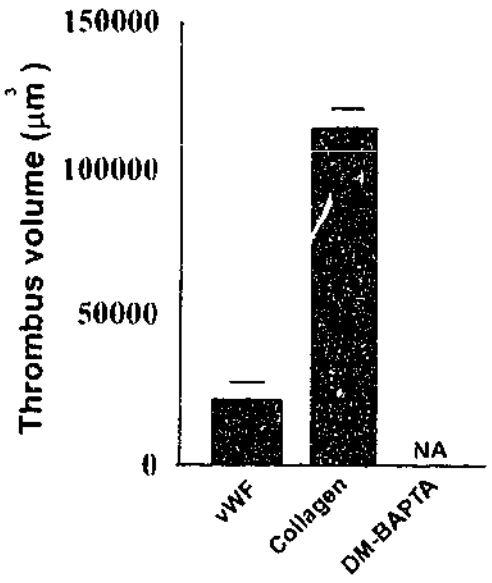
Figure 5.4 ICC Drives Thrombus Growth

Washed platelets (150 x 10^9 cells/L) were treated with vehicle (0.25% Me_2SO) or 100 μM DM-BAPTA,AM (dm-B) were indicated prior to perfusion over immobilised human vWf (100 μg/ml) or type 1 fibrillar collagen (2.5 mg/ml) 1800 s^{-1} for 5 minutes as described in section 2.6.4. Platelet thrombi formed after 5 minutes were subsequently imaged by confocal microscopy and reconstructed using image analysis software. 3D-reconstructed images (a) were created using image tool software. (b) Thrombus volumetric data demonstrating the marked difference in platelet thrombus size on the surface of immobilised vWf versus type 1 collagen. Note: in the case of DM-BAPTA-treated cells, thrombi did not form and the platelets translocated freely across the collagen surface (n=3). In (c) the percentage of platelets forming stationary adhesion contacts with vWf or collagen was quantified and expressed as the percentage of total adherent platelets (n=3).

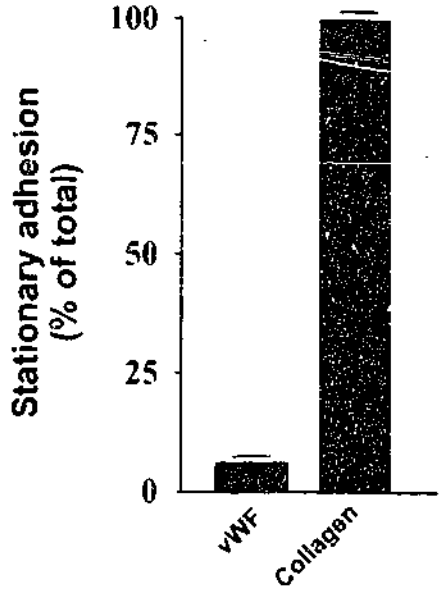
a.


vWF
Collagen
DM-BAPTA
Collagen

b.


Thrombus volume (μm³)
150000
100000
50000
0
NA
vWF
Collagen
DM-BAPTA

c.


Stationary adhesion
(% of total)
100
75
50
25
0
vWF
Collagen

platelets that tethered to type I collagen fibrils were found to form stable adhesion contacts and elicited rapid oscillatory calcium flux. Furthermore, collagen induced higher level calcium signalling than vWf. The mean $\Delta[Ca^{2+}]_c$ response in vWf adherent platelets was found to be relatively low (mean 234.5 nM; max 796 nM) compared to the calcium levels expressed in collagen adherent platelets (mean 913.3 nM; max 1,974 nM) (fig. 5.5a). More importantly, of the vWf adherent platelets that tethered to a primary adherent platelet undergoing sustained calcium oscillations, only 53% were found to form stationary contacts and display ongoing calcium flux, the remaining 47% failed to sustain this response and subsequently detached and returned to the bulk flow (fig. 5.5b and c). In contrast, on a collagen matrix, 100% of adherent platelets displayed sustained calcium oscillations resulting in the formation of extremely efficient nuclei for the subsequent recruitment of platelets from flowing blood. All platelets that subsequently tethered to the primary adherent platelets formed stable adhesion contacts and displayed sustained calcium flux themselves (fig. 5.5b and c). Another fundamental difference between the $\Delta[Ca^{2+}]_c$ observed in platelets adherent to vWf and type I collagen, is the amount of time that the platelets express a high calcium activation state. Frequency distribution analysis of platelet calcium over a 30 second period showed that the probability of any one platelet adherent to vWf expressing an elevated $\Delta[Ca^{2+}]_c$ >100nM was 0.06, compared to 0.95 on a collagen surface (fig. 5.5a). In all studies (> 100 independent experiments), there was a strict correlation between cytosolic calcium flux in the primary adherent layer of platelets and the propensity of these cells to act as nuclei for platelet aggregate formation. Chelating cytosolic calcium using DM-BAPTA-AM (100 μM) completely eliminated calcium oscillations in the primary adherent cells, and consequently inhibited platelet aggregation and thrombus growth on both a vWf and collagen matrix (fig. 5.5a). Taken together, these results demonstrate that there are significant differences in the cytosolic calcium signal

Figure 5.5 The Extent of Thrombus Growth Depends on the Level of Cytosolic Calcium Flux Induced by the Adhesive Matrix. Platelets (150 x 10^9/L) were treated with or without DM-BAPTA (dm-B) and perfused over immobilised vWf or type I collagen at 1800 s^{-1}. (a) $\Delta[Ca^{2+}]_c$ population analysis showing the distribution of platelet calcium events occurring at the surface of immobilised vWf and type 1 collagen. The grey box indicates the 100 nM calcium threshold below which platelets are considered to be in the resting state (n=3). (b) The percentage of platelets coming into contact with an initially adherent platelet and undergoing concomitant calcium oscillations (ICC), was quantified at time points where the primary adherent cell was expressing high $\Delta[Ca^{2+}]_c$. The data indicates that ICC occurs in 53 % of cells tethering to primary adherent cells at the surface of vWf, while 100% of tethering cells express sustained calcium oscillations at the surface of type 1 collagen (n = 25 platelets). (c) Oregon Green fluorescence images demonstrating intraplatelet calcium flux during real-time aggregate formation at the surface of immobilised vWf or collagen type 1. (←) Indicates the site of initial platelet adhesion to both collagen and vWF matrices. Note that the images on type I collagen show aggregate formation on a single collagen fibre in comparison to the continuous surface of immobilised vWf.

elicited in the initial layer of adherent platelets which influence the efficiency of ICC propagation and aggregate formation on a vWf versus a collagen substrate.

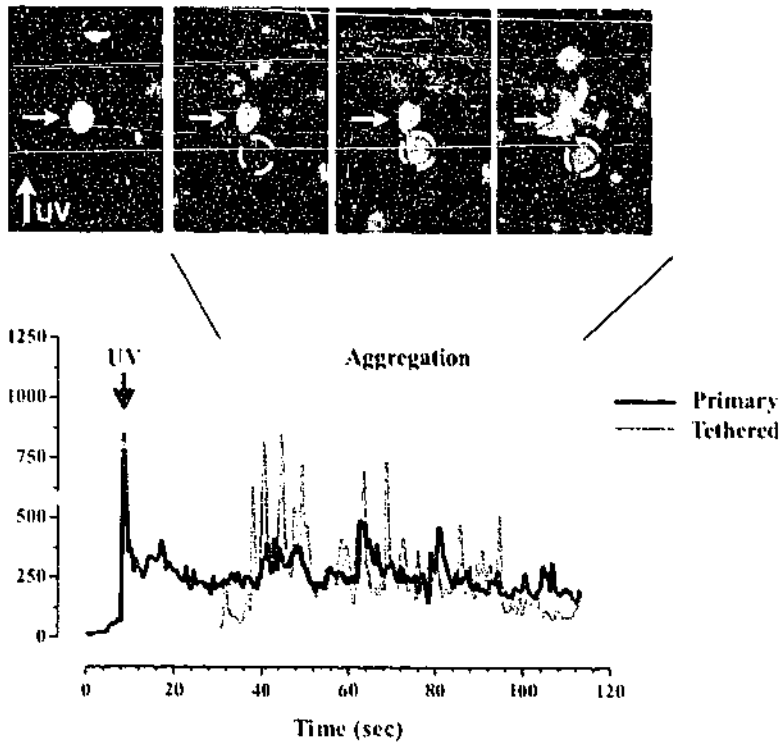
To investigate more directly the hypothesis that elevation of cytosolic calcium in an initial population of adherent platelets is required for efficient platelet aggregation and ICC on a vWf matrix, the effect of artificially inducing a transient calcium spike in translocating platelets was examined. In these studies, washed platelets were loaded with calcium indicator dyes and the caged calcium chelator NP-EGTA, prior to reconstitution (150×10^9/L) with red blood cells (50% haematocrit), and perfusion through vWf coated microcapillary slides at 1800 s^{-1}. Platelets rolling on the vWf surface were then exposed to a rapid beam of UV light which resulted in a transient but rapid increase in cytosolic calcium (Section 4.7) and (Nesbitt et al., 2002). Unlike the two-step pre-adhesion method described in section 5.3, this experimental approach allows rapid release of calcium in a discrete population of primary adherent platelets only, immediately prior to the formation of platelet-platelet adhesion contacts, thereby maximising the possibility of coordinating the initiation of calcium flux with subsequent ICC. Consistent with previous studies (Nesbitt et al., 2002), inducing transient calcium spikes in platelets that were not interacting with other translocating platelets resulted in only a temporary arrest of translocation in these cells (fig. 5.6a). However, induction of a single calcium spike at the point at which two translocating platelets come into contact with each other, resulted in the induction of a sustained oscillatory calcium flux and prolonged arrest of both platelets. Furthermore, these cells formed efficient nuclei for the recruitment of additional free flowing platelets leading to ICC and the rapid formation of platelet aggregates (fig. 5.6a and b). As demonstrated on figure 5.6c, platelet aggregate formation and ICC under these experimental conditions was completely inhibited by pre-treating the platelets with apyrase suggesting that it was completely dependent on released ADP. These results further

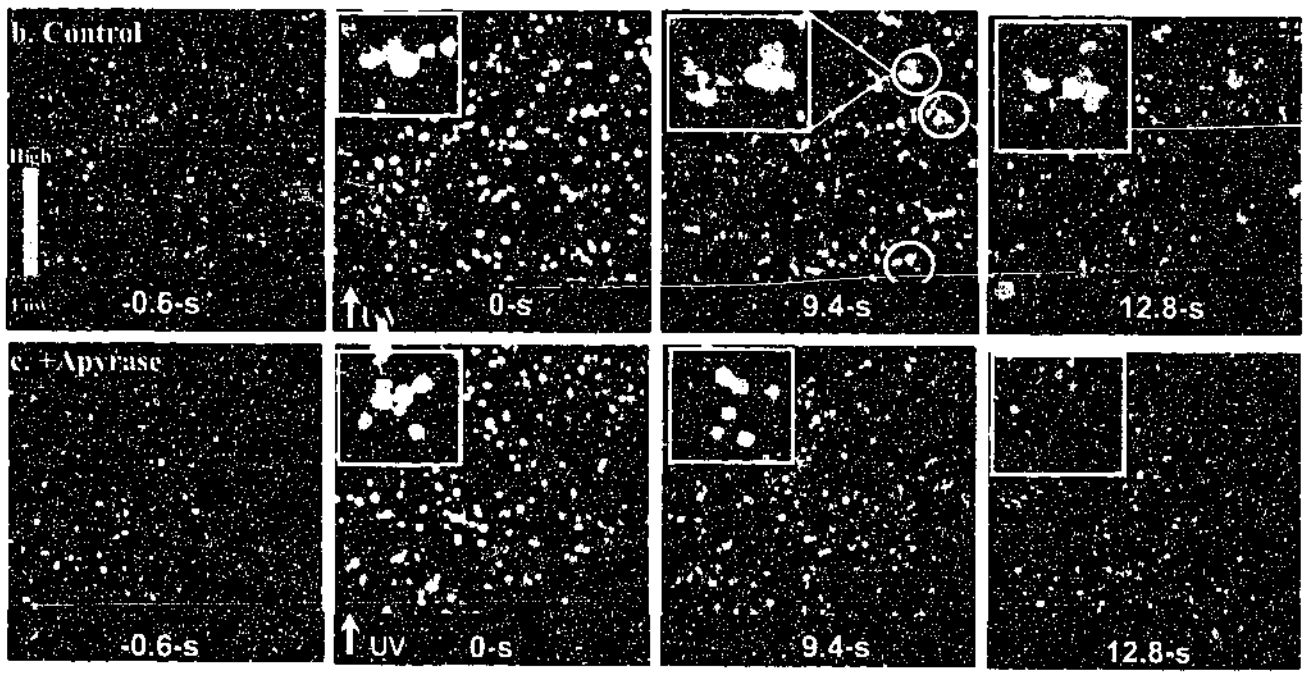
support the hypothesis that the "transmission" of calcium signals (ICC) between adjacent platelets is a major factor regulating the efficiency of platelet aggregation under flow.

Figure 5.6 NP-EGTA Uncaging Triggers ICC Events and Platelet Aggregation

Platelets (150 x 10^9/L) loaded with NP-EGTA (10 μM) were perfused through vWf (100 μg/ml) coated microcapillary tubes at a shear rate of 1800 s^{-1}. Calcium release was induced by UV uncaging as described in section 2.7.2. (a) Single channel Oregon Green fluorescence images (top panel) and real-time calcium flux recordings (lower panel) demonstrating the ICC propagation between a stationary adherent cell (→) and a tethering partner (marque), following NP-EGTA uncaging. The arrow (↑) indicates the point at which the primary stationary cell is activated by UV light. The grey box indicates the period during which the secondary platelet (red line) tethers to the primary stationary platelet (black line). (b) Control NP-EGTA-loaded platelets were allowed to tether and translocate across the vWf matrix (-1.2-s and -0.6-s) and subsequently exposed to a near UV light source (350nm ↑UV) for 0.6-s leading to release of intra-cellular calcium (0-s). The zoomed boxes highlight the occurrence of platelet aggregation and ongoing ICC following UV uncaging. Small aggregates forming on the vWf surface are highlighted by the marques. (c) NP-EGTA-loaded platelets were perfused on the vWf matrix in the presence of 1.5 U/ml apyrase. The zoomed boxes demonstrate that apyrase treatment results in a lack of ICC post UV stimulation. Platelets resume translocation following a brief period of stationary adhesion.

a. NP-EGTA
Aggregation





5.6 Discussion.

The studies in this chapter provide the first evidence that platelets are able to 'communicate' with adjacent cells through an indirect process termed inter-platelet calcium communication (ICC). These studies describe a novel co-operative signalling mechanism involving ADP-induced activation of the $P2Y_{12}$ pathway, and integrin $\alpha_{IIb}\beta_3$ signalling, during platelet adhesion and aggregation under flow conditions. Furthermore, the present studies demonstrate that the efficiency with which primary adherent platelets can promote ICC, is critical for promoting the transition from surface translocation to firm platelet adhesion and in regulating the extent of thrombus growth under flow conditions on different matrices such as vWf and collagen. These findings suggest that differences in the reactivity of these adhesive substrates may be explained by their ability to promote ICC and integrin $\alpha_{IIb}\beta_3$ activation in subsequently tethering platelet, and provide a new model for the regulation of platelet thrombus growth under high shear conditions.

It is generally accepted that the conversion of translocating platelets to stably adherent cells on the surface of thrombi is modulated by the activation status of integrin $\alpha_{IIb}\beta_3$. It is also well established that some adhesive substrates such as collagen, are more thrombogenic than other substrates such as vWf. However, an important and largely unresolved issue is the mechanism(s) by which these adhesive substrates propagate stimulatory signals to subsequent layers of aggregating platelets and induce activation of integrin $\alpha_{IIb}\beta_3$ on adjacent platelets. As demonstrated here, the ability of vWf and collagen to promote platelet thrombus formation is directly related to the level of calcium mobilisation that the respective matrices elicit in the initial layer of adherent cells. Rapid thrombus formation on a collagen matrix was associated with efficient communication of calcium signals between aggregating platelets, resulting in a high proportion of tethering

platelets forming stable adhesion contacts. In contrast, most platelets translocating rapidly on a vWf matrix do not exhibit sufficient sustained calcium oscillations. The relative inefficiency of ICC on a vWf matrix results in a high proportion of tethering platelets forming reversible adhesion contacts with the substrate and therefore limited aggregate formation.

The extent of thrombus growth and the efficiency of ICC in vivo is likely to be governed by the cumulative effect of multiple activating stimuli generated at sites of vascular injury. The data presented in this chapter excludes a role for TXA_2 in promoting calcium flux between aggregating platelets, as ICC remained unaffected in aspirin-treated platelets. However, in agreement with a number of studies supporting an important role for ADP in thrombus formation (Born, 1986; Folie and McIntire, 1989; Wagner and Hubbell, 1992b; Zawilska et al., 1982), the current studies highlight a central role for ADP in the propagation of ICC and the development of platelet aggregates under flow. It is well established in the literature that ADP is a relatively weak platelet agonist, and that ADP-induced integrin $\alpha_{IIb}\beta_3$ activation requires co-operative signalling through the platelet purinergic receptors $P2Y_1$ and $P2Y_{12}$ (Daniel et al., 1998; Gachet, 2001a; Gachet, 2001b; Geiger et al., 1998; Hechler et al., 1998a; Jin et al., 1998; Jin and Kunapuli, 1998; Savi et al., 1998). Signalling through $P2Y_1$ alone induces a Gq-dependent calcium response that leads to relatively weak platelet stimulation and unstable aggregate formation (Born, 1962; Gachet, 2001a; MacFarlane, 1987). Similarly, platelet stimulation through the Gi-linked $P2Y_{12}$ pathway alone is has been found to induce platelet shape change, but not to support integrin $\alpha_{IIb}\beta_3$ activation. Signalling through the $P2Y_{12}$ pathway is believed to be essential for the potentiation of platelet aggregation to a variety of physiological agonists including thrombin and collagen (Gachet, 2001a; Gachet, 2001b; Gachet et al., 1995) and for the stabilisation of thrombi *in vitro* and *in vivo* (Fabre et al., 1999; Gachet, 2001b; Leon et al.,

1999b; Remijn et al., 2002). Integrin $\alpha_{IIb}\beta_3$-induced outside-in signalling has been recently found to support sustained calcium oscillations and irreversible platelet activation on vWf, but only in a relatively small proportion of the platelet population (Nesbitt et al., 2002), suggesting that, like the ADP-receptors, integrin $\alpha_{IIb}\beta_3$ in isolation, is also a relatively weak platelet activator. While previous studies have demonstrated that platelet aggregation in response to ADP requires signalling through both Gq and Gi-linked surface receptors, the present studies provide evidence that co-operative signalling between the Gi-linked $P2Y_{12}$ receptor and integrin $\alpha_{IIb}\beta_3$ engagement can also lead to platelet aggregation under high shear on vWf.

The phenomenon of intercellular calcium signalling is a widespread form of cell-cell communication often used to allow co-ordination of cell proliferation, differentiation and metabolism in many cell types including osteoblasts (Jorgensen et al., 2000; Romanello and D'Andrea, 2001; Romanello et al., 2001; Saunders et al., 2001), chondrocytes (Elfervig et al., 2001; Tonon and D'Andrea, 2000), astrocytes (Fam et al., 2000; Guthrie et al., 1999; Rouach et al., 2000; Scemes, 2000; Scemes et al., 2000; Shiga et al., 2001; Verderio and Matteoli, 2001), oligodendrocytes (Verderio and Matteoli, 2001), neurons (Fam et al., 2000; Guthrie et al., 1999; Rouach et al., 2000; Scemes, 2000; Scemes et al., 2000), endothelial cells (Moerenhout et al., 2001) and retinal epithelial cells (Himpens et al., 1999). To date, at least three distinct mechanisms have been demonstrated to co-ordinate calcium signalling between cells, including: (i) autocrine activation of P2 purinergic receptors leading to calcium release from intracellular stores (Churchill and Louis, 1998; Elfervig et al., 2001; Fam et al., 2000; Jorgensen et al., 2000; Verderio and Matteoli, 2001), (ii) gap junction mediated communication leading to extracellular calcium influx (D'Andrea et al., 2000; Evans and Boitano, 2001; Fam et al., 2000; Lyng et al., 2000; Romanello and D'Andrea, 2001; Romanello et al., 2001; Tonon and D'Andrea,

2000), and (iii) by a recently identified extracellular calcium-sensing receptor that responds to changes in the calcium signalling status of neighbouring cells (Hofer et al., 2000). The results presented in this chapter demonstrate for the first time the involvement of an integrin in this process, and furthermore, establish an important role for co-operative signalling between integrin $\alpha_{IIb}\beta_3$ and purinergic receptors in regulating ICC and thrombus growth.

CHAPTER 6:

ROLE OF ADP IN PROMOTING PRIMARY PLATELET ADHESION ON vWf

Chapter 6.

Role of ADP in Promoting Primary Platelet Adhesion on vWf.

6.1 Introduction.

It is well established that vWf is the major ligand promoting platelet adhesion and aggregation under high shear conditions (Kulkarni et al., 2000; Savage et al., 1996). Studies presented earlier in this thesis (section 3.5) and by Nesbitt et al., (2002), have demonstrated that primary platelet adhesion to vWf requires the co-ordinated generation of GP Ib/V/IX-dependent calcium spikes, followed by a self-propagating calcium signal mediated by integrin $\alpha_{IIb}\beta_3$ outside-in signalling, which promotes firm platelet adhesion. These studies demonstrated that stationary adhesion on a vWf matrix at high shear occurs in the absence of soluble agonist stimulation. However, the majority of platelets that adhere to immobilised vWf under laminar flow conditions translocate over the surface with very few platelets forming stationary adhesion contacts and displaying sustained calcium oscillations. This suggests that platelet activation on vWf under these conditions is a relatively inefficient process. A similar phenomenon has been described during thrombus growth *in vivo* where the vast majority of platelets tethering to the thrombus surface through the vWf-GPIb/V/IX interaction do not form stable adhesion contacts but rather, translocate and eventually detach from the thrombus surface (Kulkarni et al., 2000).

Shear-induced platelet aggregation studies, using a cone and plate viscometer, have demonstrated dramatic vWf-induced platelet activation under high shear (Chow et al., 1992; Ikeda et al., 1991; Moake et al., 1988; Moritz et al., 1983). However, platelet aggregation in this system has been attributed largely to the accumulation of secreted ADP. Moreover, the inter-platelet calcium communication studies presented in chapter 5,

demonstrated that while a low level of stationary adhesion can occur on vWf, in the absence of ADP stimulation, subsequent aggregate formation cannot proceed (section 5.4). Together, these results suggest that ADP released from platelets activated by vWf may be an effective mechanism of increasing the efficiency of platelet activation.

The studies in chapter 5 demonstrated an important role for co-operative signalling between integrin $\alpha_{IIb}\beta_3$ and the G_i-linked, $P2Y_{12}$ receptor during platelet aggregate formation under flow conditions. Several other studies have previously demonstrated an important role for G_i-mediated signal transduction in promoting integrin $\alpha_{IIb}\beta_3$ activation. Patients with congenital defects in the G_i-coupled $P2Y_{12}$ receptor have been shown to display impaired aggregation in response to ADP stimulation and impaired thrombus growth on collagen (Cattaneo et al., 1992; Nurden et al., 1995; Remijn et al., 2002). Furthermore, ADP-induced aggregation can be blocked by selectively inhibiting the G_i linked $P2Y_{12}$ receptor in human platelets or by knocking-out this receptor in mouse platelets (Jantzen et al., 2001; Yang et al., 2000). Under these conditions, full aggregation in response to ADP could be restored by direct activation of the $G_{z\alpha}$ pathway through adrenaline binding to the α_{2a}-adrenergic receptor (Hechler et al., 1998a; Jin and Kunapuli, 1998).

The studies in this chapter investigate the role for ADP in promoting stationary adhesion on vWf. Furthermore, the functional relationship between the platelet ADP receptors and integrin $\alpha_{IIb}\beta_3$ signalling in promoting platelet adhesion on vWf are examined.

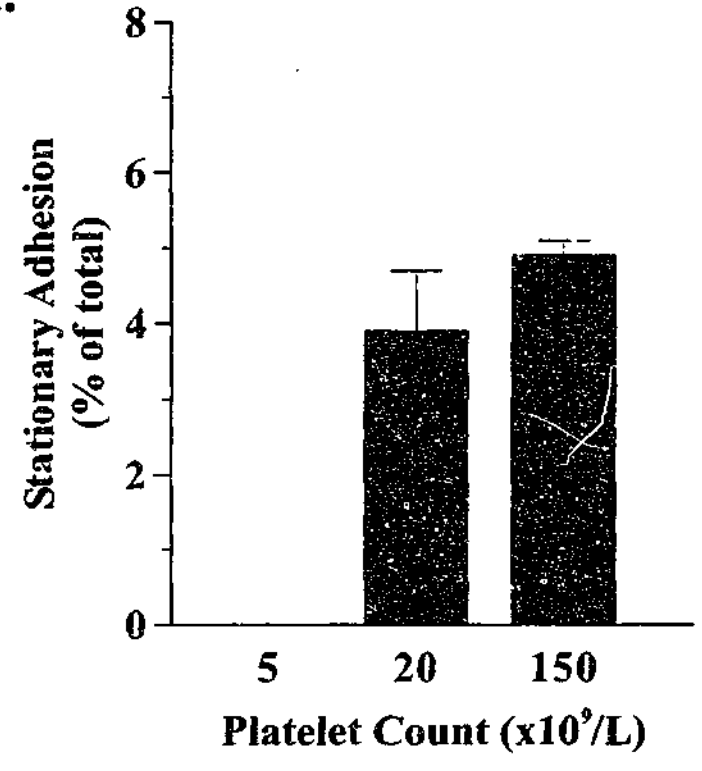
6.2 Increased Platelet Density Results in Increased Levels of Platelet Adhesion and Calcium Mobilisation on a vWf matrix Under Flow.

The demonstration that clinically low platelet counts (<20 x 10^9/L) lead to an increased risk of haemorrhage (Gaydos, 1962), suggests that platelet density may play a role in regulating platelet activation *in vivo*. To investigate the possibility that platelet density affects stationary adhesion formation on vWf, platelets were perfused at densities of 5, 20 and 150 x 10^9/L through vWf coated microcapillary tubes at 1800 s^{-1}, and the level of stationary adhesion (lasting at least 30 seconds) quantified. As shown in figure 6.1a, at 5 x 10^9/L none of the platelets tethering to the vWf surface formed stationary adhesion contacts for a period of ≥ 30 seconds. In contrast, at a platelet count of 20 x 10^9/L, 4 ± 0.85% of the tethered platelet population formed stationary adhesions for at least 30 seconds and at platelet counts of 150 x 10^9/L, 5 ± 0.14% of platelets formed stable adhesion contacts ≥30 seconds. The duration of stationary adhesion contacts in a population of vWf adherent cells was examined at 5 x 10^9/L and 150 x 10^9/L. As demonstrated in figure 6.1b. at a platelet density of 5 x 10^9/L, 66 ± 3.3% of tethered platelets exhibited stationary contact formation with the matrix lasting 1 second. The level of stationary adhesion decreased exponentially over time such that, 3.8 ± 0.8% of the platelet population formed sustained adhesion contacts for 10 seconds, and 0% formed stationary adhesion contacts lasting 30 seconds (fig. 6.1b). A similar phenomenon was observed at 150 x 10^9/L, however, the rate of decrease was significantly lower. At 150 x 10^9/L, 65 ± 3.5% of platelets formed adhesion contacts lasting at least 1 second, decreasing to 26.8 ± 3.8% at 10 seconds, and 5.2 ± 1.3% stationary platelets at 30 seconds (fig. 6.1b). The most impressive difference between high and low density platelet populations was the proportion of platelets exhibiting stationary adhesion for 10 seconds, where <4% of platelets were present at 5 x 10^9/L compared to ~26% at 150 x 10^9/L (fig. 6.1b). This data

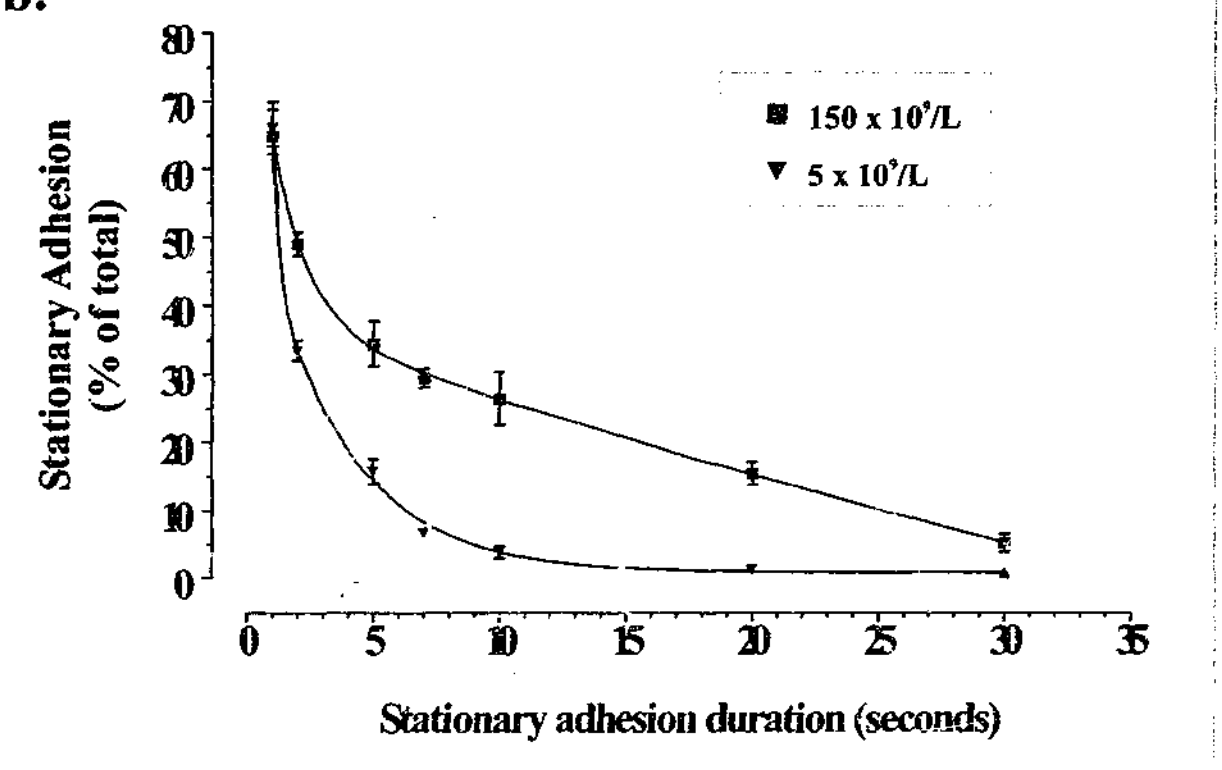
Figure 6.1 Stationary adhesion formation is modulated by platelet density under flow conditions.

Washed platelets were reconstituted at the indicated densities with 50% autologous red blood cells (RBC's). Following reconstitution, platelets were treated with or without 2U/ml apyrase and platelet counts were confirmed using a coulter counter prior to perfusion of the platelet suspensions over immobilised vWf at 1800 s^{-1} (n = 3). In (a) the proportion of platelets (expressed as % of total adherent population) forming stationary adhesions for at least 30 seconds was plotted as a function of the platelet density ($x10^9$/L) (data represents mean ± S.E.M of three separate experiments). In (b) Washed platelets were reconstituted with 50% red blood cells at 5 x 10^9/L (t), or 150 x 10^9/L prior to perfusion over vWf at 1800s^{-1} (n = 3). Real-time tethering and stationary adhesion formation was recorded by video microscopy for off line analysis (as described under methods section 2.6.3). The proportion of platelets forming stationary adhesion contacts lasting for a period of 1, 2, 5, 7, 10 and 30 seconds was assessed and expressed as a % of the total adherent population. The results represent the mean ± S.E.M from three separate experiments.

a.


Stationary Adhesion
(% of total)
8
6
4
2
0
5
20
150
Platelet Count (x10⁹/L)

b.


Stationary Adhesion
(% of total)
80
70
60
50
40
30
20
10
0
150 x 10⁹/L
5 x 10⁹/L
0
5
10
15
20
25
30
35
Stationary adhesion duration (seconds)

demonstrates that both at high and low density, the majority of platelet-vWf interactions remain transient, however increased platelet density results in greater duration of adhesion contacts.

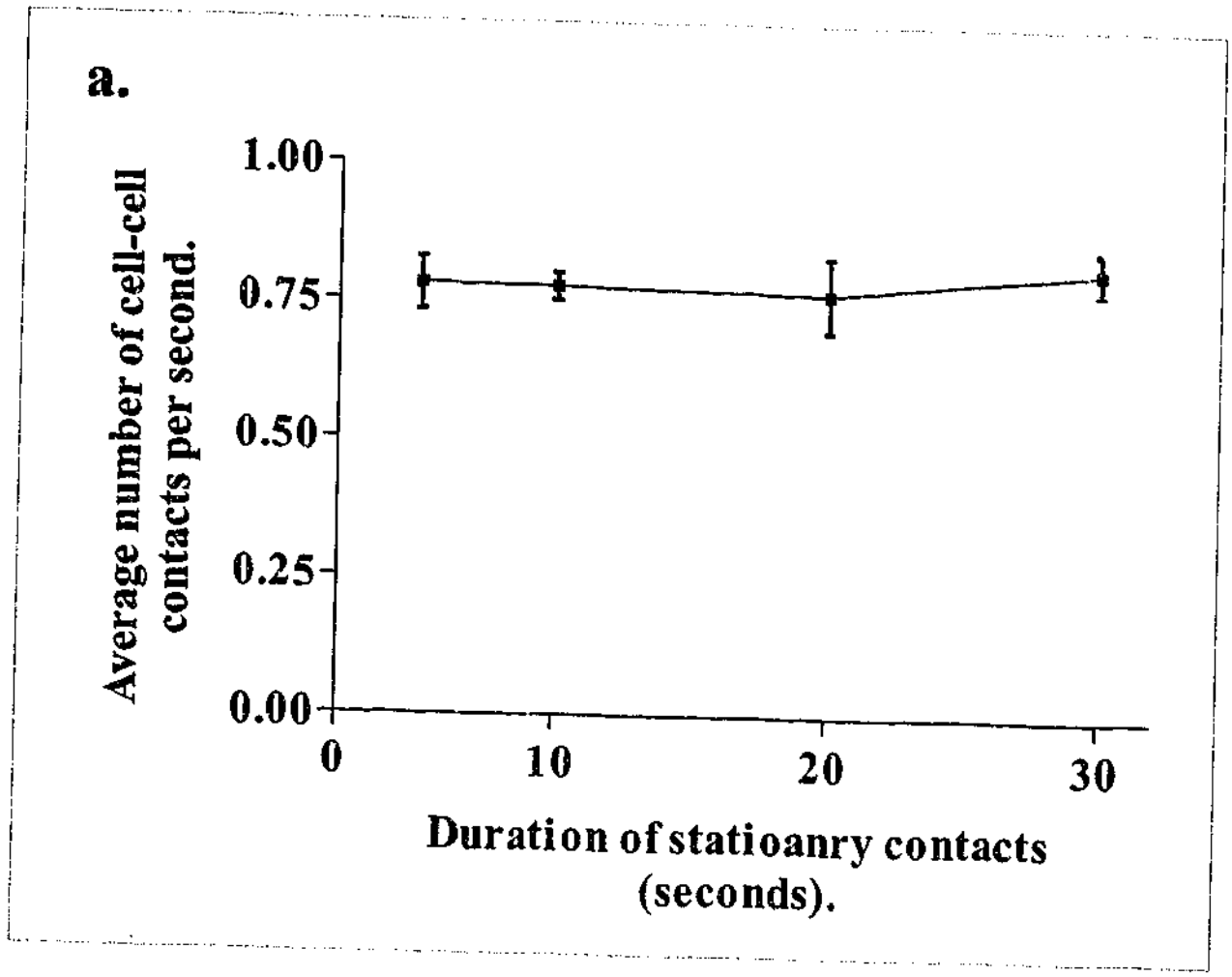
One possible explanation for the effect of density on platelet adhesion is that transient adherent platelets experience less frequent interactions with translocating platelets in the shear field than those forming more prolonged adhesion contacts. To examine this hypothesis, the number of collisions between translocating platelets and cells forming stationary adhesion contacts for 5, 10, 20 or 30 seconds was analysed, and the mean number of collisions within each group was calculated per second to give collision frequency. As demonstrated in figure 6.2a, these studies demonstrated no significant difference in the frequency of platelet-platelet collisions experienced by platelets that form stationary contacts lasting for 5, 10, 20 or 30 seconds.

An alternative hypothesis for the effect of platelet density on stationary adhesion is that the duration of the interaction between stationary and translocating platelets is longer for platelets forming prolonged stationary adhesions than for platelets forming transient stationary adhesions. To investigate this possibility, analysis of the duration of platelet-platelet adhesion contacts was performed. In these studies, interaction times were assessed for platelets that formed stationary adhesion contacts for 5, 10 and 30 seconds. As shown in figure 6.2b, the average duration of platelet-platelet contact for platelets forming stationary adhesions for 5 seconds is ~ 1 second. This increases to an average contact time of ~ 2 seconds for platelet remaining stationary for 10 seconds and to >7 seconds for stationary platelets lasting 30 seconds (fig. 6.2b). These results suggest that while the frequency of platelet interactions does not regulate stationary adhesion formation, extended contact times between stationary and translocating platelets may promote increased adhesion stability.

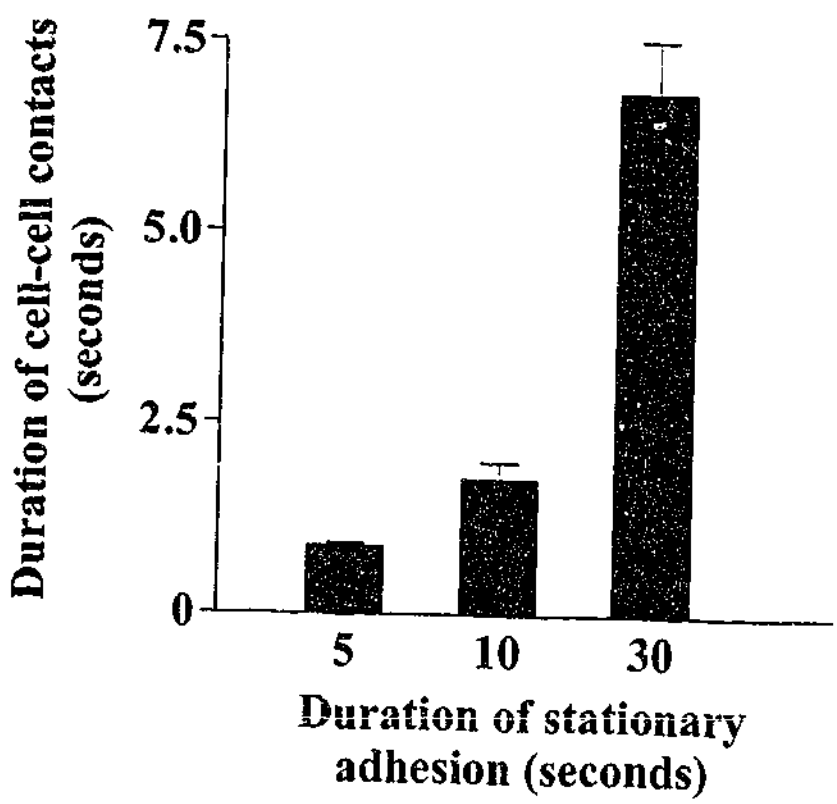
Figure 6.2 Effect of platelet-platelet interactions in promoting sustained stationary adhesion contacts on vWf under flow.

Washed platelets (150 x 10^9/L) were treated with or without 2U/ml apyrase prior to perfusion over immobilised vWf at 1800 s^{-1}. (a) The number of platelet-platelet interactions with a primary adherent platelet displaying stationary adhesion contacts lasting for either: 5, 10, 20 or 30 seconds was quantified. The graph illustrates the frequency of platelet collision (cell-cell contacts per second) experienced by platelets that form stationary adhesions for 5, 10, 20 and 30 seconds. Data is expressed as mean ± S.E.M of three independent experiments. (b) The duration of platelet-platelet interactions between translocating platelets and primary adherent platelets displaying stationary adhesion contacts lasting for 5, 10 or 30 seconds was quantified. The graph illustrates the average duration of platelet-platelet contact between translocating platelets and stationary platelets. Data is expressed as mean ± S.E.M of three independent experiments.

a.


Average number of cell-cell contacts per second.
1.00
0.75
0.50
0.25
0.00
0
10
20
30
Duration of statioanry contacts (seconds).

b.

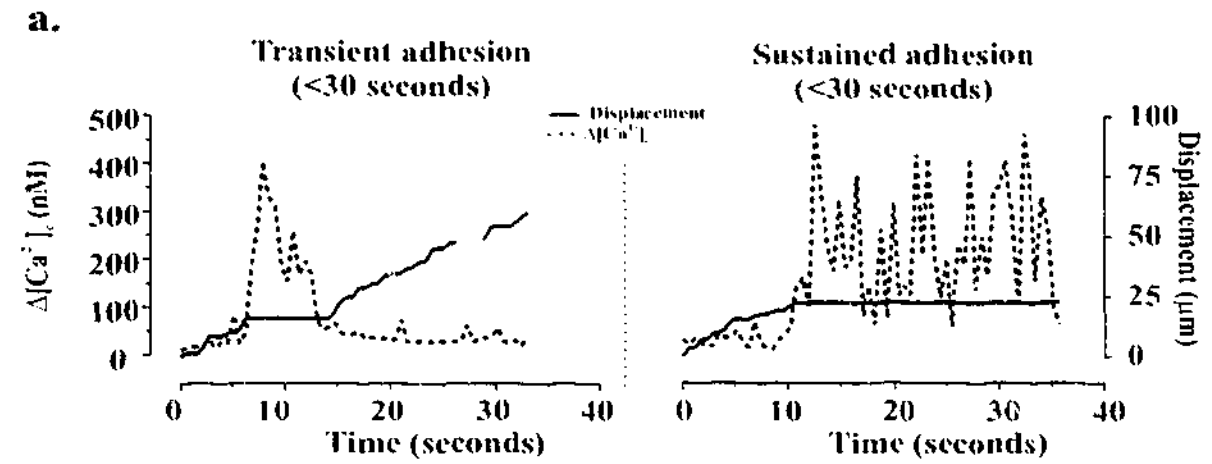

Duration of cell-cell contacts (seconds)
7.5
5.0
2.5
0
5
10
30
Duration of stationary adhesion (seconds)

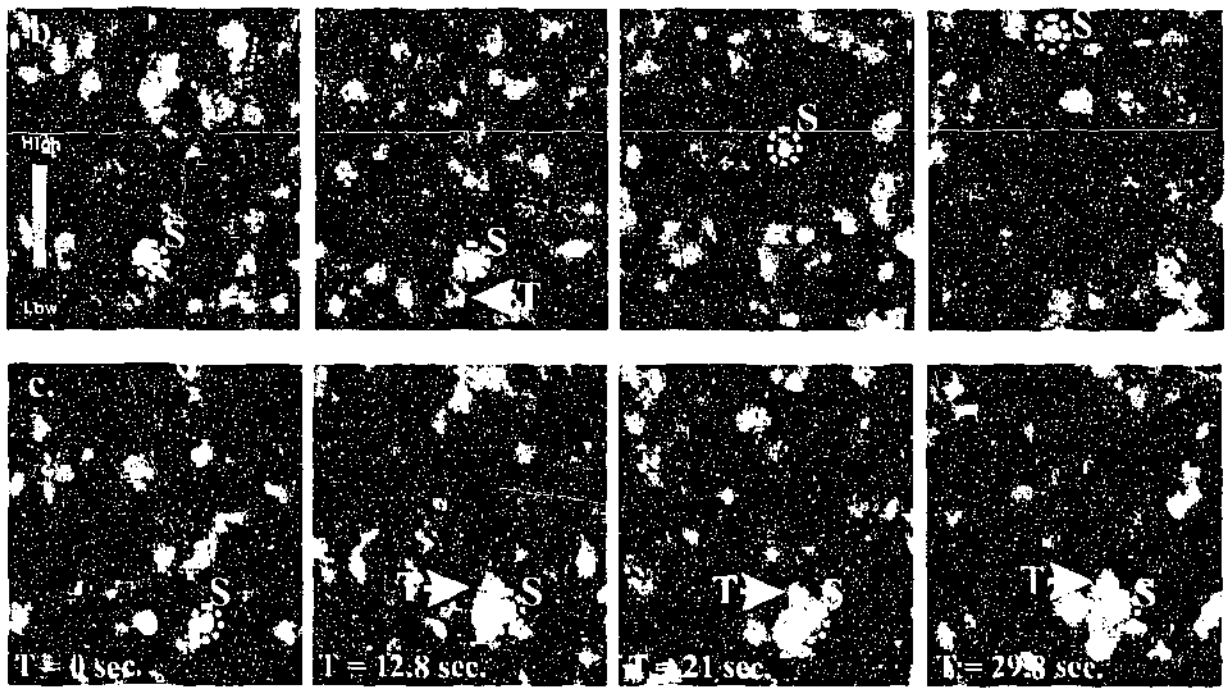
Previous studies in section 5.2 of this thesis demonstrated that sustained platelet-platelet interactions resulted in ICC and aggregate formation. In order to examine the requirement for ICC for stable adhesion formation on vWf, detailed analysis of translocation behaviour and calcium flux was carried out in primary stationary platelets and in translocating platelets that interacted with these cells. These studies demonstrated that those platelets that formed transient stationary adhesions lasting ≤10 seconds, also exhibited transient, high level, calcium flux, however these calcium signals were short-lived and upon down-regulation of the calcium response, platelets returned to translocation (fig. 6.3a). Furthermore, when analysing platelets that interacted with these cells, no calcium signal (ICC) was observed in tethering platelets (fig. 6.3b and d), and no stable aggregate formation was observed (fig. 6.3b and d). On the other hand, platelets that elicited a cytosolic calcium response in tethering platelets invariably underwent sustained calcium oscillations, resulting in the formation of stable adhesion contacts on the vWf matrix (≥30 seconds), (fig. 6.3a, c and e). This data raises the possibility that a process similar to intercellular calcium communication may not only provide a mechanism to activate translocating platelets that interact with primary stationary cells, but may also provide a mechanism to enhance the adhesion stability in primary adherent platelets.

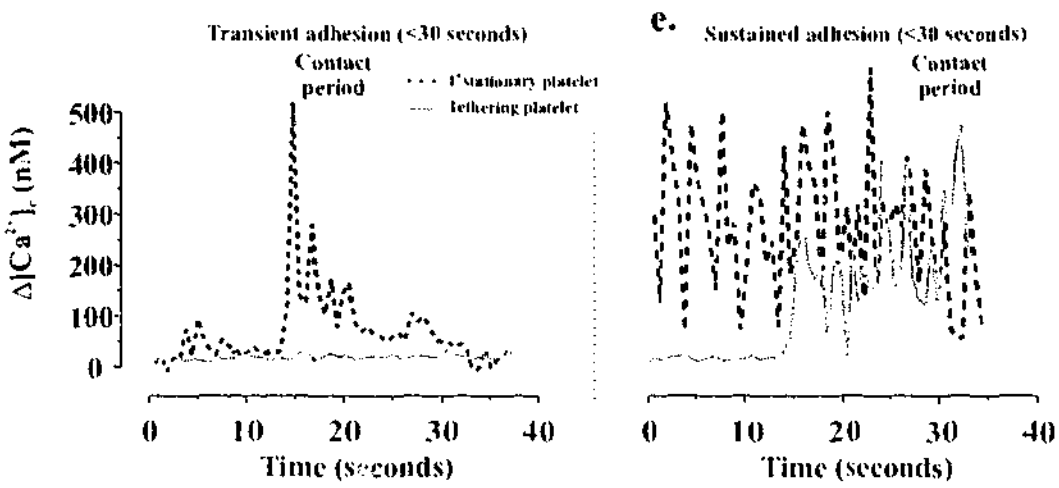
In light of previous ICC studies demonstrating that the formation of platelet aggregates occurs as a result of ADP secretion, it is possible that at high platelet density, the duration of stationary adhesion formation on vWf is increased as a consequence of ADP secretion. To investigate the role of secreted ADP in primary adhesion formation at high density (150 x 10^9/L), platelets were perfused over immobilised vWf (1800 s^{-1}) in the presence of apyrase or a combination of A3P5PS and AR-C69931MX, and the stability of stationary contact formation was assessed. Inhibition of ADP resulted in a significant reduction in the stability of stationary adhesion contacts compared to control flows. As

Figure 6.3 Effect of Calcium Communication in promoting sustained stationary adhesion contacts on vWf under flow.

Calcium dye-loaded platelets (150 x 10^9/L) were perfused over immobilised vWf at 1800 s^{-1} and cytosolic calcium flux was monitored by confocal microscopy. (a) Representative single-cell calcium recordings and translocation profiles of platelets exhibiting transient (<30 seconds) and sustained (>30 seconds) calcium flux on vWf at 1800s^{-1} (n = 25 platelets). (b) and (c) Single channel Oregon Green fluorescence images of platelets during adhesion to vWf under flow. In (b) collision of translocating platelets (**T→**) with a single platelet exhibiting transient calcium flux and stationary adhesion (<30 seconds) (**S**: demarcated by the dotted circle) does not result in ICC or stable aggregate formation. Both the translocating platelet and the stationary platelet return to surface translocation. In (c) collisions between a translocating platelet (**T→**), and a primary stationary platelet (**S**: demarcated by the dotte circle) forming stable adhesion contacts (>30 seconds) leads to ICC, stable aggregate formation and sustained stationary adhesion formation of the stationary platelet (**S**). (d) Representative calcium flux profiles of a platelet undergoing transient adhesion (<30 seconds) (dotted black line), and of a tethering platelet (solid red line) interacting with it. The contact between these two platelets does not result in ICC or stable adhesion during the contact time between these two platelets (shaded box). (e) Representative calcium flux profiles of a platelet undergoing sustained calcium flux and stationary adhesion (>30 seconds) (dotted black line), and a tethering platelet (solid red line) showing efficient ICC and aggregate formation during the contact time between the platelets (shaded box).


a.
Transient adhesion
(<30 seconds)
Sustained adhesion
(<30 seconds)
Displacement
Δ[Ca2+]c
Δ[Ca2+]c (nM)
Displacement (μm)
Time (seconds)
Time (seconds)


b.
High
Low
S
T
c.
T = 0 sec.
T = 12.8 sec.
T = 21 sec.
T = 29.8 sec.


d.
Transient adhesion (<30 seconds)
Contact period
Stationary platelet
Tethering platelet
Δ[Ca2+]c (nM)
Time (seconds)
e.
Sustained adhesion (<30 seconds)
Contact period
Time (seconds)

shown in figure 6.4a, the proportion of platelets forming stationary adhesion for 1 second is similar for control and apyrase treated platelets, 65 ± 3.5% versus 61.4 ± 3.3% respectively. However the proportion of platelets forming stationary adhesions for 10 seconds decreased from 26.8 ± 3.8% in control flows to 11 ± 1% in apyrase treated platelets, and for 30 seconds it was reduced from 5.2 ± 1.3% in control flows to 1.2 ± 0.5% in the presence of apyrase. Similar results were found in the presence of A3P5PS and AR-C69931MX (fig. 6.4a). Consistent with a role for ADP in sustaining platelet adhesive interactions, the contact time between translocating platelets and platelets that were stationary for 30 seconds was reduced from 7.06 ± 0.70 to 2.16 ± 0.24 seconds with all platelet-platelet interactions in the presence of apyrase remaining transient (fig. 6.4b). This data demonstrates that ADP plays an important role in promoting stable platelet adhesion on vWf at high platelet density.

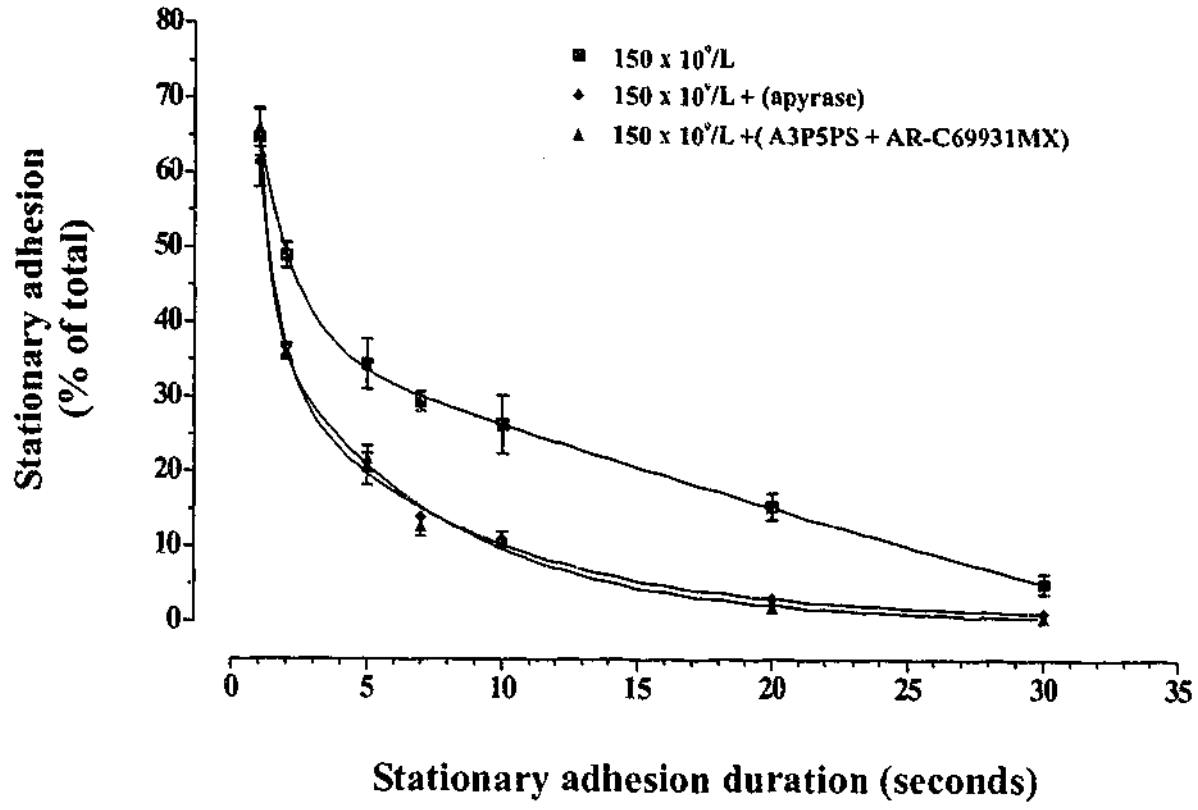
6.3 ADP and Integrin $\alpha_{IIb}\beta_3$ Act in a Co-operative Manner to Promote Sustained Calcium Flux and Firm Platelet Adhesion under Flow.

The studies presented in this thesis define an important role for ADP in promoting stationary adhesion and aggregation on a vWf matrix. Using mathematical modelling, it has previously been demonstrated that that the concentration of ADP at the surface of a developing thrombus can reach micromolar concentrations known to induce irreversible platelet activation (Folie, 1989; Hubbel JA, 1986a; Hubbel JA, 1986b; Wagner, 1992). To investigate the effect of high concentrations of ADP on the efficiency of platelet adhesion to vWf, flow assays were performed in the presence of 12.5 μM exogenous ADP. Exposure ot platelets to high concentrations of exogenous ADP (12.5 μM) during surface translocation on vWf resulted in a dramatic increase in the proportion of platelets forming stationary adhesion contacts (from <6 % up to >90 %) (fig. 6.5a). All firmly adherent

Figure 6.4 Effect of platelet density and secreted ADP on stability of stationary adhesion contacts on vWf under flow.

Washed platelets (150 x 10^9/L) were treated with vehicle (■), 2 U/ml apyrase (♦), or a combination of A3P5PS (200 μM) and AR-C69931MX (200 nM) (▲), prior to perfusion over immobilised vWf at 1800s^{-1}. (a) Real-time tethering and stationary adhesion formation was recorded by video microscopy for off line analysis (as described under methods section 2.6.3). The proportion of platelets forming stationary adhesion contacts lasting for a period of 1, 2, 5, 7, 10 and 30 seconds was assessed and expressed as a % of the total adherent population. The results represent the mean ± S.E.M from three separate experiments. (b) The duration of platelet-platelet interactions between translocating platelets and primary adherent platelets displaying stationary adhesion contacts lasting at least 30 seconds was quantified in the presence or absence of apyrase (2 U/ml). The graph illustrates the average duration of platelet-platelet contact between translocating platelets and stationary platelets. Data is expressed as mean ± S.E.M of three separate experiments.

a.


Stationary adhesion (% of total)
80
70
60
50
40
30
20
10
0
150 x 10⁹/L
150 x 10⁹/L + (apyrase)
150 x 10⁹/L +(A3P5PS + AR-C69931MX)
0
5
10
15
20
25
30
35
Stationary adhesion duration (seconds)

b.

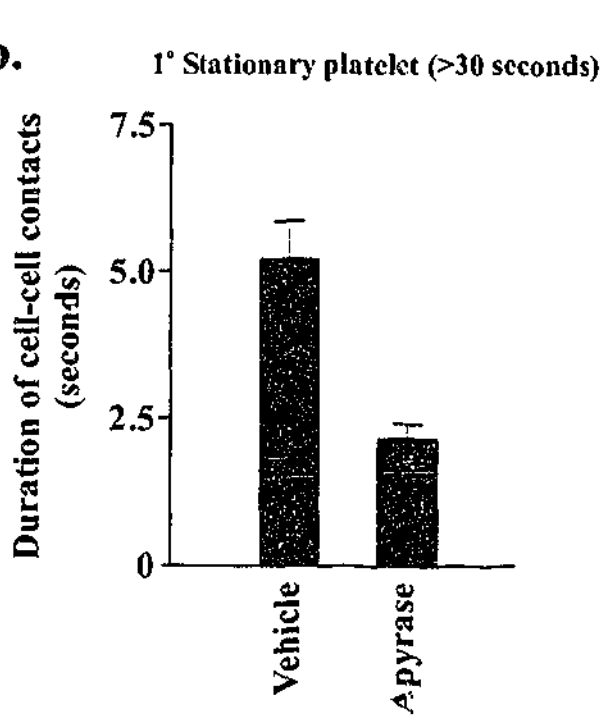
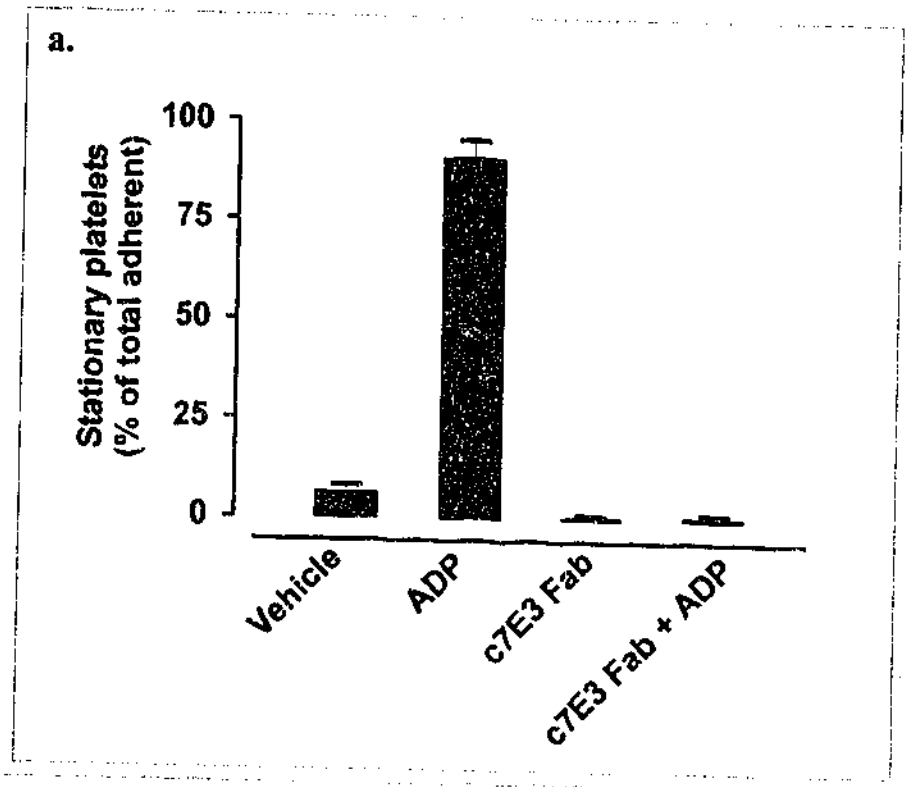
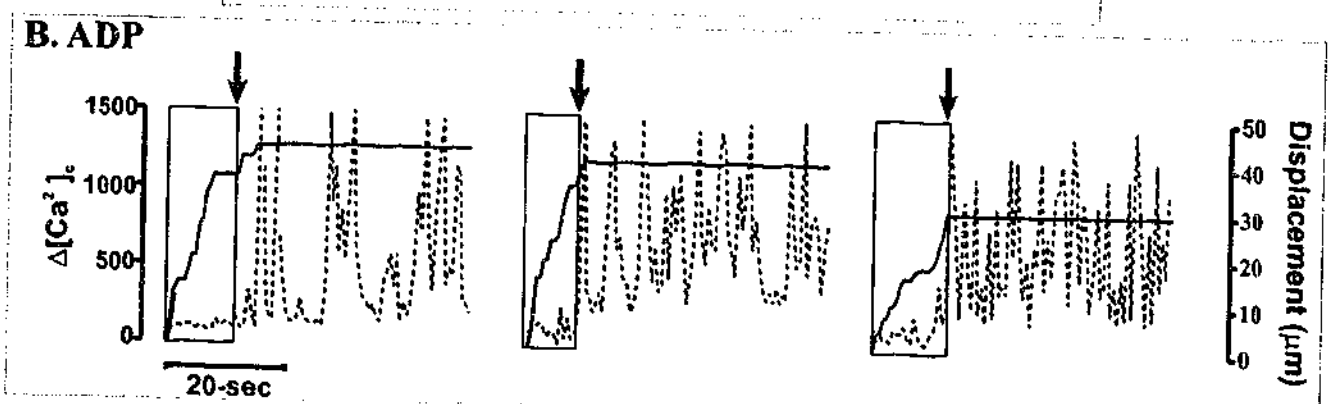
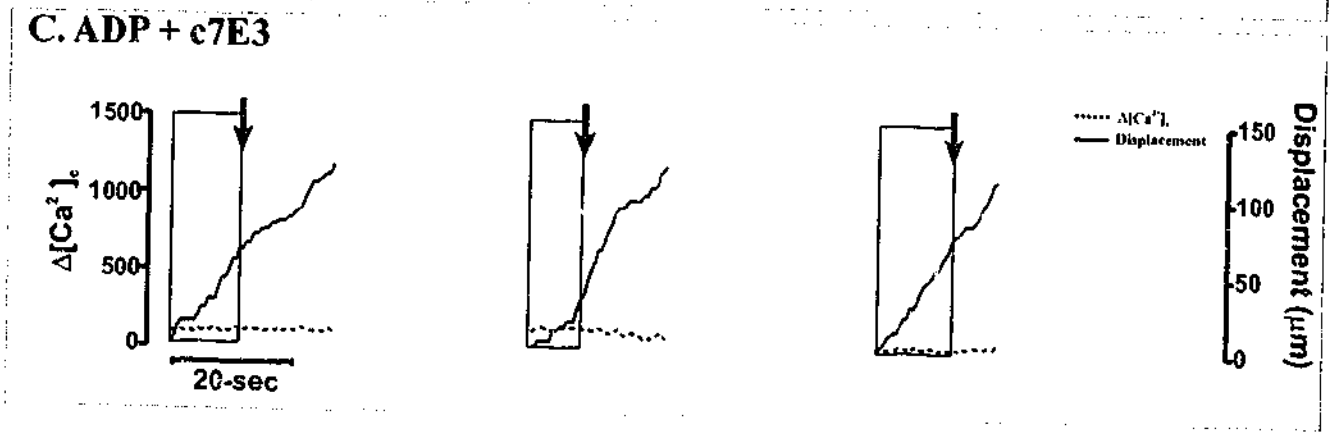

1° Stationary platelet (>30 seconds)
Duration of cell-cell contacts (seconds)
7.5
5.0
2.5
0
Vehicle
Apyrase

Figure 6.5 Co-operative signalling between integrin $\alpha_{IIb}\beta_3$ and ADP receptors is required for sustained calcium flux on vWf.

Washed platelets (5 x 10^9/L) were treated with or without c7E3 Fab (20 μg/ml) prior to perfusion over immobilised vWf (100 μg/ml) at 1800 s^{-1}, and subsequently chased with a bolus of 12.5 μM ADP. (a) The proportion of platelets forming stationary adhesions for at least 10 seconds was quantified in the presence or absence of ADP and/or c7E3 Fab (data represents mean ± S.E.M of three separate experiments). (b) Representative single-cell calcium recordings and the corresponding translocation profiles of platelets adhering to immobilised vWf at 1800s^{-1}, demonstrating the immediate onset of sustained calcium flux and sustained platelet arrest upon ADP addition. (c) Representative single-cell calcium recordings and the corresponding translocation profiles on immobilised vWf, of c7E3 Fab-treated platelets upon ADP addition at 1800 s^{-1}. The shaded boxes highlight the translocation behaviour and calcium profiles of platelets prior to ADP addition, the arrow indicates the point at which ADP is added.


a.
Stationary platelets (% of total adherent)
100
75
50
25
0
Vehicle
ADP
c7E3 Fab
c7E3 Fab + ADP


B. ADP
1500
1000
500
0
Δ[Ca²⁺]c
20-sec
50
40
30
20
10
0
Displacement (μm)


C. ADP + c7E3
1500
1000
500
0
Δ[Ca²⁺]c
20-sec
Δ[Ca²⁺]c
Displacement
150
100
50
0
Displacement (μm)

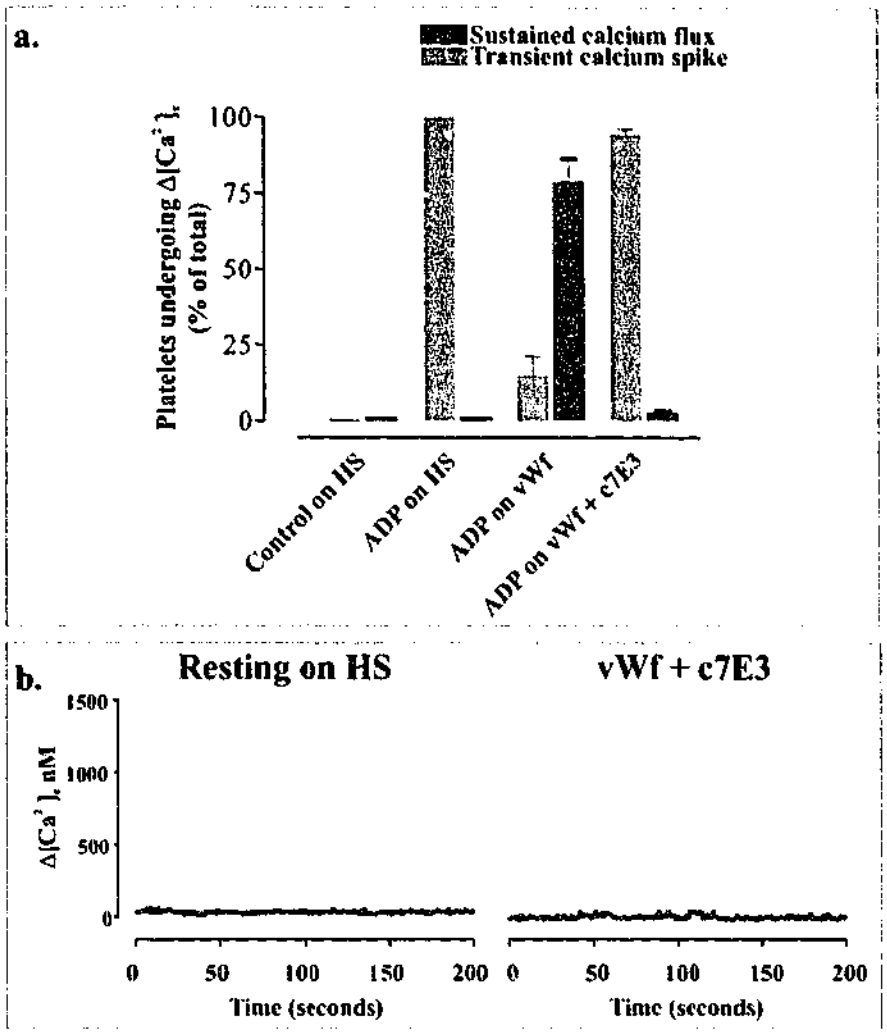
platelets elicited high-level sustained calcium oscillations and formed stationary adhesion contacts lasting ≥30 seconds (fig. 6.5a and b). This sustained oscillatory calcium flux in vWf adherent platelets was completely inhibited by pre-treating platelets with 20 μg/ml of c7E3 Fab (fig. 6.5a and c). Consistent with these observations, stimulation of platelets in suspension with 12.5 μM ADP resulted in 100 % of platelets undergoing only a transient calcium response (fig. 6.6a), that was found to peak at 1100 nM ± 300 nM (mean ± S.E.M), and return back to basal levels ($\Delta[Ca^{2+}]_c$ <40 ± 20 nM) within 8.5 ± 3.5 seconds (mean ± S.E.M) (fig. 6.6b and c). However, when platelets were allowed to interact with a vWf surface under static conditions, ADP addition resulted in >75% of the platelet population adhering firmly to the vWf matrix and exhibiting a sustained oscillatory calcium response. Similar to the flow studies, sustained calcium flux under static conditions was also sensitive to integrin $\alpha_{IIb}\beta_3$ inhibition (fig. 6.6a and c). Furthermore, of the remaining ~25% of the non-c7E3 Fab treated platelet population on vWf that did not engage the matrix, all of these exhibited a transient calcium flux (fig. 6.6a, c and data not shown). These results demonstrate that sustained calcium flux in ADP stimulated platelets is integrin $\alpha_{IIb}\beta_3$ ligation dependent, and provide further evidence for the importance of the synergistic signalling between ADP and integrin $\alpha_{IIb}\beta_3$ in regulating platelet adhesion under high shear conditions.

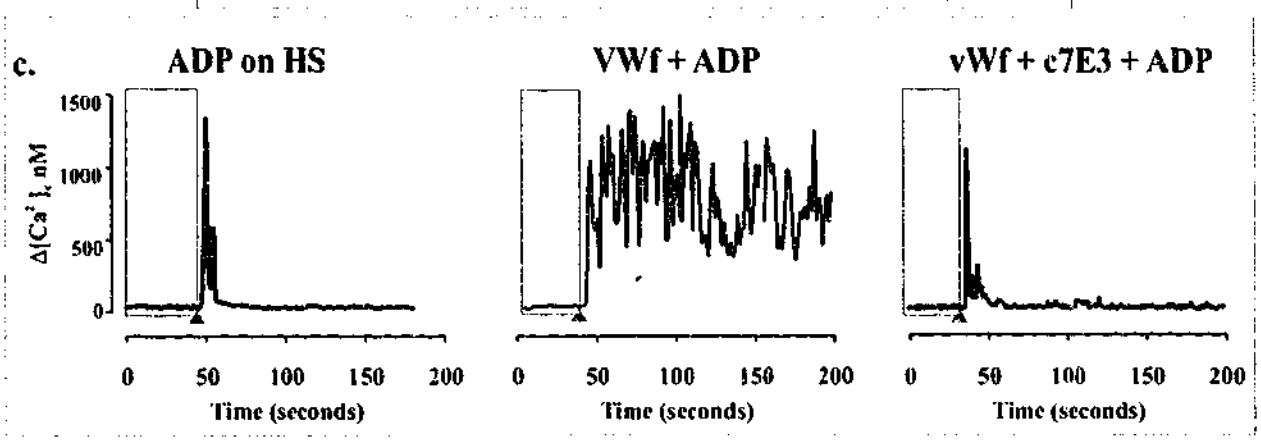
6.4 ADP-induced Integrin $\alpha_{IIb}\beta_3$ Activation and Sustained Calcium Flux under Flow Requires Signalling Through $P2Y_1$ and $P2Y_{12}$.

To investigate the individual contribution of the $P2Y_1$ and $P2Y_{12}$ signalling pathways in promoting stationary adhesion formation on vWf in the presence of high concentrations of ADP, platelets were treated with A3P5PS and AR-C69931MX, and the effects of these inhibitors on ADP-induced cytosolic calcium flux were examined under

Figure 6.6 Calcium responses induced by ADP in platelets under static conditions.

Washed platelet suspensions were treated with or without c7E3 Fab (20 μg/ml), and applied under static conditions to a non-reactive surface [10% (v/v) human serum (HS)] or immobilised vWf (100μg/ml), prior to stimulation with 12.5 μM ADP. (a) The proportion of platelets undergoing transient (grey bars), or sustained (black bars), $\Delta[Ca^{2+}]_c$ in response to ADP stimulation was quantified in a platelet population resting on HS or vWf in the presence or absence of c7E3 and or ADP. Results represent the mean ± S.E.M of three separate experiments. (b) Representative single-cell calcium recordings of platelets resting on HS, and on vWf in the presence of c7E3 Fab (vWf + c7E3). (c) Representative calcium flux profiles in platelets stimulated with ADP on human serum (ADP on HS), vWf in the absence of c7E3 Fab (vWf + ADP), and on vWf in the presence of c7E3 Fab (vWf + c7E3 + ADP). Note: the shaded box highlights the calcium status in platelets prior to ADP stimulation, the arrow indicates the point at which ADP is added.


a.
Sustained calcium flux
Transient calcium spike
Platelets undergoing Δ[Ca²⁺]ᵢ (% of total)
100
75
50
25
0
Control on HS
ADP on HS
ADP on vWf
ADP on vWf + c7E3
b.
Resting on HS
vWf + c7E3
Δ[Ca²⁺]ᵢ nM
1500
1000
500
0
0
50
100
150
200
Time (seconds)


c.
ADP on HS
VWf + ADP
vWf + c7E3 + ADP
Δ[Ca²⁺]ᵢ nM
1500
1000
500
0
0
50
100
150
200
Time (seconds)

flow conditions. Pre-treatment of platelets with AR-C69931MX prior to perfusion over a vWf matrix, inhibited stationary adhesion formation and sustained calcium oscillations following addition of high concentrations of exogenous ADP (fig. 6.7a and b). This data is consistent with earlier ICC studies (section 5.4) demonstrating an important role for $P2Y_{12}$ receptor signalling for stationary adhesion and aggregation on vWf. In contrast to ICC, treatment of platelets with A3P5PS prior to addition of high levels of exogenous ADP was found to inhibit stationary adhesion formation and sustained calcium oscillations on vWf (fig. 6.7a and b). This data suggests an equally important role for $P2Y_1$ and $P2Y_{12}$ in platelet activation by high concentrations of ADP. In control studies performed in suspension, pre-treatment with A3P5PS resulted in total inhibition of ADP induced calcium mobilisation while pre-treatment with AR-C69931MX did not effect the transient ADP-induced calcium response (fig. 6.8). These results demonstrate that at high ADP concentrations $P2Y_1$-induced calcium flux is not sufficient to promote irreversible adhesion to vWf under flow.

6.5 The $P2Y_{12}$ Pathway and Integrin $\alpha_{IIb}\beta_3$ Act in a Synergistic Manner to Promote Sustained Calcium Flux and Firm Platelet Adhesion under Flow.

The observation that calcium flux induced by ADP binding to $P2Y_1$ alone cannot promote sustained stationary adhesion on vWf suggests an important role for the $P2Y_{12}$ in sustaining calcium flux and integrin $\alpha_{IIb}\beta_3$ activation. To examine this hypothesis in more detail, flow based adhesion studies were performed using NP-EGTA-loaded platelets in which exogenous ADP (12.5 μM) was added to the bulk flow. In control studies, the ability of NP-EGTA-loaded platelets to respond normally to bolus ADP stimulation was assessed. Consistent with data presented in figure 6.6a, exposure of vWf adherent, NP-EGTA loaded platelets to ADP resulted in >90% of platelets expressing sustained cytosolic

Figure 6.7 Co-operative signalling through purinergic $P2Y_1$ and $P2Y_{12}$ receptors is required for sustained adhesion and calcium flux on vWf.

Wasned platelets (5 x 10^9/L) were treated with or without A3P5PS (200 μM) or AR-C69931MX (200 nM) prior to perfusion over vWf (100 μg/ml) at 1800 s^{-1}, and subsequently chased with 12.5 μM ADP. (a) The percentage of platelets forming stationary adhesion contacts for at least 10 seconds was quantified in the presence or absence of ADP, A3P5PS and/or AR-C69931MX (data represents mean ± S.E.M of three separate experiments). (b) Representative single-cell calcium recordings and the corresponding translocation profiles of platelets translocating on immobilised vWf at 1800s^{-1}, showing that the immediate onset of sustained calcium flux and stationary adhesion upon ADP addition is blocked by either A3P5PS or AR-C69931MX treatment (n=25 platelets).

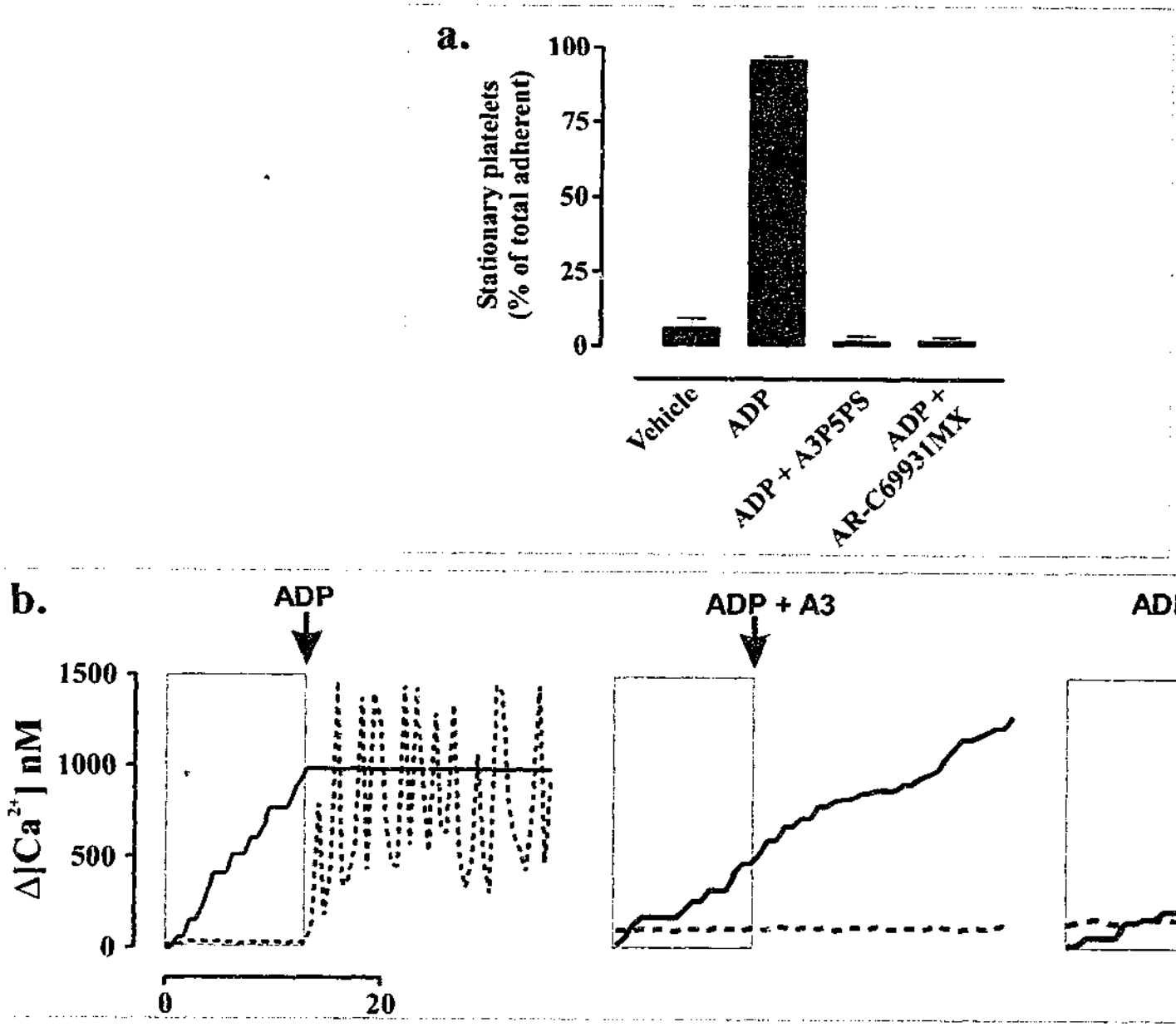
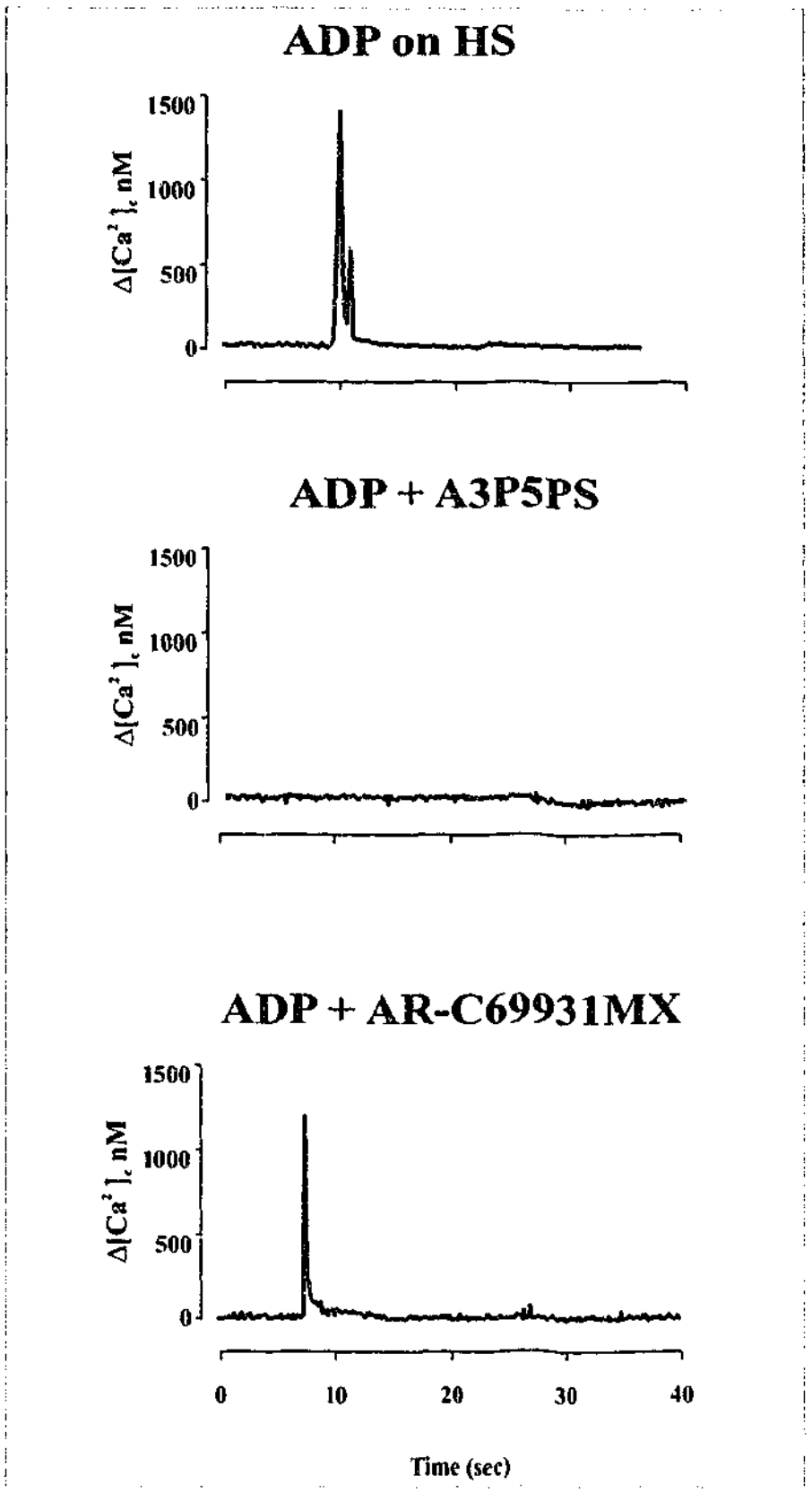

a.
Stationary platelets (% of total adherent)
100
75
50
25
0
Vehicle
ADP
ADP + A3P5PS
ADP + AR-C69931MX
b.
ADP
ADP + A3
ADP + AR-C
Δ[Ca²⁺]ᵢ
Displacement
Δ[Ca²⁺] nM
1500
1000
500
0
0
20
75
50
25
0
Displacement (μM)

Figure 6.8 Effect of A3P5PS and AR-C69931MX on platelet aggregation and calcium flux in response to ADP.

Washed platelet suspensions were treated with or without A3P5PS (200 μM) or AR-C69931MX (200nM), and applied under static conditions to a non-reactive surface (10% (v/v) HS), prior to stimulation with 12.5 μM ADP. The figure shows representative single-cell calcium recordings of ADP stimulated platelets on HS, in the presence or absence of A3P5PS or AR-C69931MX as indicated (n = 25 platelets).


ADP on HS
1500
1000
500
0
Δ[Ca²]c nM
ADP + A3P5PS
1500
1000
500
0
Δ[Ca²]c nM
ADP + AR-C69931MX
1500
1000
500
0
Δ[Ca²]c nM
0
10
20
30
40
Time (sec)

calcium flux and forming prolonged stationary contacts on vWf (data not shown). Pre-treatment with A3P5PS (200 μM) or AR-C69931MX (200 nM), prior to perfusion over vWf in the presence of ADP inhibited ADP-induced stationary adhesion formation (fig. 6.9a). Exposure of NP-EGTA-loaded platelets to UV light (300-400 nm), for 0.6 seconds, in the absence of exogenous ADP, resulted in transient calcium spikes and stationary contact formation (fig. 6.9b and c). However, when platelets were exposed to UV light in the presence of A3P5PS and ADP, the calcium response mediated by the uncaging of cytosolic calcium resulted in >90% of the translocating population forming stationary adhesion contacts and exhibiting sustained high-range calcium flux (fig. 6.9a and b). On the other hand when platelets were exposed to UV light in the presence of AR-C69931MX and ADP, calcium uncaging did not lead to the generation of sustained calcium signalling and associated platelet adhesion, rather, these platelets displayed transient stationary adhesion formation and calcium flux (fig. 6.9a and c). These observations demonstrate that the induction of a transient calcium response by NP-EGTA uncaging is sufficient to induce only transient and reversible stationary adhesion formation. However, subsequent $P2Y_{12}$ engagement and G_i-signalling is required to promote sustained stationary adhesion.

To investigate further the role of G_i-signalling in stationary adhesion formation under flow, adrenaline was used to induce G_i signal transduction through an alternative pathway to that activated by $P2Y_{12}$ ligation. Adrenaline is known to bind to α_2-adrenergic receptors on the platelet surface. This receptor is linked to an inhibitory G-protein denoted $G_{\alpha z}$, which is also coupled to adenylate cyclase (AC), leading to a reduction in cAMP levels. In these studies, NP-EGTA-loaded platelets were perfused through vWf coated microcapillary tubes in the presence of adrenaline (50 μM), prior to UV uncaging. Adrenaline (50 μM) alone did not effect the level of stationary adhesion formation on vWf under flow conditions (fig. 6.10a), and consistent with previous experiments, stimulation

Figure 6.9 Co-operative signalling through P2Y12 and integrin $\alpha_{IIb}\beta_3$ is sufficient to promote sustained adhesion on vWf in response to transient calcium release.

Platelets loaded with 10 μM of the caged calcium compound NP-EGTA were treated with or without (200 μM) A3P5PS and/or (200 nM) AR-C69931MX prior to perfusion over immobilised vWf at 1800 s^{-1}. These were subsequently chased with 12.5 μM ADP. The platelets were allowed to translocate for approximately 18 seconds before being exposed to a near UV light source (300-400 nM) for 0.6 seconds (marked by the arrow). (a) The percentage of platelets forming stationary adhesion contacts for at least 10 seconds was quantified in the presence or absence of ADP, A3P5PS and/or AR-C69931MX prior to UV stimulation (black bars) and post UV stimulation (white bars) (data represents mean ± S.E.M of three separate experiments). (b) Demonstrates the displacement vs. time graph of a representative control NP-EGTA loaded platelet exposed to UV light and two representative A3P5PS-treated platelets exposed to UV light in the presence of ADP. (c) Shows displacement vs. time graphs of a representative control NP-EGTA loaded platelet exposed to UV light and two representative AR-C69931MX-treated platelets exposed to UV light in the presence of ADP. The shaded boxes highlight the platelet translocation behaviour and calcium profiles of platelets prior to ADP addition and UV stimulation (n=25 platelets).

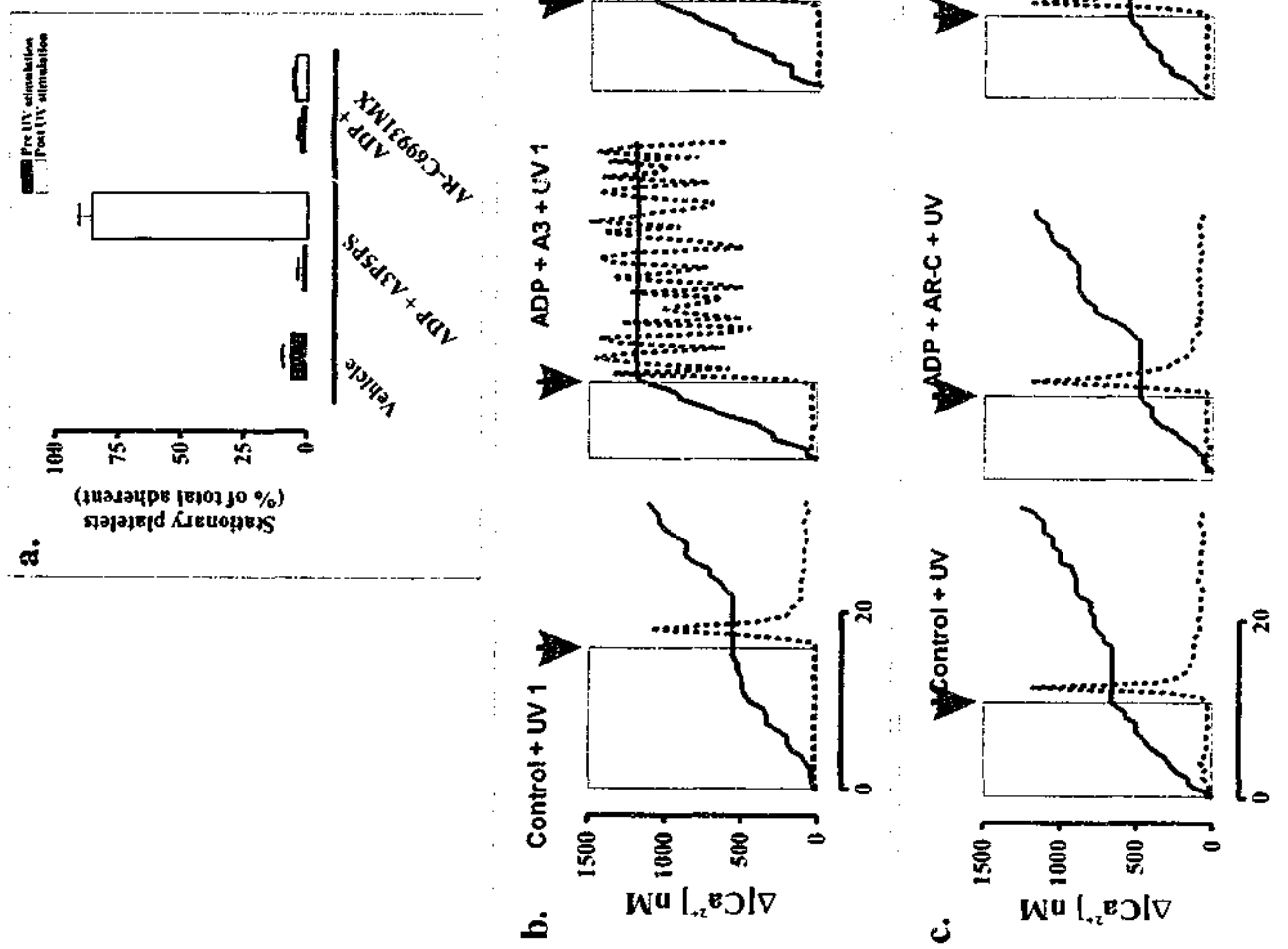
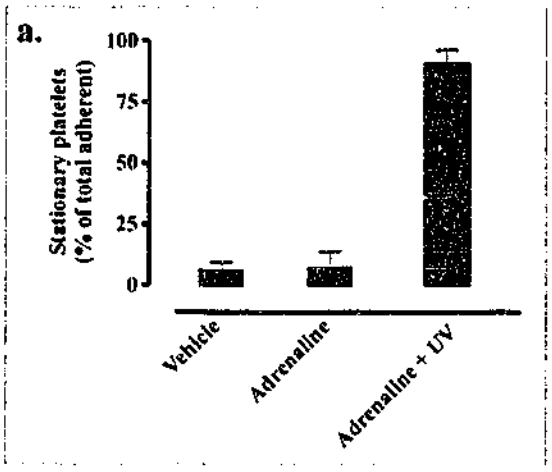
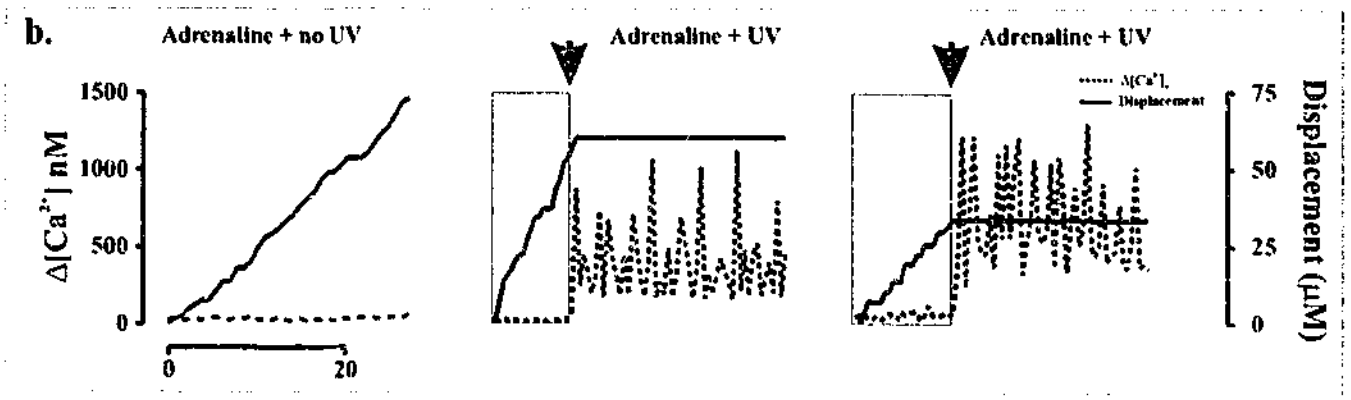

a.
Stationary platelets
(% of total adherent)
100
75
50
25
0
Vehicle
ADP +
ADP+A3PSPS
AR-C69931MX
Pre UV stimulation
Post UV stimulation
b.
Control + UV 1
ADP + A3 + UV 1
ADP + A3 + UV 2
Δ[Ca2+] nM
1500
1000
500
0
20
Displacement (μM)
75
50
25
c.
Control + UV
ADP + AR-C + UV
150
100
Displacement

Figure 6.10 Co-operative signalling through the α_2-adrenergic receptor and integrin $\alpha_{IIb}\beta_3$ is sufficient to induce sustained adhesion and calcium flux on vWf following transient calcium release.

Platelets loaded with 10μM of the caged calcium compound NP-EGTA were perfused over vWf at 1800 s^{-1} and subsequently chased with 50 μM adrenaline. The platelets were allowed to translocate for approximately 18 seconds before being exposed to a near UV light source (300-400 nM) for 0.6 seconds (marked by the arrow). (a) The percentage of platelets forming stationary adhesion contacts for at least 10 seconds was quantified in the presence or absence of adrenaline and UV stimulation (data represents mean ± S.E.M of three separate experiments). The graphs in (b) show representative displacement vs. time graph of a control NP-EGTA loaded platelet exposed to UV light and two representative platelets exposed to UV light in the presence of adrenaline. The shaded boxes highlight the platelet translocation behaviour and calcium profiles of platelets prior to UV stimulation (n=25 platelets).


a.
Stationary platelets (% of total adherent)
100
75
50
25
0
Vehicle
Adrenaline
Adrenaline + UV


b.
Adrenaline + no UV
Adrenaline + UV
Adrenaline + UV
Δ[Ca2+] nM
1500
1000
500
0
0
20
Δ[Ca2+]i
Displacement
75
50
25
0
Displacement (μM)

of NP-EGTA-loaded platelets with UV light resulted in transient stationary adhesion formation and calcium flux (fig. 6.10b). However, exposure of platelets to UV light in the presence of adrenaline resulted in sustained stationary adhesion and calcium signalling in >90% of translocating platelets (fig. 6.10a and b). This data further confirms the hypothesis that G_i-linked $P2Y_{12}$ stimulation is responsible for stabilising the interaction of $\alpha_{IIb}\beta_3$ with vWf.

6.6 Discussion.

The studies in this chapter demonstrate an important role for platelet density in regulating platelet adhesion to vWf under flow conditions. Moreover, these studies suggest that ADP is a key factor in regulating both primary stationary adhesion to vWf as well as subsequent aggregate formation under flow conditions. Consistent with previous ICC studies, the results presented in this chapter support a co-operative signalling relationship between integrin $\alpha_{IIb}\beta_3$ and ADP receptors in promoting stationary adhesion on vWf under flow. However, contrary to ICC studies demonstrating a key role only for the $P2Y_{12}$ receptor in promoting stationary adhesion and aggregate formation under flow, the present studies demonstrate an important role for both $P2Y_1$ and $P2Y_{12}$ receptors at high concentrations of ADP. This suggests different mechanisms of activation are utilised by platelets at high and low ADP concentrations.

The studies in this chapter demonstrate a significant reduction in platelet stability and in the duration of stationary contact formation with decreasing platelet counts. Consistent with previous studies showing that very few platelet form stationary adhesion contacts with vWf expressed on the surface of developing thrombi (Kulkarni et al., 2000), the present studies demonstrated that at high or low density, the majority of platelet adhesion on vWf remained reversible. However, these studies also demonstrate that higher platelet densities increase the stability of adhesion contacts. The most notable difference was seen in the proportion of platelets forming stationary adhesion contacts for 10 seconds where, at low platelet densities (5×10^9/L), only ~3% of platelets were stationary compared with >25% at 150×10^9/L. These findings have potentially important clinical significance given that a fall in platelet count to levels below 20×10^9/L have been shown to result in an increased risk of haemorrhage (Gaydos, 1962).

The effect of density on adhesion stability correlated with increases in the duration of contact between translocating platelets and stationary cells. This contact time between platelets as well as adhesion stability of primary stationary platelets were reduced by inhibiting the cellular effects of released ADP. While the exact role of secreted ADP in promoting activation of adjacent platelets remains to be established, it is feasible to suggest that ADP released from adherent platelets can have paracrine effects on adjacent platelets. Thus, binding of secreted ADP to $P2Y_1$ and/or $P2Y_{12}$ receptors on stationary and tethering platelets results in activation and stabilisation of integrin $\alpha_{IIb}\beta_3$ and subsequently, increased stationary adhesion formation. To determine the validity of this hypothesis, future studies will require the development of a detection system to directly measure the levels of secreted ADP from vWf adherent platelets. Beigi et al., (1999) have devised a surface attached luciferase assay which allows detection of local nucleotide release from activated platelets. A similar technique may allow direct measurements of ADP release from vWf adherent platelets. Such an assay system would provide significant new insight into the mechanism by which ADP regulates stationary contact formation at high platelet counts.

The present studies also demonstrate that the efficiency of platelet stationary adhesion formation on vWf is increased dramatically by co-stimulating platelets with high concentrations of exogenous ADP, supporting a co-operative signalling relationship between the purinergic ADP receptors, $P2Y_1$ and $P2Y_{12}$, and integrin $\alpha_{IIb}\beta_3$. Contrary to previous ICC studies demonstrating an important role for $P2Y_{12}$, but not $P2Y_1$ engagement in stabilising integrin $\alpha_{IIb}\beta_3$ activity on vWf (section 5.4), the results of this chapter suggest that $P2Y_1$ and $P2Y_{12}$ are equally important for promoting stationary adhesion formation on vWf when platelets are exposed to high ADP concentrations.

The key difference between these experimental systems is that in the ICC studies, ADP is likely to be present at relatively low concentrations, and the initial transient calcium signal is probably elicited by either GP Ib/V/IX or integrin $\alpha_{IIb}\beta_3$ signalling, with $P2Y_{12}$ then serving to sustain the calcium response. In contrast, the initial calcium spike in the ADP infusion experiments is mediated primarily through ADP binding to the $P2Y_1$ receptor. Based on these observations, it is conceivable that $P2Y_1$-mediated signalling is responsible for inducing an initial transient integrin $\alpha_{IIb}\beta_3$ activation, and subsequent $P2Y_{12}$ signalling acts to stabilise the integrin $\alpha_{IIb}\beta_3$–vWf interaction. This hypothesis is supported by ICC studies in which blockade of the $P2Y_{12}$ receptor inhibited platelet aggregate formation on vWf but did not inhibit the ability of platelets to undergo transient calcium spikes and associated transient stationary adhesion contacts (section 5.4). Further evidence to support the latter hypothesis comes from studies where $P2Y_1$-induced transient calcium mobilisation is mimicked under flow conditions by NP-EGTA uncaging. These studies demonstrated that reversible stationary contacts associated with transient calcium spikes could be converted to sustained stationary adhesion by stimulating platelets through G_i-linked surface receptors.

These results suggest a two-step mechanism involving $P2Y_1$ and $P2Y_{12}$ signalling leading to stationary platelet adhesion and sustained calcium flux in response to ADP under high shear conditions. In this model, an initial transient calcium response and reversible adhesion formation is mediated by $P2Y_1$ signalling. A subsequent stabilising event is mediated by synergistic $P2Y_{12}$-dependent inside-out and integrin $\alpha_{IIb}\beta_3$-dependent outside-in signalling leading to prolonged integrin $\alpha_{IIb}\beta_3$ ligation and consequently to a sustained calcium flux. The demonstration that the $P2Y_{12}$ receptor sustains integrin $\alpha_{IIb}\beta_3$-dependent calcium flux and stable platelet aggregation, is consistent with recent perfusion studies demonstrating an important role for this receptor in stabilising platelet thrombus

formation under flow (Hollopeter et al., 2001; Remijn et al., 2001; Remijn et al., 2002). The exact mechanism by which G_i signalling synergises with integrin $\alpha_{IIb}\beta_3$ to promote sustained stationary adhesion in vWf adherent platelets remains unclear. It is well established that signalling through G_i-linked receptors results in the inhibition of adenylate cyclase and the reduction of cytosolic cAMP levels. However, reduction of cAMP levels associated with G_i signalling has not been found to be sufficient to promote integrin $\alpha_{IIb}\beta_3$ activation (Lanza et al., 1988). Recent studies have demonstrated that stimulation of platelets through G_i-linked receptors results in the calcium-dependent activation of a small GTPase called Rap 1, which is known to positively modulate integrin $\alpha_{IIb}\beta_3$, suggesting a possible link between G_i signalling and integrin $\alpha_{IIb}\beta_3$ activity (Lova et al., 2002).

CHAPTER 7:

CONCLUSIONS

Chapter 7.

Conclusions.

Platelets respond to a diverse array of stimuli *in vivo*, including soluble agonists generated at the site of vascular injury (thrombin, adrenaline, ADP and TXA_2), as well as adhesive ligands present in the vessel wall (collagen and vWf). Despite the structural and functional diversity of these platelet stimuli, they all induce similar biochemical and functional changes within the cell resulting in upregulation of the adhesive capacity of membrane receptors, reorganisation of the platelet cytoskeleton, secretion of granule contents and expression of procoagulant molecules on the platelet surface. One of the key functional changes essential for the normal haemostatic function of platelets is the conversion of the major platelet integrin $\alpha_{IIb}\beta_3$ from a low affinity to a high affinity receptor capable of engaging fluid-phase adhesive proteins, including fibrinogen and vWf. Integrin $\alpha_{IIb}\beta_3$ activation is central to platelet function, as it promotes multiple responses, including platelet adhesion, aggregation, spreading, clot retraction and microvesiculation.

Many different signalling pathways have been shown to be involved in regulating integrin $\alpha_{IIb}\beta_3$ activation, including PI 3-kinase, Ras GTPases and non-receptor tyrosine kinases (Saci A, 2000; Shattil et al., 1997), however, recent studies from Gq knock-out mice have highlighted the central importance of PLC activation and phoshoinositide turnover in regulating the affinity status of integrin $\alpha_{IIb}\beta_3$ (Offermanns et al., 1997). Gq-deficient platelets fail to aggregate in response to multiple physiological agonists, and as a result, these mice suffer a severe bleeding diathesis.

PLC-mediated hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, generates two key second messengers, the water-soluble inositol 1,4,5-

trisphosphate (IP_3) and the lipid-soluble metabolite, diacylglycerol (DAG). IP_3 mobilises calcium from internal stores while DAG serves as a co-factor for the activation of conventional and novel isoforms of protein kinase C (PKC). While calcium and PKC-are important second messengers regulating integrin $\alpha_{IIb}\beta_3$ activation (Kaibuchi et al., 1983; Offermanns et al., 1997; Rink et al., 1983; Rotondo et al., 1997; Saitoh et al., 1989; Shattil, 1999; Walker and Watson, 1993; Watanabe et al., 2001; Yap et al., 2000), their absolute requirement for $\alpha_{IIb}\beta_3$ activation remains controversial.

Conflicting evidence has been presented regarding the role of cytosolic calcium for integrin $\alpha_{IIb}\beta_3$ activation. Several studies have provided evidence for a central role for calcium in regulating integrin $\alpha_{IIb}\beta_3$ activation (Lapetina et al., 1985; Quinton et al., 2002b; Saitoh et al., 1989; Walker and Watson, 1993), while others have concluded that calcium is not essential for integrin $\alpha_{IIb}\beta_3$ activation (Haimovich et al., 1996; Jen et al., 1996; Kuwahara et al., 1999; Quinton et al., 2002a; Rotondo et al., 1997; Watson and Hambleton, 1989). The results presented in this thesis demonstrate that cytosolic calcium is critical for $\alpha_{IIb}\beta_3$ activation under all experimental conditions, as pre-treatment of platelets with membrane permeable calcium chelators effectively abolishes integrin $\alpha_{IIb}\beta_3$ activation in response to soluble platelet agonists and adhesive substrates. These studies also suggest that methodological differences may account for the discrepancy between reports on the absolute requirement for calcium in integrin $\alpha_{IIb}\beta_3$ activation, demonstrating the necessity of using high concentrations of cytosolic calcium chelators (100 μM DM-BAPTA,AM) to effectively prevent increases in cytosolic calcium particularly in response to strong agonist stimulation. Furthermore, the studies presented in this thesis also highlight the importance of analysing calcium levels at the single cell level when assessing the efficiency of calcium chelators to avoid erroneous interpretation of results due to incomplete chelation of calcium in a subset of the platelet population.

Similar controversy exists with regards to the requirement for PKC in integrin $\alpha_{IIb}\beta_3$ activation with some studies suggesting an important role for PKC in this process (Kaibuchi et al., 1983; Rink et al., 1983; Rotondo et al., 1997; Saitoh et al., 1989; Walker and Watson, 1993), while others have shown integrin $\alpha_{IIb}\beta_3$ activation in the absence of PKC activity (Iorio et al., 1996; Quinton et al., 2002a). In contrast to calcium, the present studies do not establish an essential role for PKC for $\alpha_{IIb}\beta_3$ activation. According my results, PKC serves mainly to facilitate the generation of integrin $\alpha_{IIb}\beta_3$-derived outside-in signals that promote sustained calcium oscillations and mediate irreversible adhesion. Initial PKC-dependent integrin $\alpha_{IIb}\beta_3$ activation can occur in the absence of a detectable calcium rise, however, once sustained integrin $\alpha_{IIb}\beta_3$-dependent calcium flux is established and a certain cytosolic calcium threshold is achieved in the platelet, sustained platelet activation is independent of PKC activity. The exact downstream signalling events induced by PKC that give rise to integrin $\alpha_{IIb}\beta_3$ activation remain unclear.

Recent studies have demonstrated that during thrombus formation *in vivo*, very few platelets form stationary adhesion contacts on developing thrombi. The majority of platelets tethering to the developing thrombus tend to translocate, and eventually detach from the thrombus surface (Kulkarni et al., 2000). The efficiency of conversion between platelet translocation and firm adhesion is a key determinant regulating the rate and extent of thrombus growth. A key issue that remains to be clarified is the precise mechanism(s) that regulate the transition from surface translocation to firm adhesion, and the mechanism(s) governing the extent of thrombus growth on different substrates. Recent work from our laboratory has suggested that calcium signals induced by the adhesion receptors GP Ib/V/IX and integrin $\alpha_{IIb}\beta_3$ per se are sufficient to promote stationary adhesion on vWf under flow conditions in the absence of soluble agonist stimulation (Nesbitt et al., 2002; Yap et al., 2000). These studies demonstrated that GPIb binding to

vWf promotes transient, low-level, calcium spikes that are sufficient to induce reversible integrin $\alpha_{IIb}\beta_3$ activation. Subsequent integrin $\alpha_{IIb}\beta_3$-vWf binding results in integrin $\alpha_{IIb}\beta_3$-mediated outside-in signals that promote sustained calcium oscillations and firm platelet adhesion. However, the efficiency of platelet activation on vWf is low and only a small proportion of platelets are able to form stationary adhesion contacts with the matrix under flow. The studies in this thesis demonstrate that co-ordinated signalling through integrin $\alpha_{IIb}\beta_3$ and the platelet ADP receptors, $P2Y_1$ and $P2Y_{12}$, increases the stability of the integrin $\alpha_{IIb}\beta_3$–vWf interaction and is a key requirement for ICC and subsequent aggregation of platelets under flow conditions. While similar calcium communication systems have been shown to occur in other cellular systems (Jorgensen et al., 2000; Romanello and D'Andrea, 2001; Romanello et al., 2001; Saunders et al., 2001), this form of intercellular signalling has never before been demonstrated in platelets.

More specifically, studies in this thesis examining the effect of high concentrations of exogenous ADP addition on platelet activation demonstrated an important role for both $P2Y_1$ and $P2Y_{12}$ receptors as well as integrin $\alpha_{IIb}\beta_3$. In these experiments, the signalling process responsible for initial integrin $\alpha_{IIb}\beta_3$ activation and reversible platelet arrest is mediated by ADP binding to the $P2Y_1$ receptor. This, however, mediates only transient and reversible platelet arrest suggesting that activation of the PLC linked G_q pathway through $P2Y_1$ is not sufficient to promote irreversible platelet adhesion. To achieve irreversible $\alpha_{IIb}\beta_3$ activation and stable adhesion, co-stimulation through $P2Y_{12}$ is necessary, suggesting that G_i signalling is required for stabilising integrin $\alpha_{IIb}\beta_3$ activity. Consistent with this hypothesis, a key finding of the ICC studies was that the communication of the calcium signal (ICC) between adjacent platelets is mediated by a co-operative signalling mechanism involving integrin $\alpha_{IIb}\beta_3$ and the $P2Y_{12}$ receptor. The important role for the $P2Y_{12}$ receptor in integrin $\alpha_{IIb}\beta_3$ activation is also consistent with the anti-thrombotic

effectiveness of the thienopyridine class of ADP receptor antagonists, which also target the $P2Y_{12}$ purinergic receptor.

Another issue that remains unresolved is the mechanisms that underlie the differences in reactivity between collagen and vWf. Since adhesion of platelets to vWf and collagen is mediated by different platelet surface receptors, the activation of the initial layer of adherent platelets on vWf or collagen is a reflection of the signalling properties of the major receptors involved and will differ on different substrates. However, given that vWf is the major adhesive ligand mediating adhesion of subsequent layers of adhering platelets (Denis et al., 1998; Kulkarni et al., 2000; Ruggeri et al., 1999; Savage et al., 1998), it is not clear how these matrices differ in regulating activation of subsequent layers of aggregating platelets. The studies presented in this thesis raise the possibility that the level of calcium flux and the efficiency of ICC mediated by the matrix govern the extent of platelet aggregation and thrombus growth on different adhesive matrices. In light of previous studies demonstrating an important role for ADP release in platelet aggregation following vWf stimulation (Chow et al., 1992; Cachet, 2001a; Moake et al., 1988; Moritz et al., 1983; Oda et al., 1995; Peterson et al., 1987), and in thrombus formation on collagen (Folie and McIntire, 1989; Remijn et al., 2002; Turner et al., 2001; Wagner and Hubbell, 1992a; Wagner and Hubbell, 1992b), the results presented in this thesis provide a potential explanation for this difference. The release of ADP at the site of platelet-platelet contact provides a means of achieving high local concentrations of excitatory signals at sites of aggregate formation which subsequently promotes the propagation of platelet calcium signals (ICC) and sustained integrin $\alpha_{IIb}\beta_3$ activation. A likely explanation for the discrepancy between vWf and collagen is that, GPIb/V/IX and $\alpha_{IIb}\beta_3$ engagement of vWf may promote low levels of ADP release in the immediate vicinity of platelet-platelet adhesion contacts. Activation of a G_i-signalling mechanism, mediated by low levels of

released ADP, may then act to stabilise $\alpha_{IIb}\beta_3$ activation and promote stationary adhesion formation on vWf. On the other hand, given that collagen is a more potent thrombogenic matrix than vWf, collagen binding to platelet $\alpha_2\beta_1$ and GP VI may promote greater release of ADP in a localised area of platelet activation. Therefore, platelets tethering to α-granule-released vWf, expressed on the surface of collagen induced thrombi (Kulkarni et al., 2000), would also be subjected to the activating effects of large local ADP concentrations. This promotes more pronounced calcium spikes and hence a more pronounced ICC response, greater integrin $\alpha_{IIb}\beta_3$ activation and larger aggregate formation. However, further studies are required to investigate the extent of ADP release induced by ligation of the vWf receptors (GP Ib/V/IX and integrin $\alpha_{IIb}\beta_3$), and the collagen receptors ($\alpha_2\beta_1$ and GP VI).

Overall the studies in this thesis highlight a key role for calcium communication in regulating platelet adhesion and thrombus formation under flow conditions. The exact mechanisms regulating calcium signal transduction between platelets remains unknown. However, given the central role of calcium in platelet activation in response to all physiological agonists, elucidation of the key mechanisms regulating intercellular calcium communication could provide new approaches to regulate the reactivity of platelets *in vivo*.

APPENDICES

APPENDICIES.

APPENDIX A: MATERIALS.

Chemicals	Source
Acetic acid, glacial	BDH Chemicals
Acrylamide	Bio-Rad, U.S.A.
Adenosine diphosphate (ADP)	Sigma Chemical Co., U.S.A
Ammonium persulphate	BDH Chemical, Australia
β-alanine	Sigma Chemical Co., U.S.A.
β-mercaptoethanol	Sigma Chemical Co., U.S.A.
Bis-acrylamide	Bio-Rad, U.S.A.
Bovine serum albumin (BSA)	Sigma Chemical Co., U.S.A.
Bromophenol blue	Sigma Chemical Co., U.S.A.
Calcium chloride ($CaCl_2$)	Ajax Chemicals, Australia
Citric acid	Sigma Chemical Co., U.S.A.
Coomassie Brilliant Blue	Sigma Chemical Co., U.S.A.
Dimethyl sulphoxide (DMSO)	BDH Chemicals, Australia
di-potassium hydrogen orthophosphate (K_2HPO_4)	BDH Chemicals, Australia
di-sodium hydrogen orthophosphate (Na_2HPO_4)	Ajax Chemicals, Australia
DM-BAPTA, AM	Molecular Probes, U.S.A.
EDTA	Ajax Chemicals, Australia

EGTA	Sigma Chemical Co., U.S.A.
Ethanol	BDH Chemicals, Australia
Formaldehyde	BDH Chemicals, Australia
Glucose	BDH Chemicals, Australia
Glutaraldehyde	Sigma Chemical Co., U.S.A.
Glycerol	BDH Chemicals, Australia
Glycine	BDH Chemicals, Australia
HEPES	Sigma Chemical Co., U.S.A.
Hydrochloric acid (HCl)	Ajax Chemicals, Australia
Ionophore A23187	Sigma Chemical Co., U.S.A.
Magnesium chloride ($MgCl_2$)	BDH Chemicals, Australia
Methanol	Ajax Chemicals, Australia
Permafluor	Immunotech, France
Potassium chloride (KCl)	Ajax Chemicals, Australia
Ristocetin	Murex, Australia
Separarose CL-6B	Amrad Pharmacia, U.S.A.
Sodium chloride (NaCl)	Ajax Chemicals, Australia
Sodium dodecyl sulphate (SDS)	Bio-Rad, U.S.A.
Sucrose	BDH Chemicals, Australia
TEMED	Sigma Chemical Co., U.S.A.
Thapsigargin	Sigma Chemical Co., U.S.A.
Tris	Progen, Australia
Trisodium citrate	Ajax Chemicals, Australia
Triton X-100	Sigma Chemical Co., U.S.A.
Tween-20	BDH Chemicals, Australia

Adhesive matrix proteins	**Source**
Type I Collagen (Equine Tendon)	Sigma Chemical Co., U.S.A.

Soluble agonists	**Source**
ADP	Sigma Chemical Co., U.S.A.
Ionophore A23187	Sigma Chemical Co., U.S.A.
PMA (phorbol 12-myristate 13-acetate)	Sigma Chemical Co., U.S.A.
Thrombin	Park Davis Co., U.S.A.

Inhibitors	**Source**
Aggrastat	Mercke Sharpe & Dohme, U.S.A.
ALLN (Calpain inhibitor I)	Calbiochem, U.S.A.
Apyrase	Donated by Dr. Jean-Pierre Cazenave, INSERM, France
AR-C69931MX	AstraZeneca, England
A3P5PS	Sigma Chemical Co., U.S.A.
Benzamidine	Sigma Chemical Co., U.S.A.
Phenylmethylsulphonyl fluoride (PMSF)	Sigma Chemical Co., U.S.A.
ReVasc (Hirudin)	Rhône-Poulenc, Australia
Theophylline	Sigma Chemical Co., U.S.A.

Antibodies	Source
Anti-$\alpha_{IIb}\beta_3$, PAC-1	Becton Dickinson, Australia
Anti-$\alpha_{IIb}\beta_3$, c7E3 Fab	Eli-Lily, U.S.A.
Anti-GP Ibα mAb, WM23	Donated by Prof. Michael Berndt, Baker Medical Research Institute, Australia
Anti-mouse IgG, FITC-conjugated	Silenus Laboratories, Australia

Fluorescent dyes	Source
$DiOC_6$	Molecular Probes, U.S.A.
Fura Red, AM	Molecular Probes, U.S.A.
Oregon Green 488 BAPTA-1, AM	Molecular Probes, U.S.A.

Protein assay kits	Source
Bio-Rad Protein assay kit	Bio-Rad, U.S.A.

Materials	Source
Coverslips	Lomb Scientific, Australia
Microcapillary tubes	Vitro Dynamics, U.S.A.

APPENDIX B: EQUIPMENT

Hardware	Source
Amicon concentrator	Amicon, U.S.A.
Conductivity meter (CDM-80)	Radiometer A/S, Denmark
Confocal microscope TCS-SP	Leica, Germany
Dual temperature slab gel dryer (1125B)	Bio-Rad, U.S.A.
Flow cytometer (FACScalibur)	Becton Dickenson, U.S.A.
Four channel automated platelet aggregation analyser	Kyoto Daiichi, Japan
Gilson pipettes	Gilson, France
Harvard syringe pump	Harvard Apparatus, U.S.A.
ISCO retriever II fraction collector	ISCO Inc., U.S.A.
Labofuge GL centrifuge	Heraeus, Germany
MCID™ (microcomputer Imaging Device)	Bertold, Australia
Microcentrifuge, Hermle Z233M	Medos Co. Pty. Ltd., Australia
Microscope, Leica DMIRBE	Leica, Germany
Mini protean II electrophoresis cell	Bio-Rad, U.S.A.
Nutator	Proscience, U.S.A.
pH meter (D-24)	Horiba Co., Japan
Spectrophotometer (DU 530)	Beckman Instruments, U.S.A.
Tissue Homogenizer (with cutting blade fitting)	ProScience, Australia
Ultracentrifuge L8-80	Beckman Instruments, U.S.A.
Video Monitor	Sony, Australia

Video recorder	Panasonic, Australia

Analytical software packages	**Source**
Leica TCS-NT	Leica, Germany
MCID™ (MicroComputer Imaging Device)	Bertold, Australia
Voxblast	VayTek, Iowa, U.S.A
Image Tool	UTHSCSA, University of Texas, San Antonio, U.S.A.

BIBLIOGRAPHY

Bibliography.

Abrams, C., Deng, Y.J., Steiner, B., O'Toole, T. and Shattil, S.J. (1994) Determinants of specificity of a baculovirus-expressed antibody Fab fragment that binds selectively to the activated form of integrin alpha IIb beta 3. *J Biol Chem*, **269**, 18781-18788.

Adelman, B., Michelson, A.D., Loscalzo, J., Greenberg, J. and Handin, R.I. (1985) Plasmin effect on platelet glycoprotein Ib-von Willebrand factor interactions. *Blood*, **65**, 32-40.

Agranoff, B.W. (1983) Biochemical mechanisms in the phosphatidylinositol effect. *Life Sci*, **32**, 2047-2054.

Allen, R.D., Zacharski, L.R., Widirstky, S.T., Rosenstein, R., Zaitlin, L.M. and Burgess, D.R. (1979) Transformation and motility of human platelets: details of the shape change and release reaction observed by optical and electron microscopy. *J Cell Biol*, **83**, 126-142.

Alonso, M.T., Alvarez, J., Montero, M., Sanchez, A. and Garcia-Sancho, J. (1991) Agonist-induced Ca2+ influx into human platelets is secondary to the emptying of intracellular Ca2+ stores. *Biochem J*, **280 (Pt 3)**, 783-789.

Ambo, H., Kamata, T., Handa, M., Kawai, Y., Oda, A., Murata, M., Takada, Y. and Ikeda, Y. (1998a) Novel point mutations in the alphaIIb subunit (Phe289-->Ser, Glu324-->Lys and Gln747-->Pro) causing thrombasthenic phenotypes in four Japanese patients. *Br J Haematol*, **102**, 829-840.

Ambo, H., Kamata, T., Handa, M., Taki, M., Kuwajima, M., Kawai, Y., Oda, A., Murata, M., Takada, Y., Watanabe, K. and Ikeda, Y. (1998b) Three novel integrin beta3 subunit missense mutations (H280P, C560F, and G579S) in thrombasthenia, including one (H280P) prevalent in Japanese patients. *Biochem Biophys Res Commun*, **251**, 763-768.

Andrews, R.K. and Berndt, M.C. (1998) Adhesion-dependent signalling and the initiation of haemostasis and thrombosis. *Histol Histopathol*, **13**, 837-844.

Andrews, R.K. and Fox, J.E. (1991) Interaction of purified actin-binding protein with the platelet membrane glycoprotein Ib-IX complex. *J Biol Chem*, **266**, 7144-7147.

Andrews, R.K., Lopez, J.A. and Berndt, M.C. (1997) Molecular mechanisms of platelet adhesion and activation. *Int J Biochem Cell Biol*, **29**, 91-105.

Asakura, S., Niwa, K., Tomozawa, T., Lin, Y., Madoiwa, S., Sakata, Y., Sakai, T., Funayama, H. Soe, G., Forgerty, F., Hirata, H. and Matsuda, M. (1997) Fibroblasts spread on immobilized fibrin monomer by mobilizing a beta1-class integrin, together with a vitronectin receptor alphavbeta3 on their surface. *J Biol Chem*, **272**, 8824-8829.

Asazuma, N., Ozaki, Y., Satoh, K., Yatomi, Y., Handa, M., Fujimura, Y., Miura, S. and Kume, S. (1997) Glycoprotein Ib-von Willebrand factor interactions activate tyrosine kinases in human platelets. *Blood*, **90**, 4789-4798.

Asselin, J., Gibbins, J.M., Achison, M., Lee, Y.H., Morton, L.F., Farndale, R.W., Barnes, M.J. and Watson, S.P. (1997) A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C gamma2 in platelets independent of the integrin alpha2beta1. *Blood*, **89**, 1235-1242.

Bachelot, C., Cano, E., Grelac, F., Saleun, S., Druker, B.J., Levy-Toledano, S., Fischer, S. and Rendu, F. (1992) Functional implications of tyrosine protein phosphorylation in platelets. Simultaneous studies with different agonists and inhibitors. *Biochem J*, **284 (Pt 3)**, 923-928.

Badimon, L., Badimon, J.J., Galvez, A., Turitto, V. and Fuster, V. (1989a) Platelet interaction with vessel wall and collagen in pigs with homozygous von Willebrand's disease associated with abnormal collagen aggregation. *Thromb Haemost*, **61**, 57-64.

Badimon, L., Badimon, J.J., Turitto, V.T. and Fuster, V. (1989b) Role of von Willebrand factor in mediating platelet-vessel wall interaction at low shear rate; the importance of perfusion conditions. *Blood*, **73**, 961-967.

Baldassare, J.J., Henderson, P.A., Burns, D., Loomis, C. and Fisher, G.J. (1992) Translocation of protein kinase C isozymes in thrombin-stimulated human platelets. Correlation with 1,2-diacylglycerol levels. *J Biol Chem*, **267**, 15585-15590.

Baumgartner, H.R. (1973) The role of blood flow in platelet adhesion, fibrin deposition, and formation of mural thrombi. *Microvasc Res*, **5**, 167-179.

Baumgartner, H.R. (1974) The subendothelial surface and thrombosis. *Thombos Diathes haemorrh suppl*, **59**, 91-105.

Baumgartner, H.R. (1977a) [The pathogenesis of atherosclerosis. Contribution from a cytobiological viewpoint]. *Schweiz Med Wochenschr*, **107**, 717-722.

Baumgartner, H.R. (1977b) Platelet interaction with collagen fibrils in flowing blood. I. Reaction of human platelets with alpha chymotrypsin-digested subendothelium. *Thromb Haemost*, **37**, 1-16.

Baumgartner, H.R., Muggli, R., Tschopp, T.B. and Turitto, V.T. (1976) Platelet adhesion, release and aggregation in flowing blood: effects of surface properties and platelet function. *Thromb Haemost*, **35**, 124-138.

Baumgartner, H.R., Tschopp, T.B. and Weiss, H.J. (1977) Platelet interaction with collagen fibrils in flowing blood. II. Impaired adhesion-aggregation in bleeding disorders. A comparison with subendothelium. *Thromb Haemost*, **37**, 17-28.

Bell, R.L. and Majerus, P.W. (1980) Thrombin-induced hydrolysis of phosphatidylinositol in human platelets. *J Biol Chem*, **255**, 1790-1792.

Bennett, J.S. and Vilaire, G. (1979) Exposure of platelet fibrinogen receptors by ADP and epinephrine. *J Clin Invest*, **64**, 1393-1401.

Berndt, M.C. and Phillips, D.R. (1981a) Interaction of thrombin with platelets: purification of the thrombin substrate. *Ann N Y Acad Sci*, **370**, 87-95.

Berndt, M.C. and Phillips, D.R. (1981b) Purification and preliminary physicochemical characterization of human platelet membrane glycoprotein V. *J Biol Chem*, **256**, 59-65.

Berndt, M.C., Ward, C.M., Booth, W.J., Castaldi, P.A., Mazurov, A.V. and Andrews, R.K. (1992) Identification of aspartic acid 514 through glutamic acid 542 as a glycoprotein Ib-IX complex receptor recognition sequence in von Willebrand factor. Mechanism of modulation of von Willebrand factor by ristocetin and botrocetin. *Biochemistry*, **31**, 11144-11151.

Berndt, M.C., Ward, C.M., De Luca, M., Facey, D.A., Castaldi, P.A., Harris, S.J. and Andrews, R.K. (1995) The molecular mechanism of platelet adhesion. *Aust N Z J Med*, **25**, 822-830.

Berridge, M.J. (1984) Inositol trisphosphate and diacylglycerol as second messengers. *Biochem J*, **220**, 345-360.

Berridge, M.J. and Irvine, R.F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*, **312**, 315-321.

Bevers, E.M., Verhallen, P.F., Linskens, W.M., Comfurius, P. and Zwaal, R.F. (1987) Loss of phospholipid asymmetry in dilauroylphosphatidylcholine induced plasma membrane vesicles from human platelets. *Biochim Biophys Acta*, **903**, 197-205.

Billah, M.M. and Lapetina, E.G. (1982a) Degradation of phosphatidylinositol-4,5-bisphosphate is insensitive to CA2+ mobilization in stimulated platelets. *Biochem Biophys Res Commun*, **109**, 217-222.

Billah, M.M. and Lapetina, E.G. (1982b) Rapid decrease of phosphatidylinositol 4,5-bisphosphate in thrombin-stimulated platelets. *J Biol Chem*, **257**, 12705-12708.

Bird, R.B., Stewart, W.E., and Lightfoot, E.N. (1960) in transport phenomena. In. Wiley, New York, p. 42.

Bizzozero, G. (1882) Uber einen neuen formbestandteil des blutes und dessen rolle bei der thrombose und der blutgerinnung. *Virchows Arch Pathol Anat Physiol*, **90**, 261.

Blann, A.D. and Lip, G.Y. (2001) Virchow's triad revisited: the importance of soluble coagulation factors, the endothelium, and platelets. *Thromb Res*, **101**, 321-327.

Blobe, G.C., Stribling, S., Obeid, L.M. and Hannun, Y.A. (1996) Protein kinase C isoenzymes: regulation and function. *Cancer Surv*, **27**, 213-248.

Blomback, B., Hessel, B. and Hogg, D. (1976) Disulfide bridges in nh2 -terminal part of human fibrinogen. *Thromb Res*, **8**, 639-658.

Bodnar, R.J., Gu, M., Li, Z., Englund, G.D. and Du, X. (1999) The cytoplasmic domain of the platelet glycoprotein Ibalpha is phosphorylated at serine 609. *J Biol Chem*, **274**, 33474-33479.

Bonnefoy, A., Liu, Q., Legrand, C. and Frojmovic, M.M. (2000) Efficiency of platelet adhesion to fibrinogen depends on both cell activation and flow. *Biophys J*, **78**, 2834-2843.

Bootman, M.D., Berridge, M.J. and Lipp, P. (1997) Cooking with calcium: the recipes for composing global signals from elementary events. *Cell*, **91**, 367-373.

Born, G.V. (1986) Haemorheological influences on platelet thrombogenesis. *Klin Wochenschr*, **64**, 1050-1053.

Born, G.V.R. (1962) Aggregation of platelets with adenosine diphosphate and its reversal. *Nature*, **194**, 27-29.

Boyer, J.L., Romero-Avila, T., Schachter, J.B. and Harden, T.K. (1996) Identification of competitive antagonists of the P2Y1 receptor. *Mol Pharmacol*, **50**, 1323-1329.

Bradford, M. (1976) A rapid and sensitive mothod for the quantitation of microgram quantities of protein using the principals of protein dye binding. *Anal Biochem*, **72**, 248-254.

Brass, L.F., Manning, D.R., Cichowski, K. and Abrams, C.S. (1997) Signaling through G proteins in platelets: to the integrins and beyond. *Thromb Haemost*, **78**, 581-589.

Brass, L.F., Manning, D.R. and Shattil, S.J. (1991) GTP-binding proteins and platelet activation. *Prog Hemost Thromb*, **10**, 127-174.

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

Brower, M.S., Levin, R.I. and Garry, K. (1985) Human neutrophil elastase modulates platelet function by limited proteolysis of membrane glycoproteins. *J Clin Invest*, **75**, 657-666.

Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol*, **12**, 463-518.

Calderwood, D.A., Shattil, S.J. and Ginsberg, M.H. (2000) Integrins and actin filaments: reciprocal regulation of cell adhesion and signaling. *J Biol Chem*, **275**, 22607-22610.

Calverley, D.C., Kavanagh, T.J. and Roth, G.J. (1998) Human signaling protein 14-3-3zeta interacts with platelet glycoprotein Ib subunits Ibalpha and Ibbeta. *Blood*, **91**, 1295-1303.

Calvete, J.J. (1994) Clues for understanding the structure and function of a prototypic human integrin: the platelet glycoprotein IIb/IIIa complex. *Thromb Haemost*, **72**, 1-15.

Calvete, J.J. (1995) On the structure and function of platelet integrin alpha IIb beta 3, the fibrinogen receptor. *Proc Soc Exp Biol Med*, **208**, 346-360.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem*, **257**, 7847-7851.

Cattaneo, M., Canciani, M.T., Lecchi, A., Kinlough-Rathbone, R.L., Packham, M.A., Mannucci, P.M. and Mustard, J.F. (1990) Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. *Blood*, **75**, 1081-1086.

Cattaneo, M. and Gachet, C. (1999) ADP receptors and clinical bleeding disorders. *Arterioscler Thromb Vasc Biol*, **19**, 2281-2285.

Cattaneo, M., Lecchi, A., Randi, A.M., McGregor, J.L. and Mannucci, P.M. (1992) Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood*, **80**, 2787-2796.

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

Cazanave, J.P., Hemmendinger, S., Beretz, A., Sutter-Bay, A., and Launay, J. (1983) L'agregation plaquettaire: outil d'investigation clinique et d'etude pharmacologique methodologie. *Ann. Biol. Clin.*, **41**, 167-179.

Chacko, G.W., Brandt, J.T., Coggeshall, K.M. and Anderson, C.L. (1996) Phosphoinositide 3-kinase and p72syk noncovalently associate with the low affinity Fc gamma receptor on human platelets through an immunoreceptor tyrosine-based activation motif. Reconstitution with synthetic phosphopeptides. *J Biol Chem*, **271**, 10775-10781.

Chen, Y.Q., Gao, X., Timar, J., Tang, D., Grossi, I.M., Chelladurai, M., Kunicki, T.J., Fligiel, S.E., Taylor, J.D. and Honn, K.V. (1992) Identification of the alpha IIb beta 3 integrin in murine tumor cells. *J Biol Chem*, **267**, 17314-17320.

Chien, S., Usami, S., Dellenback, R.J. and Gregersen, M.I. (1970) Shear-dependent deformation of erythrocytes in rheology of human blood. *Am J Physiol*, **219**, 136-142.

Chopek, M.W., Girma, J.P., Fujikawa, K., Davie, E.W. and Titani, K. (1986) Human von Willebrand factor: a multivalent protein composed of identical subunits. *Biochemistry*, **25**, 3146-3155.

Chow, T.W., Hellums, J.D., Moake, J.L. and Kroll, M.H. (1992) Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. *Blood*, **80**, 113-120.

Chrzanowska-Wodnicka, M. and Burridge, K. (1996) Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol*, **133**, 1403-1415.

Chun, J.S., Ha, M.J. and Jacobson, B.S. (1996) Differential translocation of protein kinase C epsilon during HeLa cell adhesion to a gelatin substratum. *J Biol Chem*, **271**, 13008-13012.

Churchill, G.C. and Louis, C.F. (1998) Roles of Ca2+, inositol trisphosphate and cyclic ADP-ribose in mediating intercellular Ca2+ signaling in sheep lens cells. *J Cell Sci*, **111 (Pt 9)**, 1217-1225.

Clapham, D.E. (1995) Calcium signaling. *Cell*, **80**, 259-268.

Clark, E.A. and Brugge, J.S. (1995) Integrins and signal transduction pathways: the road taken. *Science*, **268**, 233-239.

Clark, E.A., Shattil, S.J. and Brugge, J.S. (1994) Regulation of protein tyrosine kinases in platelets. *Trends Biochem Sci*, **19**, 464-469.

Clements, J.L., Lee, J.R., Gross, B., Yang, B., Olson, J.D., Sandra, A., Watson, S.P., Lentz, S.R. and Koretzky, G.A. (1999) Fetal hemorrhage and platelet dysfunction in SLP-76-deficient mice. *J Clin Invest*, **103**, 19-25.

Clemetson, K.J. (1995) Platelet activation: signal transduction via membrane receptors. *Thromb Haemost*, **74**, 111-116.

Clemetson, K.J. (1999) Platelet collagen receptors: a new target for inhibition? *Haemostasis*, **29**, 16-26.

Clemetson, K.J. and Clemetson, J.M. (1995) Platelet GPIb-V-IX complex. Structure, function, physiology, and pathology. *Semin Thromb Hemost*, **21**, 130-136.

Clemetson, K.J., Naim, H.Y. and Luscher, E.F. (1981) Relationship between glycocalicin and glycoprotein Ib of human platelets. *Proc Natl Acad Sci U S A*, **78**, 2712-2716.

Collins, P.W., Noble, K.E., Reittie, J.R., Hoffbrand, A.V., Pasi, K.J. and Yong, K.L. (1995) Induction of tissue factor expression in human monocyte/endothelium cocultures. *Br J Haematol*, **91**, 963-970.

Colombatti, A. and Bonaldo, P. (1991) The superfamily of proteins with von Willebrand factor type A-like domains: one theme common to components of extracellular matrix, hemostasis, cellular adhesion, and defense mechanisms. *Blood*, **77**, 2305-2315.

Cooke, B.M., Usami, S., Perry, I. and Nash, G.B. (1993) A simplified method for culture of endothelial cells and analysis of adhesion of blood cells under conditions of flow. *Microvasc Res*, **45**, 33-45.

Cooney, K.A., Nichols, W.C., Bruck, M.E., Bahou, W.F., Shapiro, A.D., Bowie, E.J., Gralnick, H.R. and Ginsburg, D. (1991) The molecular defect in type IIB von Willebrand disease. Identification of four potential missense mutations within the putative Gplb binding domain. *J Clin Invest*, **87**, 1227-1233.

Cooray, P., Yuan, Y., Schoenwaelder, S.M., Mitchell, C.A., Salem, H.H. and Jackson, S.P. (1996) Focal adhesion kinase (pp125FAK) cleavage and regulation by calpain. *Biochem J*, **318 (Pt 1)**, 41-47.

Coppolino, M.G. and Dedhar, S. (2000) Bi-directional signal transduction by integrin receptors. *Int J Biochem Cell Biol*, **32**, 171-188.

Crabos, M., Fabbro, D., Stabel, S. and Erne, P. (1992) Effect of tumour-promoting phorbol ester, thrombin and vasopressin on translocation of three distinct protein kinase C isoforms in human platelets and regulation by calcium. *Biochem J*, **288 (Pt 3)**, 891-896.

Crabos, M., Imber, R., Woodtli, T., Fabbro, D. and Erne, P. (1991) Different translocation of three distinct PKC isoforms with tumor-promoting phorbol ester in human platelets. *Biochem Biophys Res Commun*, **178**, 878-883.

Crouch, M.F. and Lapetina, E.G. (1988) Spacial isolation of protein kinase C activation in thrombin stimulated human platelets. *Biochem Biophys Res Commun*, **156**, 149-156.

Cutler, L., Rodan, G. and Feinstein, M.B. (1978) Cytochemical localization of adenylate cyclase and of calcium ion, magnesium ion-activated ATPases in the dense tubular system of human blood platelets. *Biochim Biophys Acta*, **542**, 357-371.

Dalla Via, L., Stimamiglio, M., Scapin, M., Cesaro, L. and Deana, R. (1996) Correlation between cytosolic Ca2+ concentration, protein phosphorylation and platelet secretion. *Cell Calcium*, **20**, 431-440.

D'Andrea, P., Calabrese, A., Capozzi, I., Grandolfo, M., Tonon, R. and Vittur, F. (2000) Intercellular Ca2+ waves in mechanically stimulated articular chondrocytes. *Biorheology*, **37**, 75-83.

Daniel, J.L., Dangelmaier, C., Jin, J., Ashby, B., Smith, J.B. and Kunapuli, S.P. (1998) Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem*, **273**, 2024-2029.

Davie, E.W., Fujikawa, K. and Kisiel, W. (1991) The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry*, **30**, 10363-10370.

Davis, P.D., Elliott, L.H., Harris, W., Hill, C.H., Hurst, S.A., Keech, E., Kumar, M.K., Lawton, G , Nixon, J.S. and Wilkinson, S.E. (1992a) Inhibitors of protein kinase C. 2. Substituted bisindolylmaleimides with improved potency and selectivity. *J Med Chem*, **35**, 994-1001.

Davis, P.D., Hill, C.H., Lawton, G., Nixon, J.S., Wilkinson, S.E., Hurst, S.A., Keech, E. and Turner, S.E. (1992b) Inhibitors of protein kinase C. 1. 2,3-Bisarylmaleimides. *J Med Chem*, **35**, 177-184.

de Gaetano, G. (2001) Historical overview of the role of platelets in hemostasis and thrombosis. *Haemetologica*, **86**, 349-356.

De Marco, L., Girolami, A., Russell, S. and Ruggeri, Z.M. (1985a) Interaction of asialo von Willebrand factor with glycoprotein Ib induces fibrinogen binding to the glycoprotein IIb/IIIa complex and mediates platelet aggregation. *J Clin Invest*, **75**, 1198-1203.

De Marco, L., Girolami, A., Zimmerman, T.S. and Ruggeri, Z.M. (1985b) Interaction of purified type IIB von Willebrand factor with the platelet membrane glycoprotein Ib induces fibrinogen binding to the glycoprotein IIb/IIIa complex and initiates aggregation. *Proc Natl Acad Sci U S A*, **82**, 7424-7428.

De Marco, L., Mazzucato, M., Masotti, A. and Ruggeri, Z.M. (1994) Localization and characterization of an alpha-thrombin-binding site on platelet glycoprotein Ib alpha. *J Biol Chem*, **269**, 6478-6484.

Dejana, E., Languino, L.R., Polentarutti, N., Balconi, G., Ryckewaert, J.J., Larrieu, M.J., Donati, M.B., Mantovani, A. and Marguerie, G. (1985) Interaction between fibrinogen and cultured endothelial cells. Induction of migration and specific binding. *J Clin Invest*, **75**, 11-18.

Denis, C., Methia, N., Frenette, P.S., Rayburn, H., Ullman-Cullere, M., Hynes, R.O. and Wagner, D.D. (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. *Proc Natl Acad Sci U S A*, **95**, 9524-9529.

Dent, J.A., Galbusera, M. and Ruggeri, Z.M. (1991) Heterogeneity of plasma von Willebrand factor multimers resulting from proteolysis of the constituent subunit. *J Clin Invest*, **88**, 774-782.

Disatnik, M.H. and Rando, T.A. (1999) Integrin-mediated muscle cell spreading. The role of protein kinase c in outside-in and inside-out signaling and evidence of integrin cross-talk. *J Biol Chem*, **274**, 32486-32492.

Dolmetsch, R.E., Xu, K. and Lewis, R.S. (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature*, **392**, 933-936.

Dong, J.F., Sae-Tung, G. and Lopez, J.A. (1997) Role of glycoprotein V in the formation of the platelet high-affinity thrombin-binding site. *Blood*, **89**, 4355-4363.

Donne, A. (1842) De L'origine des globules du sang, de leur mode de formation et de leur fin. *Comp Rend Acad Sci*, **14**, 366.

Doolittle, R.F., Watt, K.W., Cottrell, B.A., Strong, D.D. and Riley, M. (1979) The amino acid sequence of the alpha-chain of human fibrinogen. *Nature*, **280**, 464-468.

Dopheide, S.M., Yap, C.L. and Jackson, S.P. (2001) Dynamic aspects of platelet adhesion under flow. *Clin Exp Pharmacol Physiol*, **28**, 355-363.

Du, X., Harris, S.J., Tetaz, T.J., Ginsberg, M.H. and Berndt, M.C. (1994) Association of a phospholipase A2 (14-3-3 protein) with the platelet glycoprotein Ib-IX complex. *J Biol Chem*, **269**, 18287-18290.

Eberth, J.a.S., C. (1886) Experimentelle un'ersuchungen uber thrombose. *Virchows Arch Pathol Anat Physiol*, **103**, 39.

Elfervig, M.K., Graff, R.D., Lee, G.M., Kelley, S.S., Sood, A. and Banes, A.J. (2001) ATP induces Ca(2+) signaling in human chondrons cultured in three-dimensional agarose films. *Osteoarthritis Cartilage*, **9**, 518-526.

Elzagallaai, A., Rose, S.D. and Trifaro, J.M. (2000) Platelet secretion induced by phorbol esters stimulation is mediated through phosphorylation of MARCKS: a MARCKS-derived peptide blocks MARCKS phosphorylation and serotonin release without affecting pleckstrin phosphorylation. *Blood*, **95**, 894-902.

Escolar, G., Leistikow, E. and White, J.G. (1989) The fate of the open canalicular system in surface and suspension-activated platelets. *Blood*, **74**, 1983-1988.

Esko, J.D. and Raetz, C.R. (1980) Mutants of Chinese hamster ovary cells with altered membrane phospholipid composition. Replacement of phosphatidylinositol by phosphatidylglycerol in a myo-inositol auxotroph. *J Biol Chem*, **255**, 4474-4480.

Evans, W.H. and Boitano, S. (2001) Connexin mimetic peptides: specific inhibitors of gap-junctional intercellular communication. *Biochem Soc Trans*, **29**, 606-612.

Ezumi, Y., Shindoh, K., Tsuji, M. and Takayama, H. (1998) Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets. *J Exp Med*, **188**, 267-276.

Ezumi, Y., Takayama, H. and Okuma, M. (1995) Differential regulation of protein-tyrosine phosphatases by integrin alpha IIb beta 3 through cytoskeletal reorganization and tyrosine phosphorylation in human platelets. *J Biol Chem*, **270**, 11927-11934.

Fabre, J.E., Nguyen, M., Latour, A., Keifer, J.A., Audoly, L.P., Coffman, T.M. and Koller, B.H. (1999) Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat Med*, **5**, 1199-1202.

Fagura, M.S., Dainty, I.A., McKay, G.D., Kirk, I.P., Humphries, R.G., Robertson, M.J., Dougall, I.G. and Leff, P. (1998) P2Y1-receptors in human platelets which are pharmacologically distinct from P2Y(ADP)-receptors. *Br J Pharmacol*, **124**, 157-164.

Falasca, M., Logan, S.K., Lehto, V.P., Baccante, G., Lemmon, M.A. and Schlessinger, J. (1998) Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. *Embo J*, **17**, 414-422.

Falati, S., Edmead, C.E. and Poole, A.W. (1999) Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor gamma-chain, Fyn, and Lyn to activate human platelets. *Blood*, **94**, 1648-1656.

Falk, E. (1983) Plaque rupture with severe pre-existing stenosis precipitating coronary thrombosis. Characteristics of coronary atherosclerotic plaques underlying fatal occlusive thrombi. *Br Heart J*, **50**, 127-134.

Fam, S.R., Gallagher, C.J. and Salter, M.W. (2000) P2Y(1) purinoceptor-mediated Ca(2+) signaling and Ca(2+) wave propagation in dorsal spinal cord astrocytes. *J Neurosci*, **20**, 2800-2808.

Fantl, W.J., Muslin, A.J., Kikuchi, A., Martin, J.A., MacNicol, A.M., Gross, R.W. and Williams, L.T. (1994) Activation of Raf-1 by 14-3-3 proteins. *Nature*, **371**, 612-614.

Farrell, D.H., Thiagarajan, P., Chung, D.W. and Davie, E.W. (1992) Role of fibrinogen alpha and gamma chain sites in platelet aggregation. *Proc Natl Acad Sci U S A*, **89**, 10729-10732.

Ferris, C.D., Huganir, R.L., Bredt, D.S., Cameron, A.M. and Snyder, S.H. (1991) Inositol trisphosphate receptor: phosphorylation by protein kinase C and calcium calmodulin-dependent protein kinases in reconstituted lipid vesicles. *Proc Natl Acad Sci U S A*, **88**, 2232-2235.

Fiore, L.D. and Deykin, D. (1994) Mechanisms of hemostasis and arterial thrombosis. *Cardiol Clin*, **12**, 399-409.

Folie, B.J. and McIntire, L.V. (1989) Mathematical analysis of mural thrombogenesis. Concentration profiles of platelet-activating agents and effects of viscous shear flow. *Biophys J*, **56**, 1121-1141.

Folie, B.J., McIntyre, L.V. (1989) Mathematical analysis of mural thrombogenisis. Concentration profiles of platelet-activating agents and effects of viscous shear flow. *Biophys J*, **56**, 1121-1141.

Forrester, J. (1991) Intimal disruption and coronary thrombosis: its role in the pathogenesis of human coronary disease. *Am J Cardiol*, **68**, 69B-77B.

Forrester, J.S., Litvack, F. and Grundfest, W. (1991) Initiating events of acute coronary arterial occlusion. *Annu Rev Med*, **42**, 35-45.

Fox, J.E. (1985a) Identification of actin-binding protein as the protein linking the membrane skeleton to glycoproteins on platelet plasma membranes. *J Biol Chem*, **260**, 11970-11977.

Fox, J.E. (1985b) Linkage of a membrane skeleton to integral membrane glycoproteins in human platelets. Identification of one of the glycoproteins as glycoprotein Ib. *J Clin Invest*, **76**, 1673-1683.

Fox, J.E. (1993) Regulation of platelet function by the cytoskeleton. *Adv Exp Med Biol*, **344**, 175-185.

Fox, J.E., Aggerbeck, L.P. and Berndt, M.C. (1988) Structure of the glycoprotein Ib.IX complex from platelet membranes. *J Biol Chem*, **263**, 4882-4890.

Fox, J.E., Lipfert, L., Clark, E.A., Reynolds, C.C., Austin, C.D. and Brugge, J.S. (1993) On the role of the platelet membrane skeleton in mediating signal transduction. Association of GP IIb-IIIa, pp60c-src, pp62c-yes, and the p21ras GTPase-activating protein with the membrane skeleton. *J Biol Chem*, **268**, 25973-25984.

Frangioni, J.V., Oda, A., Smith, M., Salzman, E.W. and Neel, B.G. (1993) Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets. *Embo J*, **12**, 4843-4856.

Freed, E., McCormick, F. and Ruggieri, R. (1994a) Proteins of the 14-3-3 family associate with Raf and contribute to its activation. *Cold Spring Harb Symp Quant Biol*, **59**, 187-193.

Freed, E., Symons, M., Macdonald, S.G., McCormick, F. and Ruggieri, R. (1994b) Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science*, **265**, 1713-1716.

Fu, H., Xia, K., Pallas, D.C., Cui, C., Conroy, K., Narsimhan, R.P., Mamon, H., Collier, R.J. and Roberts, T.M. (1994) Interaction of the protein kinase Raf-1 with 14-3-3 proteins. *Science*, **266**, 126-129.

Fujikawa, K., Suzuki, H., McMullen, B. and Chung, D. (2001) Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood*, **98**, 1662-1666.

Fukami, M.H. and Salganicoff, L. (1977) Human platelet storage organelles. A review. *Thromb Haemost*, **38**, 963-970.

Fuster, V. and Chesebro, J.H. (1986) Mechanisms of unstable angina. *N Engl J Med*, **315**, 1023-1025.

Gabbeta, J., Yang, X., Sun, L., McLane, M.A., Niewiarowski, S. and Rao, A.K. (1996) Abnormal inside-out signal transduction-dependent activation of glycoprotein IIb-IIIa in a patient with impaired pleckstrin phosphorylation. *Blood*, **87**, 1368-1376.

Gachet, C. (2001a) ADP receptors of platelets and their inhibition. *Thromb Haemost*, **86**, 222-232.

Gachet, C. (2001b) Identification, characterization, and inhibition of the platelet ADP receptors. *Int J Hematol*, **74**, 375-381.

Gachet, C., Cattaneo, M., Ohlmann, P., Hechler, B., Lecchi, A., Chevalier, J., Cassel, D., Mannucci, P.M. and Cazenave, J.P. (1995) Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. *Br J Haematol*, **91**, 434-444.

Gachet, C., Hechler, B., Leon, C., Vial, C., Leray, C., Ohlmann, P. and Cazenave, J.P. (1997) Activation of ADP receptors and platelet function. *Thromb Haemost*, **78**, 271-275.

Gao, J., Zoller, K.E., Ginsberg, M.H., Brugge, J.S. and Shattil, S.J. (1997) Regulation of the pp72syk protein tyrosine kinase by platelet integrin alpha IIb beta 3. *Embo J*, **16**, 6414-6425.

Gaydos, L.S., Freireich, E.J. and Mantel, N. (1962) The quantitative relationship between platelet count and haemorhage in patients with acute leukaemia. *New England Journal of Medicine*, **226**, 905-909.

Geiger, J., Honig-Liedl, P., Schanzenbacher, P. and Walter, U. (1998) Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. *Eur J Pharmacol*, **351**, 235-246.

Gekeler, V., Boer, R., Uberall, F., Ise, W., Schubert, C., Utz, I., Hofmann, J., Sanders, K.H., Schachtele, C., Klemm, K. and Grunicke, H. (1996) Effects of the selective bisindolylmaleimide protein kinase C inhibitor GF 109203X on P-glycoprotein-mediated multidrug resistance. *Br J Cancer*, **74**, 897-905.

George, J.N.A., Phillips DR. (1985) *Platelet membrane glycoproteins*. Plenium Press, New York.

Gerrard, J.M. (1986) Calcium Translocations. In Holmsen, H. (ed.), *Platelet Responses and Metabolism*. CRC Press, Florida, Vol. 3.

Gerrard, J.M. and Carroll, R.C. (1981) Stimulation of platelet protein phosphorylation by arachidonic acid and endoperoxide analogs. *Prostaglandins*, **22**, 81-94.

Gerrard, J.M. and White, J.G. (1976) The influence of aspirin and indomethacin on the platelet contractile wave. *Am J Pathol*, **82**, 513-526.

Gerrard, J.M., White, J.G. and Peterson, D.A. (1978) The platelet dense tubular system: its relationship to prostaglandin synthesis and calcium flux. *Thromb Haemost*, **40**, 224-231.

Gibbins, J., Asselin, J., Farndale, R., Barnes, M., Law, C.L. and Watson, S.P. (1996) Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets. *J Biol Chem*, **271**, 18095-18099.

Gibbins, J.M., Okuma, M., Farndale, R., Barnes, M. and Watson, S.P. (1997) Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain. *FEBS Lett*, **413**, 255-259.

Giuriato, S., Bodin, S., Erneux, C., Woscholski, R., Plantavid, M., Chap, H. and Payrastre, B. (2000) pp60c-src associates with the SH2-containing inositol-5-phosphatase SHIP1 and is involved in its tyrosine phosphorylation downstream of alphaIIbbeta3 integrin in human platelets. *Biochem J*, **348 Pt 1**, 107-112.

Golden, A., Brugge, J.S. and Shattil, S.J. (1990) Role of platelet membrane glycoprotein IIb-IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. *J Cell Biol*, **111**, 3117-3127.

Goto, S., Ikeda, Y., Saldivar, E. and Ruggeri, Z.M. (1998) Distinct mechanisms of platelet aggregation as a consequence of different shearing flow conditions. *J Clin Invest*, **101**, 479-486.

Grainick, H.R., Williams, S.B. and Coller, B.S. (1985) Asialo von Willebrand factor interactions with platelets. Interdependence of glycoproteins Ib and IIb/IIIa for binding and aggregation. *J Clin Invest*, **75**, 19-25.

Gralnick, H.R., Williams, S., McKeown, L.P., Hansmann, K., Fenton, J.W., 2nd and Krutzsch, H. (1994) High-affinity alpha-thrombin binding to platelet glycoprotein Ib alpha: identification of two binding domains. *Proc Natl Acad Sci U S A*, **91**, 6334-6338.

Gratacap, M.P., Payrastre, B., Viala, C., Mauco, G., Plantavid, M. and Chap, H. (1998) Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase C-gamma2 is an early key event in FcgammaRIIA-mediated activation of human platelets. *J Biol Chem*, **273**, 24314-24321.

Grondin, P., Plantavid, M., Sultan, C., Breton, M., Mauco, G. and Chap, H. (1991) Interaction of pp60c-src, phospholipase C, inositol-lipid, and diacylglycerol kinases with the cytoskeletons of thrombin-stimulated platelets. *J Biol Chem*, **266**, 15705-15709.

Gross, B.S., Lee, J.R., Clements, J.L., Turner, M., Tybulewicz, V.L., Findell, P.R., Koretzky, G.A. and Watson, S.P. (1999) Tyrosine phosphorylation of SLP-76 is downstream of Syk following stimulation of the collagen receptor in platelets. *J Biol Chem*, **274**, 5963-5971.

Gryglewski, R.J., Bunting, S., Moncada, S., Flower, R.J. and Vane, J.R. (1976) Arterial walls are protected against deposition of platelet thrombi by a substance (prostaglandin X) which they make from prostaglandin endoperoxides. *Prostaglandins*, **12**, 685-713.

Gu, M., Xi, X., Englund, G.D., Berndt, M.C. and Du, X. (1999) Analysis of the roles of 14-3-3 in the platelet glycoprotein Ib-IX-mediated activation of integrin alpha(IIb)beta(3) using a reconstituted mammalian cell expression model. *J Cell Biol*, **147**, 1085-1096.

Gugler, E. and Luscher, E.F. (1965) Platelet function in congenital afibrinogenemia. *Thromb Diath Haemorrh*, **14**, 361-373.

Guthrie, P.B., Knappenberger, J., Segal, M., Bennett, M.V., Charles, A.C. and Kater, S.B. (1999) ATP released from astrocytes mediates glial calcium waves. *J Neurosci*, **19**, 520-528.

Haimovich, B., Kaneshiki, N. and Ji, P. (1996) Protein kinase C regulates tyrosine phosphorylation of pp125FAK in platelets adherent to fibrinogen. *Blood*, **87**, 152-161.

Haimovich, B., Lipfert, L., Brugge, J.S. and Shattil, S.J. (1993) Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. *J Biol Chem*, **268**, 15868-15877.

Haller, H., Lindschau, C., Maasch, C., Olthoff, H., Kurscheid, D. and Luft, F.C. (1998) Integrin-induced protein kinase Calpha and Cepsilon translocation to focal adhesions mediates vascular smooth muscle cell spreading. *Circ Res*, **82**, 157-165.

Handa, M., Titani, K., Holland, L.Z., Roberts, J.R. and Ruggeri, Z.M. (1986) The von Willebrand factor-binding domain of platelet membrane glycoprotein Ib. Characterization by monoclonal antibodies and partial amino acid sequence analysis of proteolytic fragments. *J Biol Chem*, **261**, 12579-12585.

Hannun, Y.A. and Bell, R.M. (1986) Phorbol ester binding and activation of protein kinase C on triton X-100 mixed micelles containing phosphatidylserine. *J Biol Chem*, **261**, 9341-9347.

Hannun, Y.A., Loomis, C.R. and Bell, R.M. (1985) Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. *J Biol Chem*, **260**, 10039-10043.

Hannun, Y.A., Loomis, C.R. and Bell, R.M. (1986a) Protein kinase C activation in mixed micelles. Mechanistic implications of phospholipid, diacylglycerol, and calcium interdependencies. *J Biol Chem*, **261**, 7184-7190.

Hannun, Y.A., Loomis, C.R., Merrill, A.H., Jr. and Bell, R.M. (1986b) Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. *J Biol Chem*, **261**, 12604-12609.

Harmon, J.T. and Jamieson, G.A. (1986) Activation of platelets by alpha-thrombin is a receptor-mediated event. D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone-thrombin, but not N alpha-tosyl-L-lysine chloromethyl ketone-thrombin, binds to the high affinity thrombin receptor. *J Biol Chem*, **261**, 15928-15933.

Hartwig, J.H. (1992) Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol*, **118**, 1421-1442.

Hartwig, J.H., Kung, S., Kovacsovics, T., Janmey, P.A., Cantley, L.C., Stossel, T.P. and Toker, A. (1996) D3 phosphoinositides and outside-in integrin signaling by glycoprotein IIb-IIIa mediate platelet actin assembly and filopodial extension induced by phorbol 12-myristate 13-acetate. *J Biol Chem*, **271**, 32986-32993.

Haslam, R.J., Lynham, J.A. and Fox, J.E. (1979) Effects of collagen, ionophore A23187 and prostaglandin E1 on the phosphorylation of specific proteins in blood platelets. *Biochem J*, **178**, 397-406.

Hato, T., Pampori, N. and Shattil, S.J. (1998) Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of integrin alphaIIb beta3. *J Cell Biol*, **141**, 1685-1695.

Hayem, G. (1882) Sur le mechanism de l'arrest des hemorrhagies. *C. R. Acad. Sci.*, **95**, 18.

Hechler, B., Eckly, A., Ohlmann, P., Cazenave, J.P. and Gachet, C. (1998a) The P2Y1 receptor, necessary but not sufficient to support full ADP-induced platelet aggregation, is not the target of the drug clopidogrel. *Br J Haematol*, **103**, 858-866.

Hechler, B., Leon, C., Vial, C., Vigne, P., Frelin, C., Cazenave, J.P. and Gachet, C. (1998b) The P2Y1 receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. *Blood*, **92**, 152-159.

Heemskerk, J.W., Feijge, M.A., Sage, S.O. and Walter, U. (1994) Indirect regulation of Ca2+ entry by cAMP-dependent and cGMP-dependent protein kinases and phospholipase C in rat platelets. *Eur J Biochem*, **223**, 543-551.

Heemskerk, J.W., Hoyland, J., Mason, W.T. and Sage, S.O. (1992) Spiking in cytosolic calcium concentration in single fibrinogen-bound fura-2-loaded human platelets *Biochem J*, **283 (Pt 2)**, 379-383.

Heemskerk, J.W., Vis, P., Feijge, M.A., Hoyland, J., Mason, W.T. and Sage, S.O. (1993) Roles of phospholipase C and Ca(2+)-ATPase in calcium responses of single, fibrinogen-bound platelets. *J Biol Chem*, **268**, 356-363.

Hemler, M.E. (1998) Integrin associated proteins. *Curr Opin Cell Biol*, **10**, 578-585.

Henry, S.A., Atkinson, K.D., Kolat, A.I. and Culbertson, M.R. (1977) Growth and metabolism of inositol-starved Saccharomyces cerevisiae. *J Bacteriol*, **130**, 472-484.

Henschen, A. (1983) On the structure of functional sites in fibrinogen. *Thromb Res*, **Suppl 5**, 27-39.

Heynes Adu, P.B., PN. Loetter, MG. (1985) *Platelet kinetics and imaging. Techniques and normal platelet kinetics*. Boca Raton.

Himpens, B., Stalmans, P., Gomez, P., Malfait, M. and Vereecke, J. (1999) Intra- and intercellular Ca2+ signaling in retinal pigment epithelial cells during mechanical stimulation. *Faseb J*, **13 Suppl**, S63-68.

Hofer, A.M., Curci, S., Doble, M.A., Brown, E.M. and Soybel, D.I. (2000) Intercellular communication mediated by the extracellular calcium-sensing receptor. *Nat Cell Biol*, **2**, 392-398.

Hollopeter, G., Jantzen, H.M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R.B., Nurden, P., Nurden, A., Julius, D. and Conley, P.B. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature*, **409**, 202-207.

holme, R., Sixma, J.J., Murer, E.H. and Hovig, T. (1973) Demonstration of fibrinogen secretion via the surface connecting canalicular system. *Thromb. Res.*, **3**, 347.

Holmsen, H. (1986) *Platelet responses and metabolism*. CRC-Press, BOC Raton.

Holmsen, H. and Weiss, H.J. (1979) Secretable storage pools in platelets. *Annu Rev Med*, **30**, 119-134.

Hourani, S.M. and Cusack, N.J. (1991) Pharmacological receptors on blood platelets. *Pharmacol Rev*, **43**, 243-298.

Howard, M.A., Sawers, R.J. and Firkin, B.G. (1973) Ristocetin: a means of differentiating von Willebrand's disease into two groups. *Blood*, **41**, 687-690.

Huang, J., Driscoll, E.M., Gonzales, M.L., Park, A.M. and Lucchesi, B.R. (2000) Prevention of arterial thrombosis by intravenously administered platelet P2T receptor antagonist AR-C69931MX in a canine model. *J Pharmacol Exp Ther*, **295**, 492-499.

Huang, M.M., Lipfert, L., Cunningham, M., Brugge, J.S., Ginsberg, M.H. and Shattil, S.J. (1993) Adhesive ligand binding to integrin alpha IIb beta 3 stimulates tyrosine phosphorylation of novel protein substrates before phosphorylation of pp125FAK. *J Cell Biol*, **122**, 473-483.

Hubbel JA, M.L. (1986a) Platelet active concentration profiles near growing thrombi. A mathematical consideration. *Biophys J*, **50**, 937-945.

Hubbel JA, M.L. (1986b) Visualisation and analysis of mural thrombogenisis on collagen, polyurethane and nylon. *Biomaterials*, **7**, 354-363.

Huber, M., Helgason, C.D., Damen, J.E., Liu, L., Humphries, R.K. and Krystal, G. (1998a) The src homology 2-containing inositol phosphatase (SHIP) is the gatekeeper of mast cell degranulation. *Proc Natl Acad Sci U S A*, **95**, 11330-11335.

Huber, M., Helgason, C.D., Scheid, M.P., Duronio, V., Humphries, R.K. and Krystal, G. (1998b) Targeted disruption of SHIP leads to Steel factor-induced degranulation of mast cells. *Embo J*, **17**, 7311-7319.

Hughes, J. (1959) *Thromb Diath Haemorrh*, **3**, 34.

Hughes, P.E., Diaz-Gonzalez, F., Leong, L., Wu, C., McDonald, J.A., Shattil, S.J. and Ginsberg, M.H. (1996) Breaking the integrin hinge. A defined structural constraint regulates integrin signaling. *J Biol Chem*, **271**, 6571-6574.

Hughes, P.E. and Pfaff, M. (1998) Integrin affinity modulation. *Trends Cell Biol*, **8**, 359-364.

Hughes, P.E., Renshaw, M.W., Pfaff, M., Forsyth, J., Keivens, V.M., Schwartz, M.A. and Ginsberg, M.H. (1997) Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell*, **88**, 521-530.

Humphries, M.J. (1996) Integrin activation: the link between ligand binding and signal transduction. *Curr Opin Cell Biol*, **8**, 632-640.

Humphries, R.G. (2000) Pharmacology of AR-C69931MX and related compounds: from pharmacological tools to clinical trials. *Heamatologica*, **85**, 66-72.

Humphries, R.G., Robertson, M.J. and Leff, P. (1995) A novel series of P2T purinoceptor antagonists: definition of the role of ADP in arterial thrombosis. *Trends Pharmacol Sci*, **16**, 179-181.

Hynes, R.O. (1987) Integrins: a family of cell surface receptors. *Cell*, **48**, 549-554.

Hynes, R.O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*, **69**, 11-25.

Ichinohe, T., Takayama, H., Ezumi, Y., Arai, M., Yamamoto, N., Takahashi, H. and Okuma, M. (1997) Collagen-stimulated activation of Syk but not c-Src is severely compromised in human platelets lacking membrane glycoprotein VI. *J Biol Chem*, **272**, 63-68.

Ikeda, Y., Handa, M., Kamata, T., Kawano, K., Kawai, Y., Watanabe, K., Kawakami, K., Sakai, K., Fukuyama, M., Itagaki, I. and et al. (1993) Transmembrane calcium influx associated with von Willebrand factor binding to GP Ib in the initiation of shear-induced platelet aggregation. *Thromb Haemost*, **69**, 496-502.

Ikeda, Y., Handa, M., Kawano, K., Kamata, T., Murata, M., Araki, Y., Anbo, H., Kawai, Y., Watanabe, K., Itagaki, I. and et al. (1991) The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest*, **87**, 1234-1240.

Ingall, A.H., Dixon, J., Bailey, A., Coombs, M.E., Cox, D., McInally, J.I., Hunt, S.F., Kindon, N.D., Teobald, B.J., Willis, P.A., Humphries, R.G., Leff, P., Clegg, J.A., Smith, J.A. and Tomlinson, W. (1999) Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. *J Med Chem*, **42**, 213-220.

Inoue, K., Ozaki, Y., Satoh, K., Wu, Y., Yatomi, Y., Shin, Y. and Morita, T. (1999) Signal transduction pathways mediated by glycoprotein Ia/IIa in human platelets: comparison with those of glycoprotein VI. *Biochem Biophys Res Commun*, **256**, 114-120.

Iorio, P., Gresele, P., Stasi, M., Nucciarelli, F., Vezza, R., Nenci, G.G. and Goracci, G. (1996) Protein kinase C inhibitors enhance G-protein induced phospholipase A2 activation in intact human platelets. *FEBS Lett*, **381**, 244-248.

Isobe, T., Hiyane, Y., Ichimura, T., Okuyama, T., Takahashi, N., Nakajo, S. and Nakaya, K. (1992) Activation of protein kinase C by the 14-3-3 proteins homologous with Exo1 protein that stimulates calcium-dependent exocytosis. *FEBS Lett*, **308**, 121-124.

Jackobsen, E., and Koerulf, P. (1970) A modified b-alanine precipitation proceedure to prepare fibrinogen free of anti-thrombin III and plsminogen. *Thromb. Res.*, **3**, 145-149.

Jackson, S.P., Schoenwaelder, S.M., Yuan, Y., Rabinowitz, I., Salem, H.H. and Mitchell, C.A. (1994) Adhesion receptor activation of phosphatidylinositol 3-kinase. von Willebrand factor stimulates the cytoskeletal association and activation of phosphatidylinositol 3-kinase and pp60c-src in human platelets. *J Biol Chem*, **269**, 27093-27099.

Jandrot-Perrus, M., Huisse, M.G., Ternisien, C., Bezeaud, A. and Guillin, M.C. (1992) Thrombin binding to platelet membrane glycoprotein Ib. *Semin Thromb Hemost*, **18**, 261-266.

Jantzen, H.M., Gousset, L., Bhaskar, V., Vincent, D., Tai, A., Reynolds, E.E. and Conley, P.B. (1999) Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost*, **81**, 111-117.

Jantzen, H.M., Milstone, D.S., Gousset, L., Conley, P.B. and Mortensen, R.M. (2001) Impaired activation of murine platelets lacking G alpha(i2). *J Clin Invest*, **108**, 477-483.

Jen, C.J., Chen, H.I., Lai, K.C. and Usami, S. (1996) Changes in cytosolic calcium concentrations and cell morphology in single platelets adhered to fibrinogen-coated surface under flow. *Blood*, **87**, 3775-3782.

Jin, J., Daniel, J.L. and Kunapuli, S.P. (1998) Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem*, **273**, 2030-2034.

Jin, J. and Kunapuli, S.P. (1998) Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci U S A*, **95**, 8070-8074.

Jorgensen, N.R., Henriksen, Z., Brot, C., Eriksen, E.F., Sorensen, O.H., Civitelli, R. and Steinberg, T.H. (2000) Human osteoblastic cells propagate intercellular calcium signals by two different mechanisms. *J Bone Miner Res*, **15**, 1024-1032.

Jung, S.M. and Moroi, M. (1998) Platelets interact with soluble and insoluble collagens through characteristically different reactions. *J Biol Chem*, **273**, 14827-14837.

Jung, S.M. and Moroi, M. (2000a) Activation of the platelet collagen receptor integrin alpha(2)beta(1): its mechanism and participation in the physiological functions of platelets. *Trends Cardiovasc Med*, **10**, 285-292.

Jung, S.M. and Moroi, M. (2000b) Signal-transducing mechanisms involved in activation of the platelet collagen receptor integrin alpha(2)beta(1). *J Biol Chem*, **275**, 8016-8026.

Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. and Nishizuka, Y. (1983) Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. *J Biol Chem*, **258**, 6701-6704.

Kajikawa, N., Kaibuchi, K., Matsubara, T., Kikkawa, U., Takai, Y., Nishizuka, Y., Itoh, K. and Tomioka, C. (1983) A possible role of protein kinase C in signal-induced lysosomal enzyme release. *Biochem Biophys Res Commun*, **116**, 743-750.

Kaplan, K.L., Broekman, M.J., Chernoff, A., Lesznik, G.R. and Drillings, M. (1979) Platelet alpha-granule proteins: studies on release and subcellular localization. *Blood*, **53**, 604-618.

Kaser-Glanzmann, R., Jakabova, M., George, J.N. and Luscher, E.F. (1977) Stimulation of calcium uptake in platelet membrane vesicles by adenosine 3',5'-cyclic monophosphate and protein kinase. *Biochim Biophys Acta*, **466**, 429-440.

Kehrel, B. (1995) Platelet-collagen interactions. *Semin Thromb Hemost*, **21**, 123-129.

Kloczewiak, M., Timmons, S. and Hawiger, J. (1983) Recognition site for the platelet receptor is present on the 15-residue carboxy-terminal fragment of the gamma chain of human fibrinogen and is not involved in the fibrin polymerization reaction. *Thromb Res*, **29**, 249-255.

Kloczewiak, M., Timmons, S., Lukas, T.J. and Hawiger, J. (1984) Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the gamma chain. *Biochemistry*, **23**, 1767-1774.

Knezevic, I., Leisner, T.M. and Lam, S.C. (1996) Direct binding of the platelet integrin alphaIIbbeta3 (GPIIb-IIIa) to talin. Evidence that interaction is mediated through the cytoplasmic domains of both alphaIIb and beta3. *J Biol Chem*, **271**, 16416-16421.

Kroll, M.H., Harris, T.S., Moake, J.L., Handin, R.I. and Schafer, A.I. (1991) von Willebrand factor binding to platelet GpIb initiates signals for platelet activation. *J Clin Invest*, **88**, 1568-1573.

Kroll, M.H., Hellums, J.D., Guo, Z., Durante, W., Razdan, K., Hrbolich, J.K. and Schafer, A.I. (1993) Protein kinase C is activated in platelets subjected to pathological shear stress. *J Biol Chem*, **268**, 3520-3524.

Kroll, M.H., Hellums, J.D., McIntire, L.V., Schafer, A.I. and Moake, J.L. (1996) Platelets and shear stress. *Blood*, **88**, 1525-1541.

Kroll, M.H. and Schafer, A.I. (1989) Biochemical mechanisms of platelet activation. *Blood*, **74**, 1181-1195.

Kulkarni, S., Dopheide, S.M., Yap, C.L., Ravanat, C., Freund, M., Mangin, P., Heel, K.A., Street, A., Harper, I.S., Lanza, F. and Jackson, S.P. (2000) A revised model of platelet aggregation. *J Clin Invest*, **105**, 783-791.

Kuwahara, M., Sugimoto, M., Tsuji, S., Matsui, H., Mizuno, T., Miyata, S. and Yoshioka, A. (2002) Platelet shape changes and adhesion under high shear flow. *Arterioscler Thromb Vasc Biol*, **22**, 329-334.

Kuwahara, M., Sugimoto, M., Tsuji, S., Miyata, S. and Yoshioka, A. (1999) Cytosolic calcium changes in a process of platelet adhesion and cohesion on a von Willebrand factor-coated surface under flow conditions. *Blood*, **94**, 1149-1155.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Laffargue, M., Monnereau, L., Tuech, J., Ragab, A., Ragab-Thomas, J., Payrastre, B., Raynal, P. and Chap, H. (1997) Integrin-dependent tyrosine phoshorylation and cytoskeletal translocation of Tec in thrombin-activated platelets. *Biochem Biophys Res Commun*, **238**, 247-251.

Lapetina, E.G., Reep, B., Ganong, B.R. and Bell, R.M. (1985) Exogenous sn-1,2-diacylglycerols containing saturated fatty acids function as bioregulators of protein kinase C in human platelets. *J Biol Chem*, **260**, 1358-1361.

LaRosa, C.A., Rohrer, M.J., Benoit, S.E., Barnard, M.R. and Michelson, A.D. (1994) Neutrophil cathepsin G modulates the platelet surface expression of the glycoprotein (GP) Ib-IX complex by proteolysis of the von Willebrand factor binding site on GPIb alpha and by a cytoskeletal-mediated redistribution of the remainder of the complex. *Blood*, **84**, 158-168.

Law, D.A., Nannizzi-Alaimo, L. and Phillips, D.R. (1996) Outside-in integrin signal transduction. Alpha IIb beta 3-(GP IIb IIIa) tyrosine phosphorylation induced by platelet aggregation. *J Biol Chem*, **271**, 10811-10815.

Lefkovits, J., Plow, E.F. and Topol, E.J. (1995) Platelet glycoprotein IIb/IIIa receptors in cardiovascular medicine. *N Engl J Med*, **332**, 1553-1559.

Leon, C., Hechler, B., Freund, M., Eckly, A., Vial, C., Ohlmann, P., Dierich, A., LeMeur, M., Cazenave, J.P. and Gachet, C. (1999a) Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest*, **104**, 1731-1737.

Leon, C., Vial, C., Gachet, C., Ohlmann, P., Hechler, B., Cazenave, J.P., Lecchi, A. and Cattaneo, M. (1999b) The P2Y1 receptor is normal in a patient presenting a severe deficiency of ADP-induced platelet aggregation. *Thromb Haemost*, **81**, 775-781.

Lerea, K.M., Cordero, K.P., Sakariassen, K.S., Kirk, R.I. and Fried, V.A. (1999) Phosphorylation sites in the integrin beta3 cytoplasmic domain in intact platelets. *J Biol Chem*, **274**, 1914-1919.

Li, R.Y., Gaits, F., Ragab, A., Ragab-Thomas, J.M. and Chap, H. (1994) Translocation of an SH2-containing protein tyrosine phosphatase (SH-PTP1) to the cytoskeleton of thrombin-activated platelets. *FEBS Lett*, **343**, 89-93.

Li, W., Llopis, J., Whitney, M., Zlokarnik, G. and Tsien, R.Y. (1998) Cell-permeant caged InsP3 ester shows that Ca2+ spike frequency can optimize gene expression. *Nature*, **392**, 936-941.

Liu, Q., Rooney, M.M., Kasirer-Friede, A., Brown, E., Lord, S.T. and Frojmovic, M.M. (1998) Role of the gamma chain Ala-Gly-Asp-Val and Aalpha chain Arg-Gly-Asp-Ser sites of fibrinogen in coaggregation of platelets and fibrinogen-coated beads. *Biochim Biophys Acta*, **1385**, 33-42.

Liu, S., Calderwood, D.A. and Ginsberg, M.H. (2000) Integrin cytoplasmic domain-binding proteins. *J Cell Sci*, **113**, 3563-3571.

Lopez, J.A. (1994) The platelet glycoprotein Ib-IX complex. *Blood Coagul Fibrinolysis*, **5**, 97-119.

Lopez, J.A., Chung, D.W., Fujikawa, K., Hagen, F.S., Papayannopoulou, T. and Roth, G.J. (1987) Cloning of the alpha chain of human platelet glycoprotein Ib: a transmembrane

protein with homology to leucine-rich alpha 2-glycoprotein. *Proc Natl Acad Sci U S A*, **84**, 5615-5619.

Lopez, J.A. and Dong, J.F. (1997) Structure and function of the glycoprotein Ib-IX-V complex. *Curr Opin Hematol*, **4**, 323-329.

Lowe, G.D. (1999) Rheological influences on thrombos ·. *Baillieres Best Pract Res Clin Haematol*, **12**, 435-449.

Lusher, E.F. (1960) *Vox Sang. (Basel)*, 259.

Lyng, F.M., Jones, G.R. and Rommerts, F.F. (2000) Rapid androgen actions on calcium signaling in rat sertoli cells and two human prostatic cell lines: similar biphasic responses between 1 picomolar and 100 nanomolar concentrations. *Biol Reprod*, **63**, 736-747.

Maalej, N., Holden, J.E. and Folts, J.D. (1998) Effect of Shear Stress on Acute Platelet Thrombus Formation in Canine Stenosed Carotid Arteries: An In Vivo Quantitative Study. *J Thromb Thrombolysis*, **5**, 231-238.

MacFarlane, D.E. (1987) Agonists and receptors: adenosine diphosphate. In Raton, B. (ed.), *In: Platelet responses and metabolism*. CRC-Press, Florida, pp. 19-36.

MacKenzie, A.B., Mahaut-Smith, M.P. and Sage, S.O. (1996) Activation of receptor-operated cation channels via P2X1 not P2T purinoceptors in human platelets. *J Biol Chem*, **271**, 2879-2881.

Majerus, P.W., Neuf ·d, E.J. and Wilson, D.B. (1984) Production of phosphoinositide-derived messengers. *Cell*, **37**, 701-703.

Mannucci, P.M. and Shar·, A.A. (1967) Platelet volume and shape in relation to aggregation and adhesion. *Br J Haematol*, **13**, 604-617.

Marguerie, G.A., Edgington, T.S. and Plow, E.F. (1980) Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. *J Biol Chem*, **255**, 154-161.

Marguerie, G.A., Plow, E.F. and Edgington, T.S. (1979) Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J Biol Chem*, **254**, 5357-5363.

Marshall, S.J., Asazuma, N., Best, D., Wonerow, P., Salmon. G., Andrews, R.K. and Watson, S.P. (2002) Glycoprotein IIb-IIIa-dependent aggregation by glycoprotein Ibalpha is reinforced by a Src family kinase inhibitor (PP1)-sensitive signalling pathway. *Biochem J*, **361**, 297-305.

Marti, T., Rosselet, S.J., Titani, K. and Walsh, K.A. (1987) Identification of disulfide-bridged substructures within human von Willebrand factor. *Biochemistry*, **26**, 8099-8109.

Mauco, G., Chap, H. and Douste-Blazy, L. (1979) Characterization and properties of a phosphatidylinositol phosphodiesterase (phospholipase C) from platelet cytosol. *FEBS Lett*, **100**, 367-370.

Mauco, G., Dajeans, P., Chap, H. and Douste-Blazy, L. (1987) Subcellular localization of inositol lipids in blood platelets as deduced from the use of labelled precursors. *Biochem J*, **244**, 757-761.

Mazzucato, M., Spessotto, P., Masotti, A., De Appollonia, L., Cozzi, M.R., Yoshioka, A., Perris, R., Colombatti, A. and De Marco, L. (1999) Identification of domains responsible for von Willebrand factor type VI collagen interaction mediating platelet adhesion under high flow. *J Biol Chem*, **274**, 3033-3041.

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

Merchant, J., and Parker, I. (2001) Role of elementary calcium puffs in generating repetitive calcium oscillations. *Embo J*, **20**, 65-76.

Merchant, J., Callamaras, N., and Parker, I. (1999) Initiation of IP3-mediated calcium waves in Xenopus oocytes. *Embo J*, **18**, 5285-5299.

Meyer, D. and Girma, J.P. (1993) von Willebrand factor: structure and function. *Thromb Haemost*, **70**, 99-104.

Michell, R.H. (1975) Inositol phospholipids and cell surface receptor function. *Biochim Biophys Acta*, **415**, 81-47.

Michelson, A.D. and Barnard, M.R. (1990) Plasmin-induced redistribution of platelet glycoprotein Ib. *Blood*, **76**, 2005-2010.

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

Miranti, C.K., Leng, L., Maschberger, P., Brugge, J.S. and Shattil, S.J. (1998) Identification of a novel integrin signaling pathway involving the kinase Syk and the guanine nucleotide exchange factor Vav1. *Curr Biol*, **8**, 1289-1299.

Moake, J.L., Rudy, C.K., Troll, J.H., Weinstein, M.J., Colannino, N.M., Azocar, J., Seder, R.H., Hong, S.L. and Deykin, D. (1982) Unusually large plasma factor VIII:von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Engl J Med*, **307**, 1432-1435.

Moake, J.L., Turner, N.A., Stathopoulos, N.A., Nolasco, L. and Hellums, J.D. (1988) Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. *Blood*, **71**, 1366-1374.

Moerenhout, M., Vereecke, J. and Himpens, B. (2001) Mechanism of intracellular Ca(2+)-wave propagation elicited by mechanical stimulation in cultured endothelial CPAE cells. *Cell Calcium*, **29**, 117-123.

Mohri, H., Fujimura, Y., Shima, M., Yoshioka, A., Houghten, R.A., Ruggeri, Z.M. and Zimmerman, T.S. (1988) Structure of the von Willebrand factor domain interacting with glycoprotein Ib. *J Biol Chem*, **263**, 17901-17904.

Mohri, H., Yoshioka, A., Zimmerman, T.S. and Ruggeri, Z.M. (1989) Isolation of the von Willebrand factor domain interacting with platelet glycoprotein Ib, heparin, and collagen and characterization of its three distinct functional sites. *J Biol Chem*, **264**, 17361-17367.

Montgomery, R.R. and Zimmerman, T.S. (1978) von Willebrand's disease antigen II. A new plasma and platelet antigen deficient in severe von Willebrand's disease. *J Clin Invest*, **61**, 1498-1507.

Moritz, M.W., Reimers, R.C., Baker, R.K., Sutera, S.P. and Joist, J.H. (1983) Role of cytoplasmic and releasable ADP in platelet aggregation induced by laminar shear stress. *J Lab Clin Med*, **101**, 537-544.

Moroi, M., Jung, S.M., Shinmyozu, K., Tomiyama, Y., Ordinas, A. and Diaz-Ricart, M. (1996) Analysis of platelet adhesion to a collagen-coated surface under flow conditions: the involvement of glycoprotein VI in the platelet adhesion. *Blood*, **88**, 2081-2092.

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

Morton, L.F., McCulloch, I.Y. and Barnes, M.J. (1993) Platelet aggregation by a collagen-like synthetic peptide. *Thromb Res*, **72**, 367-372.

Morton, L.F., Peachey, A.R. and Barnes, M.J. (1989) Platelet-reactive sites in collagens type I and type III. Evidence for separate adhesion and aggregatory sites. *Biochem J*, **258**, 157-163.

Mosesson, M.W., Siebenlist, K.R. and Meh, D.A. (2001) The structure and biological features of fibrinogen and fibrin. *Ann N Y Acad Sci*, **936**, 11-30.

Mould, A.P., Akiyama, S.K. and Humphries, M.J. (1996) The inhibitory anti-beta1 integrin monoclonal antibody 13 recognizes an epitope that is attenuated by ligand occupancy. Evidence for allosteric inhibition of integrin function. *J Biol Chem*, **271**, 20365-20374.

Naik, U.P. and Parise, L.V. (1997) Structure and function of platelet alpha IIb beta 3. *Curr Opin Hematol*, **4**, 317-322.

Naik, U.P., Patel, P.M. and Parise, L.V. (1997) Identification of a novel calcium-binding protein that interacts with the integrin alphaIIb cytoplasmic domain. *J Biol Chem*, **272**, 4651-4654.

Nakamura, S. and Yamamura, H. (1989) Thrombin and collagen induce rapid phosphorylation of a common set of cellular proteins on tyrosine in human platelets. *J Biol Chem*, **264**, 7089-7091.

Nakamura, T., Kambayashi, J., Okuma, M. and Tandon, N.N. (1999) Activation of the GP IIb-IIIa complex induced by platelet adhesion to collagen is mediated by both alpha2beta1 integrin and GP VI. *J Biol Chem*, **274**, 11897-11903.

Narumiya, S. (1996) The small GTPase Rho: cellular functions and signal transduction. *J Biochem (Tokyo)*, **120**, 215-228.

Nesbitt, W.S., Kulkarni, S., Giuliano, S., Goncalves, I., Dopheide, S.M., Yap, C.L., Harper, I.S., Salem, H.H. and Jackson, S.P. (2002) Distinct glycoprotein Ib/V/IX and integrin alpha IIbbeta 3-dependent calcium signals cooperatively regulate platelet adhesion under flow. *J Biol Chem*, **277**, 2965-2972.

Newton, A.C. (1997) Regulation of protein kinase C. *Curr Opin Cell Biol*, **9**, 161-167.

Ni, H., Denis, C.V., Subbarao, S., Degen, J.L., Sato, T.N., Hynes, R.O. and Wagner, D.D. (2000) Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *J Clin Invest*, **106**, 385-392.

Ni, H., Ramakrishnan, V., Ruggeri, Z.M., Papalia, J.M., Phillips, D.R. and Wagner, D.D. (2001) Increased thrombogenesis and embolus formation in mice lacking glycoprotein V. *Blood*, **98**, 368-373.

Nieswandt, B., Bergmeier, W., Schulte, V., Rackebrandt, K., Gessner, J.E. and Zirngibl, H. (2000) Expression and function of the mouse collagen receptor glycoprotein VI is strictly dependent on its association with the FcRgamma chain. *J Biol Chem*, **275**, 23998-24002.

Nieswandt, B., Brakebusch, C., Bergmeier, W., Schulte, V., Bouvard, D., Mokhtari-Nejad, R., Lindhout, T., Heemskerk, J.W., Zirngibl, H. and Fassler, R. (2001a) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. *Embo J*, **20**, 2120-2130.

Nieswandt, B., Schulte, V., Bergmeier, W., Mokhtari-Nejad, R., Rackebrandt, K., Cazenave, J.P., Ohlmann, P., Gachet, C. and Zirngibl, H. (2001b) Long-term antithrombotic protection by in vivo depletion of platelet glycoprotein VI in mice. *J Exp Med*, **193**, 459-469.

Nieuwenhuis, H.K., Akkerman, J.W., Houdijk, W.P. and Sixma, J.J. (1985) Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature*, **318**, 470-472.

Nieuwenhuis, H.K., Sakariassen, K.S., Houdijk, W.P., Nievelstein, P.F. and Sixma, J.J. (1986) Deficiency of platelet membrane glycoprotein Ia associated with a decreased platelet adhesion to subendothelium: a defect in platelet spreading. *Blood*, **68**, 692-695.

Nishizuka, Y. (1988a) The heterogeneity and differential expression of multiple species of the protein kinase C family. *Biofactors*, **1**, 17-20.

Nishizuka, Y. (1988b) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature*, **334**, 661-665.

Nunn, D.L. and Watson, S.P. (1987) A diacylglycerol kinase inhibitor, R59022, potentiates secretion by and aggregation of thrombin-stimulated human platelets. *Biochem J*, **243**, 809-813.

Nurden, P., Savi, P., Heilmann, E., Bihour, C., Herbert, J.M., Maffrand, J.P. and Nurden, A. (1995) An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. *J Clin Invest*, **95**, 1612-1622.

Oda, A., Druker, B.J., Ariyoshi, H., Smith, M. and Salzman, E.W. (1993) pp60src is an endogenous substrate for calpain in human blood platelets. *J Biol Chem*, **268**, 12603-12608.

Oda, A., Ikeda, Y., Ochs, H.D., Druker, B.J., Ozaki, K., Handa, M., Ariga, T., Sakiyama, Y., Witte, O.N. and Wahl, M.I. (2000) Rapid tyrosine phosphorylation and activation of Bruton's tyrosine/Tec kinases in platelets induced by collagen binding or CD32 cross-linking. *Blood*, **95**, 1663-1670.

Oda, A., Yokoyama, K., Murata, M., Tokuhira, M., Nakamura, K., Handa, M., Watanabe, K. and Ikeda, Y. (1995) Protein tyrosine phosphorylation in human platelets during shear stress-induced platelet aggregation (SIPA) is regulated by glycoprotein (GP) Ib/IX as well as GP IIb/IIIa and requires intact cytoskeleton and endogenous ADP. *Thromb Haemost*, **74**, 736-742.

Offermanns, S. (2000) The role of heterotrimeric G proteins in platelet activation. *Biol Chem*, **381**, 389-396.

Offermanns, S., Toombs, C.F., Hu, Y.H. and Simon, M.I. (1997) Defective platelet activation in G alpha(q)-deficient mice. *Nature*, **389**, 183-186.

Ohlmann, P., Eckly, A., Freund, M., Cazenave, J.P., Offermanns, S. and Gachet, C. (2000) ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. *Blood*, **96**, 2134-2139.

Ohlmann, P., Laugwitz, K.L., Nurnberg, B., Spicher, K., Schultz, G., Cazenave, J.P. and Gachet, C. (1995) The human platelet ADP receptor activates Gi2 proteins. *Biochem J*, **312 (Pt 3)**, 775-779.

O'Rourke, F., Zavoico, G.B., Smith, L.H., Jr. and Feinstein, M.B. (1987) Stimulus-response coupling in a cell-free platelet membrane system. GTP-dependent release of Ca2+ by thrombin, and inhibition by pertussis toxin and a monoclonal antibody that blocks calcium release by IP3. *FEBS Lett*, **214**, 176-180.

Parise, L.V. (1989) The structure and function of platelet integrins. *Curr Opin Cell Biol*, **1**, 947-952.

Pasquet, J.M., Bobe, R., Gross, B., Gratacap, M.P., Tomlinson, M.G., Payrastre, B. and Watson, S.P. (1999a) A collagen-related peptide regulates phospholipase Cgamma2 via phosphatidylinositol 3-kinase in human platelets. *Biochem J*, **342 (Pt 1)**, 171-177.

Pasquet, J.M., Gross, B., Quek, L., Asazuma, N., Zhang, W., Sommers, C.L., Schweighoffer, E., Tybulewicz, V., Judd, B., Lee, J.R., Koretzky, G., Love, P.E., Samelson, L.E. and Watson, S.P. (1999b) LAT is required for tyrosine phosphorylation of phospholipase cgamma2 and platelet activation by the collagen receptor GPVI. *Mol Cell Biol*, **19**, 8326-8334.

Pasquet, J.M., Quek, L., Pasquet, S., Poole, A., Matthews, J.R., Lowell, C. and Watson, S.P. (2000) Evidence of a role for SHP-1 in platelet activation by the collagen receptor glycoprotein VI. *J Biol Chem*, **275**, 28526-28531.

Paul, B.Z., Jin, J. and Kunapuli, S.P. (1999) Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. *J Biol Chem*, **274**, 29108-29114.

Payrastre, B., Missy, K., Trumel, C., Bodin, S., Plantavid, M. and Chap, H. (2000) The integrin alpha IIb/beta 3 in human platelet signal transduction. *Biochem Pharmacol*, **60**, 1069-1074.

Pearson, T.C. (1997) Hemorheologic considerations in the pathogenesis of vascular occlusive events in polycythemia vera. *Semin Thromb Hemost*, **23**, 433-439.

Peerschke, E.I. (1995) Regulation of platelet aggregation by post-fibrinogen binding events. Insights provided by dithiothreitol-treated platelets. *Thromb Haemost*, **73**, 862-867.

Peterson, D.M., Stathopoulos, N.A., Giorgio, T.D., Hellums, J.D. and Moake, J.L. (1987) Shear-induced platelet aggregation requires von Willebrand factor and platelet membrane glycoproteins Ib and IIb-IIIa. *Blood*, **69**, 625-628.

Phillips, D.R., Charo, I.F. and Scarborough, R.M. (1991) GPIIb-IIIa: the responsive integrin. *Cell*, **65**, 359-362.

Ponting, C.P. and Benjamin, D.R. (1996) A novel family of Ras-binding domains. *Trends Biochem Sci*, **21**, 422-425.

Poole, A., Gibbins, J.M., Turner, M., van Vugt, M.J., van de Winkel, J.G., Saito, T., Tybulewicz, V.L. and Watson, S.P. (1997) The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *Embo J*, **16**, 2333-2341.

Pulcinelli, F.M., Pesciotti, M., Pignatelli, P., Riondino, S. and Gazzaniga, P.P. (1998) Concomitant activation of Gi and Gq protein-coupled receptors does not require an increase in cytosolic calcium for platelet aggregation. *FEBS Lett*, **435**, 115-118.

Qiao, J.H. and Fishbein, M.C. (1991) The severity of coronary atherosclerosis at sites of plaque rupture with occlusive thrombosis. *J Am Coll Cardiol*, **17**, 1138-1142.

Quek, L.S., Bolen, J. and Watson, S.P. (1998) A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen. *Curr Biol*, **8**, 1137-1140.

Quinton, T.M., Kim, S., Dangelmaier, C., Dorsam, R.T., Jin, J., Daniel, J.L. and Kunapuli, S.P. (2002a) Protein kinase C- and calcium-regulated pathways independently synergize with Gi pathways in agonist-induced fibrinogen receptor activation. *Biochem J*, **368**, 535-543.

Quinton, T.M., Ozdener, F., Dangelmaier, C., Daniel, J.L. and Kunapuli, S.P. (2002b) Glycoprotein VI-mediated platelet fibrinogen receptor activation occurs through calcium-sensitive and PKC-sensitive pathways without a requirement for secreted ADP. *Blood*, **99**, 3228-3234.

Randi, A.M., Rabinowitz, I., Mancuso, D.J., Mannucci, P.M. and Sadler, J.E. (1991) Molecular basis of von Willebrand disease type IIB. Candidate mutations cluster in one

disulfide loop between proposed platelet glycoprotein Ib binding sequences. *J Clin Invest*, **87**, 1220-1226.

Rao, G.H. (1993) Physiology of blood platelet activation. *Indian J Physiol Pharmacol*, **37**, 263-275.

Rauterberg, J., Jaeger, E. and Althaus, M. (1993) Collagens in atherosclerotic vessel wall lesions. *Curr Top Pathol*, **87**, 163-192.

Razdan, K., Hellums, J.D. and Kroll, M.H. (1994) Shear-stress-induced von Willebrand factor binding to platelets causes the activation of tyrosine kinase(s). *Biochem J*, **302 (Pt 3)**, 681-686.

Remijn, J.A., Wu, Y.P., Ijsseldijk, M.J., Zwaginga, J.J., Sixma, J.J. and de Groot, P.G. (2001) Absence of fibrinogen in afibrinogenemia results in large but loosely packed thrombi under flow conditions. *Thromb Haemost*, **85**, 736-742.

Remijn, J.A., Wu, Y.P., Jeninga, E.H., MJ, I.J., van Willigen, G., de Groot, P.G., Sixma, J.J., Nurden, A.T. and Nurden, P. (2002) Role of ADP receptor P2Y(12) in platelet adhesion and thrombus formation in flowing blood. *Arterioscler Thromb Vasc Biol*, **22**, 686-691.

Reuther, G.W., Fu, H., Cripe, L.D., Collier, R.J. and Pendergast, A.M. (1994) Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. *Science*, **266**, 129-133.

Rink, T.J. and Sanchez, A. (1984) Effects of prostaglandin I2 and forskolin on the secretion from platelets evoked at basal concentrations of cytoplasmic free calcium by thrombin, collagen, phorbol ester and exogenous diacylglycerol. *Biochem J*, **222**, 833-836.

Rink, T.J., Sanchez, A. and Hallam, T.J. (1983) Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature*, **305**, 317-319.

Rittenhouse, S.E. (1983) Human platelets contain phospholipase C that hydrolyzes polyphosphoinositides. *Proc Natl Acad Sci U S A*, **80**, 5417-5420.

Romanello, M. and D'Andrea, P. (2001) Dual mechanism of intercellular communication in HOBIT osteoblastic cells: a role for gap-junctional hemichannels. *J Bone Miner Res*, **16**, 1465-1476.

Romanello, M., Moro, L., Pirulli, D., Crovella, S. and D'Andrea, P. (2001) Effects of cAMP on intercellular coupling and osteoblast differentiation. *Biochem Biophys Res Commun*, **282**, 1138-1144.

Rosado, J.A., Graves, D. and Sage, S.O. (2000a) Tyrosine kinases activate store-mediated Ca2+ entry in human platelets through the reorganization of the actin cytoskeleton. *Biochem J*, **351 Pt 2**, 429-437.

Rosado, J.A., Jenner, S. and Sage, S.O. (2000b) A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Evidence for conformational coupling. *J Biol Chem*, **275**, 7527-7533.

Rosado, J.A. and Sage, S.O. (2001) Activation of store-mediated calcium entry by secretion-like coupling between the inositol 1,4,5-trisphosphate receptor type II and human transient receptor potential (hTrp1) channels in human platelets. *Biochem J*, **356**, 191-198.

Roskam, J.H., J. Bounameaux, Y. (1961) L'hemostase spontanee. Etude synthetique et analytique. *J physiol*, **53**, 175-183.

Roth, G.J., Titani, K., Hoyer, L.W. and Hickey, M.J. (1986) Localization of binding sites within human von Willebrand factor for monomeric type III collagen. *Biochemistry*, **25**, 8357-8361.

Rotondo, S., Evangelista, V., Manarini, S., de Gaetano, G. and Cerletti, C. (1997) Different requirement of intracellular calcium and protein kinase C for arachidonic acid release and serotonin secretion in cathepsin G-activated platelets. *Thromb Haemost*, **78**, 919-925.

Rouach, N., Glowinski, J. and Giaume, C. (2000) Activity-dependent neuronal control of gap-junctional communication in astrocytes. *J Cell Biol*, **149**, 1513-1526.

Ruggeri, Z., M. (1993a) Mechanisms of shear induced platelet aggregation. *Thomb. Haemost.*, **78**, 611-616.

Ruggeri, Z.M. (1991) The platelet glycoprotein Ib-IX complex. *Prog Hemost Thromb*, **10**, 35-68.

Ruggeri, Z.M. (1993b) von Willebrand factor and fibrinogen. *Curr Opin Cell Biol*, **5**, 898-906.

Ruggeri, Z.M. (1999) Structure and function of von Willebrand factor. *Thromb Haemost*, **82**, 576-584.

Ruggeri, Z.M. (2000) Role of von Willebrand factor in platelet thrombus formation. *Ann Med*, **32 Suppl 1**, 2-9.

Ruggeri, Z.M., De Marco, L., Gatti, L., Bader, R. and Montgomery, R.R. (1983) Platelets have more than one binding site for von Willebrand factor. *J Clin Invest*, **72**, 1-12.

Ruggeri, Z.M., Dent, J.A. and Saldivar, E. (1999) Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood*, **94**, 172-178.

Ruggeri, Z.M., Pareti, F.I., Mannucci, P.M., Ciavarella, N. and Zimmerman, T.S. (1980) Heightened interaction between platelets and factor VIII/von Willebrand factor in a new subtype of von Willebrand's disease. *N Engl J Med*, **302**, 1047-1051.

Ruggeri, Z.M. and Ware, J. (1993) von Willebrand factor. *Faseb J*, **7**, 308-316.

Ruggeri, Z.M. and Zimmerman, T.S. (1987) von Willebrand factor and von Willebrand disease. *Blood*, **70**, 895-904.

Ruoslahti, E. (1991) Integrins. *J Clin Invest*, **87**, 1-5.

Ruoslahti, E. and Pierschbacher, M.D. (1986) Arg-Gly-Asp: a versatile cell recognition signal. *Cell*, **44**, 517-518.

Saci A, R.F., Bachelot-Loza C. (2000) Platelet alpha IIb-beta 3 integrin engagement induces tyrosine kinase phosphorylation of Cbl and its association with phosphoinositide 3-kinase and Syk. *Biochem J*, **351**, 669-676.

Sage, S.O., Pintado, E., Mahaut-Smith, M.P. and Merritt, J.E. (1990) Rapid kinetics of agonist-evoked changes in cytosolic free Ca2+ concentration in fura-2-loaded human neutrophils. *Biochem J*, **265**, 915-918.

Sage, S.O., Sargeant, P., Heemskerk, J.W. and Mahaut-Smith, M.P. (1993) Calcium influx mechanisms and signal organisation in human platelets. *Adv Exp Med Biol*, **344**, 69-82.

Saitoh, M., Salzman, E.W., Smith, M. and Ware, J.A. (1989) Activation of protein kinase C in platelets by epinephrine and A23187: correlation with fibrinogen binding. *Blood*, **74**, 2001-2006.

Salari, H., Duronio, V., Howard, S., Demos, M. and Pelech, S.L. (1990) Translocation-independent activation of protein kinase C by platelet-activating factor, thrombin and prostacyclin. Lack of correlation with polyphosphoinositide hydrolysis in rabbit platelets. *Biochem J*, **267**, 689-696.

Santoro, S.A., Walsh, J.J., Staatz, W.D. and Baranski, K.J. (1991) Distinct determinants on collagen support alpha 2 beta 1 integrin-mediated platelet adhesion and platelet activation. *Cell Regul*, **2**, 905-913.

Saunders, M.M., You, J., Trosko, J.E., Yamasaki, H., Li, Z., Donahue, H.J. and Jacobs, C.R. (2001) Gap junctions and fluid flow response in MC3T3-E1 cells. *Am J Physiol Cell Physiol*, **281**, C1917-1925.

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

Savage, B., Saldivar, E. and Ruggeri, Z.M. (1996) Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*, **84**, 289-297.

Savage, B., Shattil, S.J. and Ruggeri, Z.M. (1992) Modulation of platelet function through adhesion receptors. A dual role for glycoprotein IIb-IIIa (integrin alpha IIb beta 3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. *J Biol Chem*, **267**, 11300-11306.

Savi, P., Beauverger, P., Labouret, C., Delfaud, M., Salel, V., Kaghad, M. and Herbert, J.M. (1998) Role of P2Y1 purinoceptor in ADP-induced platelet activation. *FEBS Lett*, **422**, 291-295.

Scemes, E. (2000) Components of astrocytic intercellular calcium signaling. *Mol Neurobiol*, **22**, 167-179.

Scemes, E., Suadicani, S.O. and Spray, D.C. (2000) Intercellular communication in spinal cord astrocytes: fine tuning between gap junctions and P2 nucleotide receptors in calcium wave propagation. *J Neurosci*, **20**, 1435-1445.

Schror, K. (1991) [Endothelial factors and thrombocyte function]. *Z Kardiol*, **80 Suppl 5**, 3-6.

Schror, K. (1995) Antiplatelet drugs. A comparative review. *Drugs*, **50**, 7-28.

Schror, K. (1998) Clinical pharmacology of the adenosine diphosphate (ADP) receptor antagonist, clopidogrel. *Vasc Med*, **3**, 247-251.

Scott, J.P., Montgomery, R.R. and Retzinger, G.S. (1991) Dimeric ristocetin flocculates proteins, binds to platelets, and mediates von Willebrand factor-dependent agglutination of platelets. *J Biol Chem*, **266**, 8149-8155.

Selbie, L.A., Schmitz-Peiffer, C., Sheng, Y. and Biden, T.J. (1993) Molecular cloning and characterization of PKC iota, an atypical isoform of protein kinase C derived from insulin-secreting cells. *J Biol Chem*, **268**, 24296-24302.

Shapiro, A.L., Vinuela, E., and Maizel, J.P. (1976) Molecular weight estimation of polypeptide chains by electrophosesis in SDS-polyacrylamaide gels. *Biochem Biophys Res Commun*, **28**, 815-820.

Shattil, S.J. (1995) Function and regulation of the beta 3 integrins in hemostasis and vascular biology. *Thromb Haemost*, **74**, 149-155.

Shattil, S.J. (1999) Signaling through platelet integrin alpha IIb beta 3: inside-out, outside-in, and sideways. *Thromb Haemost*, **82**, 318-325.

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

Shattil, S.J. and Brugge, J.S. (1991) Protein tyrosine phosphorylation and the adhesive functions of platelets. *Curr Opin Cell Biol*, **3**, 869-879.

Shattil, S.J., Gao, J. and Kashiwagi, H. (1997) Not just another pretty face: regulation of platelet function at the cytoplasmic face of integrin alpha IIb beta 3. *Thromb Haemost*, **78**, 220-225.

Shattil, S.J., Haimovich, B., Cunningham, M., Lipfert, L., Parsons, J.T., Ginsberg, M.H. and Brugge, J.S. (1994) Tyrosine phosphorylation of pp125FAK in platelets requires coordinated signaling through integrin and agonist receptors. *J Biol Chem*, **269**, 14738-14745.

Shattil, S.J., Kashiwagi, H. and Pampori, N. (1998) Integrin signaling: the platelet paradigm. *Blood*, **91**, 2645-2657.

Shattil, S.J., O'Toole, T., Eigenthaler, M., Thon, V., Williams, M., Babior, B.M. and Ginsberg, M.H. (1995) Beta 3-endonexin, a novel polypeptide that interacts specifically with the cytoplasmic tail of the integrin beta 3 subunit. *J Cell Biol*, **131**, 807-816.

Shen. Y., Romo, G.M., Dong, J.F., Schade, A., McIntire, L.V., Kenny, D., Whisstock, J.C., Berndt, M.C., Lopez, J.A. and Andrews, R.K. (2000) Requirement of leucine-rich repeats of glycoprotein (GP) Ibalpha for shear-dependent and static binding of von Willebrand factor to the platelet membrane GP Ib-IX-V complex. *Blood*, **95**, 903-910.

Shiga, H., Tojima, T. and Ito, E. (2001) Ca2+ signaling regulated by an ATP-dependent autocrine mechanism in astrocytes. *Neuroreport*, **12**, 2619-2622.

Siess, W. and Lapetina, E.G. (1989a) Platelet aggregation induced by alpha 2-adrenoceptor and protein kinase C activation. A novel synergism. *Biochem J*, **263**, 377-385.

Siess, W. and Lapetina, E.G. (1989b) Prostacyclin inhibits platelet aggregation induced by phorbol ester or Ca2+ ionophore at steps distal to activation of protein kinase C and Ca2+-dependent protein kinases. *Biochem J*, **258**, 57-65.

Sims, P.J., Ginsberg, M.H., Plow, E.F. and Shattil, S.J. (1991) Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. *J Biol Chem*, **266**, 7345-7352.

Sixma, J.J., Hindriks, G., Van Breugel, H., Hantgan, R. and de Groot, P.G. (1991) Vessel wall proteins adhesive for platelets. *J Biomater Sci Polym Ed*, **3**, 17-26.

Sixma, J.J. and Molenaar, I. (1966) Microtubules and microfibrils in human platelets. *Thromb Diath Haemorrh*, **16**, 153-162.

Slater, S.J., Milano, S.K., Stagliano, B.A., Gergich, K.J., Curry, J.P., Taddeo, F.J. and Stubbs, C.D. (2000) Interaction of protein kinase C with filamentous actin: isozyme specificity resulting from divergent phorbol ester and calcium dependencies. *Biochemistry*, **39**, 271-280.

Sloan, D.C. and Haslam, R.J. (1997) Protein kinase C-dependent and Ca2+-dependent mechanisms of secretion from streptolysin O-permeabilized platelets: effects of leakage of cytosolic proteins. *Biochem J*, **328 (Pt 1)**, 13-21.

Springer, T.A. (1990) The sensation and regulation of interactions with the extracellular environment: the cell biology of lymphocyte adhesion receptors. *Annu Rev Cell Biol*, **6**, 359-402.

Stahl, K., Themann, H. and Dame, W.R. (1978) Ultrastructural morphometric investigations on normal human platelets. *Haemostasis*, **7**, 242-251.

Sugimoto, M., Mohri, H., McClintock, R.A. and Ruggeri, Z.M. (1991a) Identification of discontinuous von Willebrand factor sequences involved in complex formation with botrocetin. A model for the regulation of von Willebrand factor binding to platelet glycoprotein Ib. *J Biol Chem*, **266**, 18172-18178.

Sugimoto, M., Ricca, G., Hrinda, M.E., Schreiber, A.B., Scarfoss, G.H., Bottini, E. and Ruggeri, Z.M. (1991b) Functional modulation of the isolated glycoprotein Ib binding domain of von Willebrand factor expressed in Escherichia coli. *Biochemistry*, **30**, 5202-5209.

Sullam, P.M., Hyun, W.C., Szollosi, J., Dong, J., Foss, W.M. and Lopez, J.A. (1998) Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor FcgammaRIIA on the platelet plasma membrane. *J Biol Chem*, **273**, 5331-5336.

Sun, B., Li, J. and Kambayashi, J. (1999) Interaction between GPIbalpha and FcgammaIIA receptor in human platelets. *Biochem Biophys Res Commun*, **266**, 24-27.

Tangelder, G.J., oude Egbrink, M.G., Slaaf, D.W. and Reneman, R.S. (1989) Blood platelets: an overview. *J Reconstr Microsurg*, **5**, 167-171.

Tanji, M., Horwitz, R., Rosenfeld, G. and Waymire, J.C. (1994) Activation of protein kinase C by purified bovine brain 14-3-3: comparison with tyrosine hydroxylase activation. *J Neurochem*, **63**, 1908-1916.

Tavassoli, M. and Aoki, M. (1981) Migration of entire megakaryocytes through the marrow--blood barrier. *Br J Haematol*, **48**, 25-29.

Titani, K., Takio, K., Handa, M. and Ruggeri, Z.M. (1987) Amino acid sequence of the von Willebrand factor-binding domain of platelet membrane glycoprotein Ib. *Proc Natl Acad Sci U S A*, **84**, 5610-5614.

Toker, A. (1998) Signaling through protein kinase C. *Front Biosci*, **3**, D1134-1147.

Toker, A., Ellis, C.A., Sellers, L.A. and Aitken, A. (1990) Protein kinase C inhibitor proteins. Purification from sheep brain and sequence similarity to lipocortins and 14-3-3 protein. *Eur J Biochem*, **191**, 421-429.

Tonon, R. and D'Andrea, P. (2000) Interleukin-1beta increases the functional expression of connexin 43 in articular chondrocytes: evidence for a Ca2+-dependent mechanism. *J Bone Miner Res*, **15**, 1669-1677.

Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F. and et al. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem*, **266**, 15771-15781.

Tovey, S.C., de Smet, P., Lipp, P., Thomas, D., Young, K.W., Missiaen, L., De Smedt, H., Parys, J.B., Berridge, M.J., Thuring, J., Holmes, A. and Bootman, M.D. (2001) Calcium puffs are generic InsP(3)-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses. *J Cell Sci*, **114**, 3979-3989.

Tsai, H.M. (1996) Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood*, **87**, 4235-4244.

Tschopp, T.B. and Baumgartner, H.R. (1977) Defective platelet adhesion and aggregation on subendothelium exposed in vivo or in vitro to flowing blood of fawn-hooded rats and storage pool disease. *Thromb Haemost*, **38**, 620-629.

Tschopp, T.B., Weiss, H.J. and Baumgartner, H.R. (1974) Decreased adhesion of platelets to subendothelium in von Willebrand's disease. *J Lab Clin Med*, **83**, 296-300.

Tsuji, M., Ezumi, Y., Arai, M. and Takayama, H. (1997) A novel association of Fc receptor gamma-chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J Biol Chem*, **272**, 23528-23531.

Tsuji, S., Sugimoto, M., Miyata, S., Kuwahara, M., Kinoshita, S. and Yoshioka, A. (1999) Real-time analysis of mural thrombus formation in various platelet aggregation disorders: distinct shear-dependent roles of platelet receptors and adhesive proteins under flow. *Blood*, **94**, 968-975.

Turitto, V.T. and Baumgartner, H.R. (1974) Effect of temperature on platelet interaction with subendothelium exposed to flowing blood. *Haemostasis*, **3**, 224-236.

Turitto, V.T. and Baumgartner, H.R. (1975) Platelet interaction with subendothelium in a perfusion system: physical role of red blood cells. *Microvasc Res*, **9**, 335-344.

Turitto, V.T.a.B., H.R. (1974) Effect of physical factors on platelet adherence to subendothelium. *Thrombo Diath haemorrh suppl*, **60**, 17-24.

Turner, N.A., Moake, J.L. and McIntire, L.V. (2001) Blockade of adenosine diphosphate receptors P2Y(12) and P2Y(1) is required to inhibit platelet aggregation in whole blood under flow. *Blood*, **98**, 3340-3345.

Ugarova, T.P., Budzynski, A.Z., Shattil, S.J., Ruggeri, Z.M., Ginsberg, M.H. and Plow, E.F. (1993) Conformational changes in fibrinogen elicited by its interaction with platelet membrane glycoprotein GPIIb-IIIa. *J Biol Chem*, **268**, 21080-21087.

van Zanten, G.H., de Graaf, S., Slootweg, P.J., Heijnen, H.F., Connolly, T.M., de Groot, P.G. and Sixma, J.J. (1994) Increased platelet deposition on atherosclerotic coronary arteries. *J Clin Invest*, **93**, 615-632.

Vaughan, P.F., Walker, J.H. and Peers, C. (1998) The regulation of neurotransmitter secretion by protein kinase C. *Mol Neurobiol*, **18**, 125-155.

Verderio, C. and Matteoli, M. (2001) ATP mediates calcium signaling between astrocytes and microglial cells: modulation by IFN-gamma. *J Immunol*, **166**, 6383-6391.

Verheugt, F.W. and Brugada, P. (1991) Sudden death after acute myocardial infarction: the forgotten thrombotic view. *Am J Cardiol*, **67**, 1130-1134.

Verkleij, M.W., Morton, L.F., Knight, C.G., de Groot, P.G Barnes, M.J. and Sixma, J.J. (1998) Simple collagen-like peptides support platelet adhesion under static but not under flow conditions: interaction via alpha2 beta1 and von Willebrand factor with specific sequences in native collagen is a requirement to resist shear forces. *Blood*, **91**, 3808-3816.

Virchow, R. (1856) Gesammelte Abhandlungen zur wissenschaftlichen medizin. *Frankfurt am Main. Meidinger U Comp.*

von Andrian, U.H., Chambers, J.D., McEvoy, L.M., Bargatze, R.F., Arfors, K.E. and Butcher, E.C. (1991) Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins in vivo. *Proc Natl Acad Sci U S A*, **88**, 7538-7542.

Vuori, K. and Ruoslahti, E. (1993) Activation of protein kinase C precedes alpha 5 beta 1 integrin-mediated cell spreading on fibronectin. *J Biol Chem*, **268**, 21459-21462.

Wagner, R.W.H., J.A. (1992) ADP receptor antagonists and converting enzyme systems reduce platelet deposition onto collagen. *Thomb. Haemost.*, **67**, 461-467.

Wagner, W.R. and Hubbell, J.A. (1992a) ADP receptor antagonists and converting enzyme systems reduce platelet deposition onto collagen. *Thromb Haemost*, **67**, 461-467.

Wagner, W.R. and Hubbell, J.A. (1992b) Evidence for a role in thrombus stabilization for thromboxane A2 in human platelet deposition on collagen. *J Lab Clin Med*, **119**, 690-697.

Walker, J.M., Homan, E.C. and Sando, J.J. (1990) Differential activation of protein kinase C isozymes by short chain phosphatidylserines and phosphatidylcholines. *J Biol Chem*, **265**, 8016-8021.

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

Wang, F., Naik, U.P., Ehrlich, Y.H., Freyberg, Z., Osada, S., Ohno, S., Kuroki, T., Suzuki, K. and Kornecki, E. (1993) A new protein kinase C, nPKC eta', and nPKC theta are expressed in human platelets: involvement of nPKC eta' and nPKC theta in signal transduction stimulated by PAF *Biochem Biophys Res Commun*, **191**, 240-246.

Wang, F., Naik, U.P., Ehrlich, Y.H., Osada, S., Ohno, S. and Kornecki, E. (1995) Stimulatory antibody-induced activation and selective translocation of protein kinase C isoenzymes in human platelets. *Biochem J*, **311 (Pt 2)**, 401-406.

Wang, R., Peterson, J., Aster, R.H. and Newman, P.J. (1997) Disruption of a long-range disulfide bond between residues Cys406 and Cys655 in glycoprotein IIIa does not affect the function of platelet glycoprotein IIb-IIIa. *Blood*, **90**, 1718-1719.

Watanabe, Y., Ito, M., Kataoka, Y., Wada, H., Koyama, M., Feng, J., Shiku, H. and Nishikawa, M. (2001) Protein kinase C-catalyzed phosphorylation of an inhibitory phosphoprotein of myosin phosphatase is involved in human platelet secretion. *Blood*, **97**, 3798-3805.

Watson, S.P., Asazuma, N., Atkinson, B., Berlanga, O., Best, D., Bobe, R., Jarvis, G., Marshall, S., Snell, D., Stafford, M., Tulasne, D., Wilde, J., Wonerow, P. and Frampton, J. (2001) The role of ITAM- and ITIM-coupled receptors in platelet activation by collagen. *Thromb Haemost*, **86**, 276-288.

Watson, S.P. and Hambleton, S. (1989) Phosphorylation-dependent and -independent pathways of platelet aggregation. *Biochem J*, **258**, 479-485.

Watson, S.P., McNally, J., Shipman, L.J. and Godfrey, P.P. (1988) The action of the protein kinase C inhibitor, staurosporine, on human platelets. Evidence against a regulatory role for protein kinase C in the formation of inositol trisphosphate by thrombin. *Biochem J*, **249**, 345-350.

Weiss, H.J. (1975a) Abnormalities of factor VIII and platelet aggregation--use of ristocetin in diagnosing the von Willebrand syndrome. *Blood*, **45**, 403-412.

Weiss, H.J. (1975b) Platelet physiology and abnormalities of platelet function (first of two parts). *N Engl J Med*, **293**, 531-541.

Weiss, H.J. (1995) Flow-related platelet deposition on subendothelium. *Thromb Haemost*, **74**, 117-122.

Weiss, H.J., Hawiger, J., Ruggeri, Z.M., Turitto, V.T., Thiagarajan, P. and Hoffmann, T. (1989) Fibrinogen-independent platelet adhesion and thrombus formation on subendothelium mediated by glycoprotein IIb-IIIa complex at high shear rate. *J Clin Invest*, **83**, 288-297.

Weiss, H.J., Hoffmann, T., Yoshioka, A. and Ruggeri, Z.M. (1993) Evidence that the arg1744 gly1745 asp1746 sequence in the GPIIb-IIIa-binding domain of von Willebrand factor is involved in platelet adhesion and thrombus formation on subendothelium. *J Lab Clin Med*, **122**, 324-332.

Weiss, H.J., Tschopp, T.B. and Baumgartner, H.R. (1975) Impaired interaction (adhesion-aggregation) of platelets with the subendothelium in storage-pool disease and after aspirin ingestion. A comparison with von Willebrand's disease. *N Engl J Med*, **293**, 619-623.

Weiss, H.J., Tschopp, T.B., Baumgartner, H.R., Sussman, II, Johnson, M.M. and Egan, J.J. (1974) Decreased adhesion of giant (Bernard-Soulier) platelets to subendothelium. Further implications on the role of the von Willebrand factor in hemostasis. *Am J Med*, **57**, 920-925.

Weiss, H.J., Turitto, V.T. and Baumgartner, H.R. (1978) Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. I. Shear rate--dependent decrease of adhesion in von Willebrand's disease and the Bernard-Soulier syndrome. *J Lab Clin Med*, **92**, 750-764.

Werner, G. and Morgenstern, E. (1980) Three-dimensional reconstruction of human blood platelets using serial sections. *Eur J Cell Biol*, **20**, 276-282.

White, J.G. (1981) Morphological studies of platelets and platelet reactions. *Vox Sang*, **40 Suppl 1**, 8-17.

White, J.G. (1987) An overview of platelet structural physiology. *Scanning Microsc*, **1**, 1677-1700.

White, J.G. and Clawson, C.C. (1980) The surface-connected canalicular system of blood platelets--a fenestrated membrane system. *Am J Pathol*, **101**, 353-364.

White, J.G. and Gerrard, J.M. (1976) Ultrastructural features of abnormal blood platelets. A review. *Am J Pathol*, **83**, 589-632.

White, J.G. and Gerrard, J.M. (1982) Anatomy and Structural Organization of the Platelet. In Colman, R.W., Hirsh, J., Marder, V.J. and Salzman, E.W. (eds.), *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. J.B Lippincott Company, Philadelphia.

Wicki, A.N. and Clemetson, K.J. (1985) Structure and function of platelet membrane glycoproteins Ib and V. Effects of leukocyte elastase and other proteases on platelets response to von Willebrand factor and thrombin. *Eur J Biochem*, **153**, 1-11.

Williams, A.G., Woolkalis, M.J., Poncz, M., Manning, D.R., Gewirtz, A.M. and Brass, L.F. (1990) Identification of the pertussis toxin-sensitive G proteins in platelets, megakaryocytes, and human erythroleukemia cells. *Blood*, **76**, 721-730.

Williamson, D., Pikovski, I., Cranmer, S.L., Mangin, P., Mistry, N., Domagala, T., Chehab, S., Lanza, F., Salem, H.H. and Jackson, S.P. (2002) Interaction between platelet glycoprotein Ibalpha and filamin-1 is essential for glycoprotein Ib/IX receptor anchorage at high shear. *J Biol Chem*, **277**, 2151-2159.

Yanabu, M., Ozaki, Y., Nomura, S., Miyake, T., Miyazaki, Y., Kagawa, H., Yamanaka, Y., Asazuma, N., Satoh, K., Kume, S., Komiyama, Y. and Fukuhara, S. (1997) Tyrosine phosphorylation and p72syk activation by an anti-glycoprotein Ib monoclonal antibody. *Blood*, **89**, 1590-1598.

Yanaga, F., Poole, A., Asselin, J., Blake, R., Schieven, G.L., Clark, E.A., Law, C.L. and Watson, S.P. (1995) Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fc gamma-IIA receptor. *Biochem J*, **311 (Pt 2)**, 471-478.

Yang, J., Wu, J., Kowalska, M.A., Dalvi, A., Prevost, N., O'Brien, P.J., Manning, D., Poncz, M., Lucki, I., Blendy, J.A. and Brass, L.F. (2000) Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs *Proc Natl Acad Sci U S A*, **97**, 9984-9989.

Yap, C.L., Hughan, S.C., Cranmer, S.L., Nesbitt, W.S., Rooney, M.M., Giuliano, S., Kulkarni, S., Dopheide, S.M., Yuan, Y., Salem, H.H. and Jackson, S.P. (2000) Synergistic adhesive interactions and signaling mechanisms operating between platelet glycoprotein Ib/IX and integrin alpha IIbbeta 3. Studies in human platelets ans transfected Chinese hamster ovary cells. *J Biol Chem*, **275**, 41377-41388.

Yoshida, K., Stark, F. and Nachmias, V.T. (1988) Comparison of the effects of phorbol 12-myristate 13-acetate and prostaglandin E1 on calcium regulation in human platelets. *Biochem J*, **249**, 487-493.

Yuan, Y., Dopheide, S.M., Ivanidis, C., Salem, H.H. and Jackson, S.P. (1997) Calpain regulation of cytoskeletal signaling complexes in von Willebrand factor-stimulated platelets. Distinct roles for glycoprotein Ib-V-IX and glycoprotein IIb-IIIa (integrin alphaIIbbeta3) in von Willebrand factor-induced signal transduction. *J Biol Chem*, **272**, 21847-21854.

Yuan, Y., Kulkarni, S., Ulsemer, P., Cranmer, S.L., Yap, C.L., Nesbitt, W.S., Harper, I., Mistry, N., Dopheide, S.M., Hughan, S.C., Williamson, D., de la Salle, C., Salem, H.H., Lanza, F. and Jackson, S.P. (1999) The von Willebrand factor-glycoprotein Ib/V/IX interaction induces actin polymerization and cytoskeletal reorganization in rolling platelets and glycoprotein Ib/V/IX-transfected cells. *J Biol Chem*, **274**, 36241-36251.

Yurchenco, P.D. (1990) Assembly of basement membranes. *Ann N Y Acad Sci*, **580**, 195-213.

Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. *Faseb J*, **4**, 1577-1590.

Zaffran, Y., Meyer, S.C., Negrescu, E., Reddy, K.B. and Fox, J.E. (2000) Signaling across the platelet adhesion receptor glycoprotein Ib-IX induces alpha IIbbeta 3 activation both in platelets and a transfected Chinese hamster ovary cell system. *J Biol Chem*, **275**, 16779-16787.

Zawilska, K.M., Born, G.V. and Begent, N.A. (1982) Effect of ADP-utilizing enzymes on the arterial bleeding time in rats and rabbits. *Br J Haematol*, **50**, 317-325.

Zhang, C., Qiu, H.E., Krafft, G.A. and Klein, W.L. (1996) Protein kinase C and F-actin are essential for stimulation of neuronal FAK tyrosine phosphorylation by G-proteins and amyloid beta protein. *FEBS Lett*, **386**, 185-188.

Zheng, X., Chung, D., Takayama, T.K., Majerus, E.M., Sadler, J.E. and Fujikawa, K. (2001) Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *J Biol Chem*, **276**, 41059-41063.

Zheng, X., Majerus, E.M. and Sadler, J.E. (2002) ADAMTS13 and TTP. *Curr Opin Hematol*, **9**, 389-394.

Zucker-Franklin, D. (1996) Megakaryocyte and platelet structure in thrombocytopoiesis: the effect of cytokines. *Stem Cells*, **14 Suppl 1**, 1-17.

PUBLICATIONS

Publications.

Abstracts.

Giuliano, S., Nesbitt, W.S., Rooney, M.M., and Jackson S.P. (2000) Calcium and PKC requirement for integrin $\alpha_{IIB}\beta_3$ activation. The Australian Vascular Biology Society, 6th Annual Scientific Meeting, Adelaide, Australia.

Giuliano, S., Nesbitt, W.S., Rooney, M.M., and Jackson S.P. (2001) Calcium and PKC requirement for integrin $\alpha_{IIB}\beta_3$ activation. The XVIIth Congress for the International Society of Thrombosis and Haemostasis, Paris, France.

Nesbitt, W.S., **Giuliano, S.**, Kulkarni, S., Dopheidi, S.M., Harper, I.S., and Jackson, S.P. (2001) The integrin $\alpha_{IIb}\beta_3$-calcium axis drives thrombus growth. The XVIIth Congress for the International Society of Thrombosis and Haemostasis, Paris, France.

Kulkarni, S., Nesbitt, W.S., **Giuliano, S.**, and Jackson, S.P. (2002) Inter-platelet calcium communication drives platelet aggregate formation in a shear field. Gordon Research Conference on Haemostasis, Colby College, Maine, USA.

Publications.

Giuliano, S*., Nesbitt, W.S., Kulkarni, S., Dopheide, S.M., Harper, I. Jackson, S.P. (2003) Intercellular Calcium Communication regulates platelet aggregation and thrombus growth. *J. Cell. Biol,* **In Press.**

*** Denotes equal first authors.**

Giuliano, S., Nesbitt, W.S., Rooney, M. and Jackson, S.P. (2003) Bi-directional integrin $\alpha_{IIb}\beta_3$ signalling regulating platelet adhesion under flow: Contribution of protein kinase C. *Biochem J*, **In Press**.

Nesbitt, W.S., Kulkarni, S., **Giuliano, S.**, Goncalves, I., Dopheide, S.M., Yap, C.L., Harper, I.S., Salem, H.H. and Jackson, S.P. (2002) Distinct glycoprotein Ib/V/IX and integrin $\alpha_{IIb}\beta_3$-dependent calcium signals cooperatively regulate platelet adhesion under flow. *J Biol Chem*, **277**, 2965-2972.

Yap, C.L., Hughan, S.C., Cranmer, S.L., Nesbitt, W.S., Rooney, M.M., **Giuliano, S.**, Kulkarni, S., Dopheide, S.M., Yuan, Y., Salem, H.H. and Jackson, S.P. (2000) Synergistic adhesive interactions and signalling mechanisms operating between platelet glycoprotein Ib/IX and integrin $\alpha_{IIb}\beta_3$. Studies in human platelets and transfected Chinese hamster ovary cells. *J Biol Chem*, **275**, 41377-41388.

Mistry, N., Cranmer, S.L., Yuan, Y., Mangin, P., Dopheide, S.M., Harper, I., **Giuliano, S.**, Dunstan, D.E., Lanza, F., Salem, H.H. and Jackson, S.P. (2000) Cytoskeletal regulation of the platelet glycoprotein Ib/V/IX-von willebrand factor interaction. *Blood*, **96**, 3480-3489.

Williamson, D., **Giuliano, S.** and Jackson, S.P. (1999) Platelet adhesion receptors: novel targets for anti-thrombotic therapy. *Aust N Z J Med*, **29**, 452-461.