SECOND MESSENGER SYSTEMS IN COLLAGEN-INDUCED PLATELET ACTIVATION

By

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A Thesis Submitted to the Faculty of Graduate Studies
In Partial Fulfilment of the Requirements
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ABSTRACT

It is well established that release of arachidonic acid is a key component in platelet activation in response to low concentrations (1-20 μg/ml) of collagen, since cyclooxygenase inhibition eliminates aggregation and inhibits a number of signalling processes. This thesis used inhibitors of phospholipase C (PLC), protein kinase C (PKC) and cytosolic phospholipase A_2 ($cPLA_2$) to evaluate the role of signalling pathways in low dose collagen-induced platelet activation. The specificity and limitations of the $cPLA_2$ ($AACOCF_3$) and PKC (Rö31-8220) inhibitors have previously been established. The initial step in this thesis was to establish the limitation of U73122 specificity as a selective PLC inhibitor. Platelets were stimulated with collagen (via PLCγ), the stable thromboxane mimetic U46619 (via PLC-β), or phorbol myristate acetate (PMA) via PKC. Consistent with an inhibition of PLC, U73122 inhibited platelet aggregation and [^{3}H]-serotonin release in response to collagen and U46619 in a concentration-dependent manner. U73122 had no effect on PMA-induced [^{3}H]-serotonin release, or intracellular vacuole area, but the average number of vacuoles was significantly increased. However, U73122 did inhibit PMA-induced platelet aggregation and fibrinogen binding. Overall, these results suggest that U73122, in addition to its inhibition of PLC, also affects PLC-independent events that interfere with platelet aggregation, likely downstream of arachidonic acid release.

The effects of U73122, Rö-31-8220 and $AACOCF_3$ were evaluated on collagen (5μg/ml) induced arachidonic acid release in platelets preincubation with the dual cyclooxygenase/lipoxygenase inhibitor BW755C. U73122 blocked collagen-induced aggregation and reduced arachidonic acid release. $AACOCF_3$ blocked collagen-induced aggregation, but had no significant effect on arachidonic acid release. In contrast, pre-treatment with Rö31-8220 inhibited collagen-induced aggregation, but significantly potentiated arachidonic acid release. These results suggest that $cPLA_2$ does not play a role in the arachidonic acid release in response to low dose collagen. The data are consistent with PLCγ2 playing a role in an intricately controlled pathway, or multiple pathways, mediating the release of arachidonic acid in collagen-stimulated platelets.

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ABREVIATIONS

Abbreviation	Definition
5LO	5-lipoxygenase
$\alpha_5\alpha_1$	fibronectin receptor
$AACOCF_3$	Arachidonyl trifluoromethyl ketone
ACD	Acid, citrate, dextrose anticoagulant
ADP	Adenosine diphosphate
ADT	Adenosine triphosphate
ARF	ADP-ribosylation factors
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phosphoilpase A_2
CRP	Collagen-related peptides
CRP-XL	Cross-linked collagen-related peptides
DG	diacylglycerol
DTS	Dense tubular system
ERK	Extracellular signal regulating kinase
FAK	focal adhesion kinase
GAP	GTPase-activating proteins
GP	glycoprotein
GDP	guanosine diphosphate
GDT	guanosine triphosphate
G-protein	GTP-binding protein
HPLC	High pressure liquid chromatography
IP_3	inositol trisphosphate
ITAM	imunoreceptor tyrosine-based activation motif
JNK	c-jun N-terminal kinases
lyso-PAF	alkyl-lysophosphatidylcholine
MAP	mitogen-activated protein
MAPK	MAP kinase
MARKS	myristoylated alanine-rich C kinase substrate
MLCK	Ca^{2+} /calmodulin-dependent myosin light chain kinase

Abbreviation	Definition
PA	Phosphatidic acid
PAF	platelet activating factor
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PH	pleckstrin homology
PI	Phosphatidylinositol
PIP_3	phosphatidylinositol 3,4,5-trisphosphate,
$PIP_{2,}$	phosphatidylinositol 4,5 bisphosphate,
PIP	phosphatidylinositol 4-monophosphate,
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	phospholipase D
PMA	phorbol-12 myristate-13-acetate
PS	Phosphatidylserine
PTK	Protein tyrosine kinase
SERCA	smooth endoplasmic reticulum Ca^{2+} ATPase
SMCE	store-mediated Ca^{2+} entry
SH	src homology
Sos	son of sevenless
SAPK	stress activated protein kinases
TBHQ	2,5-di(tert-butyl)hydroquinone
Trp	Transient receptor potential
Tx A_2	thromboxane A_2
GFOGER	triple helical peptide
UV	ultraviolet
vWF	von Willebrand Factor

1 INTRODUCTION

1.1 Platelet Morphology

Platelets are the smallest cells in circulating blood with a diameter of 2-3 μm. Derived from precursor megakaryocytes in bone marrow, platelets are anucleate cells with no proliferative abilities, and a life span of 10-12 days. Platelets have a number of unique features to their morphology: a biconvex discoid shape, with a plasma membrane underlain by a cytoskeleton; a dense tubular system (DTS) instead of endoplasmic reticulum; a surface-connected canalicular system; and several types of storage granules. Energy requirements are principally derived from oxidative phosphorylation in mitochondria and glycolysis in the cytosol (McNicol and Gerrard, 1997).

The plasma membrane is an asymmetric phospholipid bilayer with an inner leaflet enriched in phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). The outler leaflet is enriched in sphingomyelin, and phosphatidylcholine (PC) is evenly distributed between both leaflets. The membrane is imbedded with proteins and glycoproteins that serve as receptors, ion channels and other functions (McNicol and Gerrard, 1997).

The cytoskeleton of unstimulated platelets consists of a membrane skeleton, a cytoplasmic skeleton and a peripheral microtubule coil that are linked together. The membrane skeleton coats the plasma membrane, supporting it and regulating membrane properties such as its contours and stabilises (Fox, 1985; Fox, 1993 a & b; Pavalko and Otey, 1994). The cytoplasmic skeleton is a network of cytoplasmic actin filaments that fill the cytoplasm and mediate contractile events. The circumferential band of microtubules is composed primarily of polymerized tubulin bound in a coil around the periphery of the platelet, giving the resting platelet its discoid shape (Fox, 1993 a & b; McNicol and Gerrard, 1997).

Actin, and its polymerization, is supremely important to both structure and signal trafficking within the platelet. Changes in platelet shape, translocation of cytosolic components to the plasma membrane, exocytosis of granule contents and even clustering of receptors and substrates are dependent on control of actin polymerization. In resting platelets approximately 40% of actin is filamentous. The remaining actin monomers are

prevented from polymerising by monomer-sequestering proteins such as thymosin β4 and by barbed-end-capping proteins such as gelsolin and CapG. Since much of monomeric actin is recovered in association with thymosin β4, it may regulate monomeric actin's ability to polymerize onto actin filaments. Gelsolin is also involved in regulating actin polymerization as it binds to the barbed ends of actin filaments, preventing addition of actin monomers to the filament ends (Fox, 1993 a & b; Pavalko and Otey, 1994).

1.1.1 Membrane Skeleton:

Electron microscopy shows the membrane skeleton to be a tightly woven planar sheet composed of a spectrin-rich network of short actin filaments connected to the central cytoplasmic core network by radial actin filaments. Spectrin supports this structure by attaching one end of the radial filaments (Fox, 1993 a & b).

The membrane skeleton is physically associated with a number of proteins including structural proteins (such as actin, myosin, filamin, spectrin, talin, vinculin, dystrophin-related protein), signalling molecules (p120Ras, GTPase-activating protein (GAP), Src and Yes), receptors (GP Ib-IX) and integrins (GPIIb-IIIa or αIIb/β3), as well as other unidentified proteins (Fox, 1985; Fox, 1993 a & b; Pavalko and Otey, 1994).

Talin, vinculin and α-actinin in particular are associated with focal adhesions of the membrane cytoskeleton and the cytoplasmic face of the plasma membrane. Talin is primarily localized to sites where actin filaments attach to the membrane (Beckerie *et al.*, 1989). Talin and vinculin are likely responsible for the association of a pool of αIIb/β3 with the membrane skeleton as talin can interact with both vinculin and the cytoplasmic tail of β_3, while vinculin binds to F-actin. *In vitro*, talin has been shown to bind to the fibronectin receptor ($\alpha_5\beta_1$) and α-actinin has been shown to bind to the cytoplasmic domain of the β_1-integrin subunit. Studies in fibroblasts have suggested that α-actinin is necessary for the maintenance of established adhesions, whereas talin's role may be in the initial formation of new adhesions but may not be required for the maintenance of an established adhesion (Pavalko and Burridge, 1991; Nuckolls *et al.*, 1992; Fox, 1993 a & b).

Profilin is a protein with a low affinity for actin and a high affinity for PI 4,5-bisphosphate [PIP_2] suggesting it is involved in regulation of PIP_2. It has been calculated

that there is sufficient PIP_2 present in platelets to bind all profilin, making it unlikely that profilin is involved in the regulation of actin polymerization (Fox, 1993 a & b).

1.1.2 Cytoplasmic Skeleton:

In unstimulated platelets, the cytoplasmic cytoskeleton consists of networks of cross-linked actin filaments. Also present are actin binding protein and α-actinin, which cross-link with the actin filaments. Tropomyosin, caldesmon and VASP bind to actin filaments and may play a role in regulating the interaction of filaments with other proteins (Fox *et al.*, 1993 a & b).

1.1.3 Dense Tubular System:

The dense tubular system (DTS) is a derivative of the megakaryocyte smooth endoplasmic reticulum. The DTS is the major Ca^{2+} sequestration organelle in platelets and the source of Ca^{2+} mobilization upon stimulation. Unsurprisingly the DTS membrane contains receptors for inositol trisphosphate (IP_3), the major intracellular signal for platelet Ca^{2+} mobilization. Unlike other cells, platelets do not have ryanodine receptors since cyclic ADP-ribose, the endogenous activator of the ryanodine receptor in the smooth endoplasmic reticulum of other cells, has no effect in platelets (McNicol & Gerrard, 1997).

A number of endogenous peroxidases are associated with the DTS. Both cyclooxygenase and thromboxane synthetase are found there in conjunction with several components of the arachidonic acid-liberating pathway, including diglyceride lipase and phospholipase A_2 (PLA_2). There is also evidence of thromboxane A_2 (TxA_2) receptors in the DTS, although the function of internal receptors is unknown (McNicol & Gerrard, 1997).

1.1.4 Surface-connected Canalicular System:

The surface-connected canalicular system, also referred to as the open canalicular system, is continuous with the plasma membrane and intercalates with the cytosol (McNicol and Gerrard, 1997). The system serves as a conduit for soluble stimulators to access additional pools of receptors and as a channel for the flow of granular contents

during exocytosis. Pools of ADP ($P2Y_1$) and TxA_2 receptors have been identified in the surface-connected canalicular system (Nurden *et al.*, 2003).

1.1.5 Storage Granules:

Storage granules in platelets consist mainly of α-granules, dense granules and lysosomes. Granular contents are secreted upon platelet activation and play critical haemostatic functions. Granule membrane proteins also function as a reservoir of platelet adhesive receptors that redistribute to the surface following exocytosis thereby up-regulating the adhesive capacity (Zucker & Nachmias, 1985; McNicol & Gerrard, 1997).

Alpha granules are the largest and most numerous of the storage granules. They contain adhesive molecules, cytokines, coagulation factors and growth factors. Adhesive molecules include fibrinogen, fibronectin and thrombospondin. Cytokines include platelet factor 4, neutrophil-activating peptide-II, platelet basic protein, and its derivative β-thromboglobulin, and connective tissue activation-protein-III. Growth factors include platelet derived growth factor, β-transforming growth factor and coagulation factors V and VIII (Zucker & Nachmias, 1985; McNicol & Gerrard, 1997).

The membranes of α-granules contain binding sites for adhesive proteins GMP-33, P-selectin and αIIb/β3 (Johnston *et al.*, 1989; Disdier et. al, 1992). Sequencing of GMP-33 has demonstrated that it is the N-terminal part of thrombospondin and is N-glycosylated and bound to heparin (Damas *et al.*, 2001). P-selectin (also known as CD62 or GMP-140) is a membrane glycoprotein that binds carbohydrate structures to mediate cell:cell adhesion (Disdier *et al.*, 1992). P-selectin-mediated platelet-endothelial interaction may be important for haemostasis as suggested by a 40% prolongation of bleeding time and greater haemorrhage induced by the Shwartzman reaction in P-selectin knockout mice (Frenette and Wagner, 1997). αIIb/β3 is the fibrinogen receptor and is integral for platelet-to-platelet interactions as well as platelet interaction with the vessel endothelium (Gerrard *et al.*, 1985). A low molecular mass GTP-binding protein, G_n24, is also present in the membrane although its function is unknown (van der Meulen *et al.*, 1991; McNicol & Gerrard, 1997). The α-granule membrane has been identified as a significant pool of $P2Y_1$ and thromboxane prostanoid (TPα) receptors (Nurden *et al.*,

2003). The small GTPase Rab4 regulates alpha-granule secretion (van der Meulen *et al.*, 1991; Resmi & Krishnan, 2002; Yoshioka *et al.*, 2001).

Dense granules contain serotonin, a non-metabolic adenine nucleotide pool of ATP and ADP, Ca^{2+} and pyrophosphate (Zucker & Nachmias, 1985; McNicol & Gerrard, 1997). Platelets cannot synthesize serotonin, but acquire and package it during transit through the intestinal circulation. Adenine nucleotides are thought to form a complex with the serotonin, preventing its diffusion out of the granule (Wojenski and Schick, 1993).

Dense granule membranes contain ionic pumps that maintain a low luminal pH, which may play a role in the accumulation of serotonin (McNicol & Gerrard, 1997). P-selectin has also been found in dense granule membranes in resting platelets, which redistributes to the plasma membrane upon activation and has been implicated in platelet:leukocyte and platelet:microvesicle interactions. Granulophysin (CD63) is present in the dense granule membrane and has been implicated in granule membrane fusion (Disdier *et al.*, 1992; Frenette and Wagner, 1997).

Lysosomal granules contain various acid proteases, acid glycosidases, acid phosphatases and aryl sulphatases which have roles in clot lysis, clearing of platelet thrombi, microbial defence, degradation of subendothelial matrix and leukotriene metabolism following release (Rendu & Brohard-Bohn, 2001).

1.2 PLATELET ACTIVATION

During haemostasis platelets undergo a variety of processes: adhesion to exposed subendothelial surfaces; shape change; activation/spreading; aggregation; and procoagulant activity. These stages can be broadly divided into two categories: reversible and irreversible activation. Reversible activation includes responses such as adhesion, shape change and primary aggregation. Irreversible activation consists of secretion, procoagulant activity and secondary aggregation and defines the haemostatic function of the platelet.

1.2.1 Adhesion

Adhesion is the initial event leading to platelet activation. *In vivo*, adhesion is initiated by contact with exposed collagen from a damaged vessel wall. Collagen and von Willebrand Factor (vWF) from the injury site interact with receptors such as $\alpha_2\beta_1$, GPVI and GPIb (see 1.3.1) to form the initial adhesion and start the activation process (Eigenthaler and Shattil, 1996; McNicol and Gerrard, 1997; McNicol, 2003).

1.2.2 Shape Change

In the aggregation-independent phase of activation platelets undergo a change of shape from discoid to spherical. F-actin and the bulk of talin form networks at the cell periphery, while filamin and tropomyosin concentrate with bundles of actin filaments within the developing filopodia. At this time, 60-70% of total α-actin becomes polymerized into filaments. A combination of filament severing and the release of barbed-end-capping proteins generates free barbed ends on the actin filaments that serve as nuclei to promote actin polymerisation. The affinity of actin monomers for barbed filaments ends is greater than that for thymosin β4, therefore exposure of barbed filament ends will result in dissociation of actin monomers from thymosin β4 and polymerization of filaments. Filament severing is mediated in a Ca^{2+}-dependent manner by gelsolin and other severing proteins while the release of barbed-end-capping proteins appears to be mediated by phosphoinositides (Fox, 1993 a & b).

The surface area of the plasma membrane also increases, probably by evagination of the surface-connected canalicular system. Myosin associates with the cytoplasmic actin filament network, while at the same time granules are centralized in preparation for secretion (Siess, 1989; Fox *et al.*, 1993).

1.2.3 Secretion

Upon activation, cytoskeletal reorganization causes constriction of the microtubules by actin-myosin, centralizating intracellular organelles in the proximity of the surface-connected canalicular system. The two membranes fuse, incorporating α-granule and dense granule membranes in the surface-connected canalicular membrane,

and secreting the granular contents into the extracellular space (see 1.1.5 Storage Granules) (Zucker & Nachmias, 1985; McNicol & Gerrard, 1997).

Secretion is dependent on protein kinase C (PKC) activity. Histological studies found that phorbol esters changed the number of intramembranous particles in platelets, suggesting that PKC activation causes alterations in the platelet membranes making them prone to cohesion in the presence of lower concentrations of Ca^{2+} and facilitating secretion (Jerushalmy *et al.*, 1988; Walker and Watson, 1993).

1.2.4 Procoagulant expression

During activation, the platelet membrane forms small blebs that are subsequently shed from the membrane surface. These particles are enriched with PS, providing a surface that enhances coagulation factor binding and activation, which generate the pro-aggregatory mediator thrombin (McNicol & Gerrard, 1997; McNicol, 2005). This provides a link between the platelet activation process and the coagulation cascade demonstrating an interdependence of these two haemostatic processes.

1.2.5 Aggregation:

Under resting conditions αIIb/β3 is in a "closed" conformation during which time the binding domain is hidden. Platelet activation initiates signalling leading to a conformational change in αIIb/β3 and expression of the high affinity binding sites on the receptor. Aggregation occurs when fibrinogen and other adhesive proteins containing the arginine-glycine-aspartic acid (RGD) motif, such as vWF, fibronectin and vitronectin, interact with the exposed binding site to form a clot composed of clumps or aggregates of platelets (Fox, 1993 a & b; Eigenthaler and Shattil, 1996).

Fibrinogen binding to αIIb/β3 results in the activation of a number of intracellular processes, including the activation of calpain, which cleaves actin-binding protein, talin and spectrin (Fox, 1993 a & b). A number of signalling pathways are invoked: tyrosine phosphorylation and activation of the protein tyrosine kinase (PTK) $pp72^{Syk}$ and $pp60^{Src}$; activation of $p120^{Ras}$ GAP; and activation of p42 mitogen-activated protein kinase. There is also a significant molecular redistribution from the membrane skeleton to the cytoskeleton. Proteins such as αIIb/β3, spectrin, dystrophin-related protein, vinculin,

talin, $p120^{Ras}$ GAP, Src, Yes, $pp60^{Src}$, $pp72^{Syk}$, $pp125^{FAK}$, PKC, PI-3K, phosphotyrosine phosphatase 1B and 1C, PLCγ1, GPT-binding proteins (RhoA, Rac, CDC42Hs, Rap1B) and calpain, as well as signalling molecules such as 1,2 diacylglycerol (DG) all redistribute. Finally granule secretion and TxA_2 formation/release are also associated with aggregation.

In most cases, protein redistribution is dependent on fibrinogen binding since function-blocking antibodies to αIIb/β3 or RGD peptides inhibit translocation. Actin polymerisation is also required since protein redistribution is inhibited by pre-treatment with cytochalasin B, C or E, which prevents actin monomer deposition onto actin filament barbed ends (Fox *et al.*, 1993). Cytoskeletal reorganization during aggregation may also promote receptor clustering (Fox, 1993 a & b).

1.3 RECEPTORS

The numerous platelet cell surface receptors (see Table 1) are integral to the functioning of platelets. Of particular interest in the thesis is the role of the collagen and IgG (FcγRIIA) receptors in collagen-induced platelet activation. The remaining receptors have limited relevance to this thesis and are not specifically reviewed.

Table 1 Receptors currently identified in human platelets (McNicol, 2005)

Agonist	Receptor	Effect
Von Willebrand Factor	GPIb/V/IX	Adhesion
Collagen	$\alpha_2\beta_1$	Adhesion
	GPVI	Adhesion/activation
IgG	FcγRIIA	Activation
ADP	$P2Y_1$	Activation
	$P2Y_{12}$	Activation
	$P2X_1$	Amplification
TxA_2	TP	Activation
Thrombin	PAR1	Activation
	PAR4	Activation
	GPIb	Activation
PAF	PAF receptor	Activation

Agonist	Receptor	Effect
Serotonin	5-HT_2	Activation
Vasopressin	V_1	Activation
Epinephrine	α_2	Activation/amplification
Prostacylcin	IP	Inhibition
PGD_2	DP	Inhibition
Adenosine	A_2	Inhibition

1.3.1 Collagen Receptors

There are 18 different types of collagen identified, of which types I, II, III, IV, V, VI, VIII and sometimes XI occur in blood vessel walls (Sixma *et al.*, 1995). Adherence has been demonstrated for types II, III, IV, V, VI, VII, VIII, and I. The collagen types I, II, III and IV are most reactive with platelets and are capable of inducing adhesion and aggregation at high shear rates of 1500 sec^{-1}. Types V, VI, VII and VIII are less reactive, functioning only at low shear rates or static conditions, and do not induce aggregation. Type IV collagen is a non-fibrillar collagen that is found in the basement membrane (Staatz *et al.*, 1990; Sixma *et al.*, 1997; Barnes *et al.*, 1998).

Collagen molecules typically contain one or more regions of triple helical structure formed by the winding of three α-chains about one another into a coiled coil structure. This tertiary (triple-helical) and quaternary (polymeric) structure is required for collagen-induced platelet activation. Each type of collagen is composed of distinct α-chains (Staatz *et al.*, 1990; Kehrel *et al.*, 1998).

Monomeric collagen is capable of adhering to platelets but does not cause activation. Collagen-related peptides (CRPs) are repeating gly-pro-hyp peptides that mimic the collagen tertiary structure and can mimic collagen quaternary structure when cross-linked via either lysyl or cystine residues (CRP-XL). These peptides are more reactive than collagen fibres in activating platelets, but do not support adhesion under flow conditions. The non-cross-linked peptides antagonize platelet activation stimulated by native collagen or CRP-XL. CRPs are selective ligands for GPVI, without any association with other collagen receptors. GFOGER is triple helical peptide that contains a copy of a $\alpha_2\beta_1$ recognition motif and is selective for the $\alpha_2\beta_1$ receptor (Staatz *et al.*, 1990; Kehrel *et al.*, 1998).

Collagen adheres to a number of integrins and other receptors on the platelet surface. Proposed receptors include glycoprotein (GP) Ia-IIa ($\alpha_2\beta_1$), GPIV (CD36), GPVI, gC1qR/p33, a recently cloned 65 kDa protein, p61 and an uncharacterised 85-90 kDa protein. $\alpha_2\beta_1$ is generally accepted as the receptor primarily responsible for adhesion. GPVI is strongly associated with activation, but its occupation does not completely account for the major aspects of collagen-induced activation (Watson and Gibbins, 1998).

Evidence that platelet adhesion and activation by collagen are mediated by different receptors is overwhelming. The current model proposes that adhesion to collagen brings a second site in the collagen molecule into the vicinity of a low-affinity receptor responsible for activation. While the receptors principally involved in adhesion have been characterized, the nature of the relationship between receptor(s) responsible for activation has not been categorically established (Watson and Gibbins, 1998).

1.3.1.1 $\alpha_2\beta_1$

The integrin $\alpha_2\beta_1$ is a general receptor for all collagens and in some cell types also functions as a laminin receptor, although not in platelets. Its primary role is platelet adhesion (Santoro and Zutter, 1995; Watson and Gibbins, 1998), although a supporting role in platelet activation has been demonstrated. The $\alpha_2\beta_1$ is an important receptor under flow conditions but may not be necessary for aggregation under static conditions (Morton *et al.*, 1995; Watson and Gibbins, 1998). Either of the α1(I) or α2(I) chains of collagen fibres (see 1.3.1) support platelet adhesion via α_2 in a Mg^{2+}-dependent manner (Watson and Gibbins, 1998).

The α_2 subunit is composed of a single polypeptide chain that contains a transmembrane domain with a small intracellular domain. The large N-terminal extracellular domain has three metal-binding domains, a characteristic common to integrin proteins, and an adjacent A-domain. The A domain contains a groove able to accommodate a collagen triple helix, which is critical to collagen binding. It also has homology with the A-domain of vWF, CR3, LFA-1 and other adhesive molecules (Santoro and Zutter, 1995; Barnes *et al.*, 1998; Moroi and Jung, 1997; Sixma *et al.*, 1997; Watson and Gibbins, 1998).

The β_1 subunit has a short cytoplasmic domain, a hydrophobic transmembrane domain that spans the membrane once and a large extracellular domain. The extracellular domain contains an epitope recognized by both activating and inhibitory monoclonal antibodies to α_2 integrins. The cytoplasmic domain appears largely responsible for mediating the interactions of integrins with the actin cytoskeleton via interactions with other cytoskeletal components such as α-actinin and talin (Santoro and Zutter, 1995).

The role of $\alpha_2\beta_1$ in collagen-induced platelet activation has been evaluated using $\alpha_2\beta_1$-directed monoclonal antibodies, such as mAb 6F1 and MoAb13, platelets deficient in various glycoproteins and GFOGER. $\alpha_2\beta_1$ clearly has a primary role in platelet adhesion to collagen and initiation of spreading. Blockade of $\alpha_2\beta_1$ with mAB 6F1 decreased the number of platelets that showed adhesion and spreading, indicating a direct role for the receptor. Further, mAB 6F1 blocked platelet adhesion to GFOGER (Watson and Gibbins, 1998; Inoue *et al.,* 2003). Patients with a platelet deficiency of α_2 show reduced Mg^{2+}-dependent adhesion to collagen under both static and flow conditions. Similarly, $\alpha_2\beta_1$ deficient platelets show reduced adhesion to collagen, and platelets that do adhere do not spread (Santoro and Zutter, 1995).

$\alpha_2\beta_1$ also induces some collagen-induced tyrosine phosphorylation. Inoue *et al.*, (2003) showed stimulation of $\alpha_2\beta_1$ with immobilized GFOGER induced spreading by a pathway involving Src, Sky, SLP-76, PLCγ2, FAK, PMAC and Ca^{2+} mobilization This does not occur under shear conditions, suggesting a mechanism to protect against platelet activation within the blood vessel in the absence of injury. Other investigators used antibodies to $\alpha_2\beta_1$ in collagen-stimulated GPVI-deficient platelets to show that $\alpha_2\beta_1$ induces activation of c-Src and tyrosine phosphorylates cortactin and several other substrates, but does not activate Sky or tyrosine phosphorylate PLCγ2 or Vav (Ichinohe *et al.*, 1997; Watson and Gibbins, 1998). This limited activation is more likely since collagen interaction of $\alpha_2\beta_1$ alone does not completely activate platelets.

1.3.1.2 gC1qR/p33

C1q is a complement protein that has been identified at sites of vascular injury and inflammation as well as in atherosclerotic lesions. It modulates platelet interactions

with collagen and immune complexes and may be involved in the clustering of $\alpha_2\beta_1$. This 68 kDa protein has a triple-helical structure similar to collagen. Antibodies to the C1q receptor block collagen-induced aggregation and secretion without affecting platelet adhesion to collagen. One of the C1q binding sites expressed on platelet surfaces is gC1qR/p33, is a protein that binds to a number of ligands and is found in a number of cellular compartments (Sixma *et al.*, 1995; Peerschke and Gheberhiwet *et al.*, 2001).

1.3.1.3 Glycoprotein IV (CD36)

GPIV is an 85 kDa transmembrane glycoprotein. Purified GPIV shows binding affinity for collagen and Fab fragments of a GPIV antibody inhibits collagen-induced platelet adhesion and aggregation. GPIV-deficient platelets show decreased initial adhesion under static conditions and lack reactivity towards type V collagen. However, under flow conditions, there is no evidence of impaired adhesion (Sixma *et al.*, 1995).

Under basal conditions GPIV is associated with the Src tyrosine kinases Yes, Fyn, and Hck, which probably accounts for the underlying protein tyrosine phosphorylation in GPVI-deficient platelets. The effects of GPIV deficiencies are considerably weaker than those of $\alpha_2\beta_1$ and GPVI deficiencies, indicating that GPIV is probably not an essential factor in platelet interaction with collagen. In fact, a small percentage (up to 5%) of the Japanese and other East Asian populations lack the GPIV receptor but have no obvious bleeding disorder (Moroi and Jung, 1997; Sixma *et al.*, 1997; Kehrel *et al.*, 1998; Watson, 1999).

1.3.1.4 Glycoprotein VI (p62)

Evidence for a role for GPVI as a collagen receptor involved in platelet activation comes from studies in GPVI deficient platelets. In normal platelets, antibody mediated GPVI cross-linking is sufficient to stimulate cAMP-insensitive tyrosine phosphorylation of Syk, PLCγ2 and Vav. However, in GPVI-deficient platelets, stimulation with either the GPVI selective CRP-XL ligand or collagen does not induce aggregation, mobilize Ca^{2+}, or stimulate TxA_2 production, and protein tyrosine phosphorylation of PLCγ2, Fak, and Vav is absent while that of Syk is decreased (Arai *et al.*, 1995; Ichinohe *et al.*, 1995; Ichinohe *et al.*, 1997; Kehrel *et al.*, 1998). Fibrinogen binding is absent in the presence of

CRP-XL and decreased with stimulation by collagen. In keeping with GPVI's selectivity for collagen, deficient platelets remain responsive to other platelets agonists, such as ADP (Sixima *et al.*, 1997; Ichinohe *et al.*, 1997; Moroi and Jung, 1997; Kehrel *et al.*, 1998).

Under flow conditions, GPVI is occupied only after primary adhesion via $\alpha_2\beta_1$ and consequently deficient platelets show defective second phase adhesion (Barnes *et al.*, 1998 Inoue *et al.*, 2003). Therefore, it is not surprising that several other events seem to be dependent on $\alpha_2\beta_1$ activation but are regulated by GPVI. For example, in GPVI-deficiency, activation of c-Src is 1.5 times normal levels. Tyrosine phosphorylation of major protein bands is still present, but time-dependent dephosphorylation is absent (Ichinohe *et al.*, 1997; Inoue *et al.*, 2003).

More recently the association of GPVI with the Fc receptor γ–chain and their role in collagen-induced platelet activation has emerged. Evidence for this relationship comes from the fact that platelets deficient in GPVI are also deficient in the Fc receptor γ–chain. Conversely, antibodies to GPVI can precipitate the FcRγ-chain (Watson and Gibbins, 1998; Watson, 1999). Further, it appears that GPVI receptor clustering is required for activation since a dimeric form of GPVI fused with the Fc domain will interact with collagen fibrils whereas monomeric GPVI will not (Moroi and Jung, 1997; Barnes *et al.*, 1998; Takahaski *et al.*, 2002). This association is reviewed below (see 1.3.2.1).

1.3.1.5 p65

p65 is a 65 kDa protein that has three transmembrane-spanning regions but no distinguishing motif to indicate a likely mechanism of intracellular signalling. p65 binds type I collagen, but not type III collagen, which activates platelets. Therefore it is not likely a major collagen receptor (Watson and Gibbins, 1998; Watson, 1999; Chiang and Takayama, 2002).

1.3.2 IgG Receptor

1.3.2.1 FcγRIIA

Although not a collagen receptor per se, FcγRIIA is a low affinity surface receptor for immune complexes in platelets, with an inseparable relationship with GPVI in activation by collagen. Evidence for the relationship between GPVI and FcγRIIA comes from a variety of sources. Platelets that are deficient in FcRγ-chain also lack GPVI. Selective stimulation of GPVI with CRPs or cross-linking FcγRIIA mimics the tyrosine phosphorylation patterns of collagen and FcγRIIA stimulation (Tsuji *et al.*, 1997; Watson, 1999).

FcγRIIA is thought to be activated through its phosphorylation by one of the Src family of tyrosine kinases. This tyrosine phosphorylation of FcγRIIA results in the recruitment, and activation, of Syk. In turn, Syk is linked to downstream activation of PLCγ2, as well as tyrosine phosphorylation or co-precipitation of a number of proteins implicated in immune receptor signalling, including: the adapter proteins Cbl, Crkl, Lat, SLP-76 and SLAP-13; the cytoskeletal proteins, cortactin, HS-1, and WASP; the GTP exchange factor Vav; the phosphoinositide 5' phosphatase SHIP; the protein tyrosine phosphatases, SHP-1 and SHP-2; and the tyrosine kinases Btk, Fyn and Lyn. Proteins that co-precipitate with some of the above include Grb2 and the p85 subunit of PI 3-kinase, which associate with the adapter Lat in platelets activated by GPVI (Yanaga *et al.*, 1995; Tsuji *et al.*, 1997; Watson and Gibbins, 1998; Watson, 1999; Larson *et al.*, 2003).

1.3.3 von Willebrand Factor Receptor

The von Willebrand Factor (vWF) receptor is essential for the initial adhesion of platelets to collagen at high shear rates. It consists of a multimers of identical 220 kDa subunits held together with disulfied bonds. Primarily released by endotheial cells, vWF can also come from plasma or activated platelets. vWF has the capacity to bind to different types of collagen as well as GPIb and αIIb/β3 on platelets (Siess, 1989). Collagen binding occurs at separate sites from platelet glycoprotein binding. vWF contains has two collagen binding sites, one each in the A1 and A3 domains. The binding of types I and III collagen to vWF occurs primarily through the A3 domain of vWF,

while type VI is reported to bind to the A1 domain of vWF (Siess, 1989; Clemetson, 1997; Sixma *et al.*, 1997; Barnes *et al.*, 1998).

1.3.3.1 GPIb-V-IX (CD42)

The GPIb-V-IX (CD42) complex is another major, non-collagen, platelet adhesion receptor. The receptor is a complex of four chains: GPIbα (150 kDa) and GPIbβ (27 kDa) linked by a disulphide bond; GPIX (22 kDa) strongly non-covalently bound in a 1:1 ratio; and GPV (82 kDa) weakly non-covalently associated with the complex in a 1:2 ratio (GPV:GPIb). The GPIba chain contains the binding sites for vWF and thrombin in the N–terminal region and for filamin in its cytoplasmatic domains (Clemetson, 1997).

GPIb-V-IX binds platelets to vWF under high shear stress conditions, temporarily slowing the platelet in the vicinity of the injured vessel wall to allow the formation of stable adhesion with collagen receptors. vWF dissociates rapidly from GPIb-V-IX and interaction with other subendothelial proteins, notably collagen fibres and fibrinogen, are required to hold the platelet at the site of damage (Barnes *et al.*, 1998; Clemetson, 1997; Sixma *et al.*, 1995; Sixma *et al.*, 1997; Watson and Gibbins, 1998).

1.3.4 ADP Receptors

In platelets, adenosine diphosphate (ADP) is stored in dense granules. ADP induces platelet aggregation, shape change, secretion, influx and intracellular mobilization of Ca^{2+}, and inhibition of the adenylyl cyclase stimulated by prostaglandins (Gachet, 2001; Nurden *et al.*, 2003). Removal of ADP does not affect the maximal elevation in Ca^{2+} but does increase the time to reach the maximum (Murphy *et al.*, 1996). Selective inhibition of lipoxygenase with BW A4C has no effect on the increase in Ca^{2+} induced by ADP indicating that the arachidonic acid lipoxygenase metabolites, primarily 12-HETE, do not have any role in ADP-evoked rise in Ca^{2+}. Cyclooxygenase blockade with aspirin or indomethacin causes a small inhibition of ADP-evoked rise in Ca^{2+} (Vindlacheruvu *et al.*, 1991). Platelets contain two G-protein-coupled ADP receptors, $P2Y_1$ and $P2Y_{12}$.

1.3.4.1 $P2Y_1$

ADP is a specific ligand for $P2Y_1$. Immuno-electron microscopy shows $P2Y_1$ to be in the plasma membrane, membranes of α-granules and the surface-connected canicular system (Gachet, 2001; Nurden *et al.*, 2003). $P2Y_1$ is coupled by Gq to PLCβ leading to increases in IP_3 and IP_4, Ca^{2+} mobilisation, shape change and the initiation of aggregation. A Ca^{2+}-induced exocytotic release of ADP constitutes a feed back mechanism to strengthen or sustain a stimulus (Haynes, 1993). Desensitisation is mediated by G-protein coupled receptor kinases 2 and 6 (Hardy *et al.*, 2005).

1.3.4.2 $P2Y_{12}$

$P2Y_{12}$ is coupled to Gαi2 and is necessary for formation and stabilization of large aggregates (Gachet, 2001; Nurden *et al.*, 2003). Desensitisation is dependent on PKC activity (Hardy *et al.*, 2005).

1.3.4.3 $P2X_1$

Although not an ADP receptor, platelets have a third type of P2 receptor. $P2X_1$ is an ATP-gated cation channel mediating rapid Ca^{2+}-influx, but its role is not yet completely known (Hoylaerts *et al.*, 2000; Gachet, 2001).

1.4 PLATELET STIMULUS RESPONSE COUPLING

There is a multitude of second messengers and other signal transduction mechanisms that link receptor occupation to the stimulus response. In platelets, collagen stimulation leads to release of arachidonic acid and subsequent production of TxA_2. The interaction of TxA_2 with its cell surface receptor leads to a separate cascade of signalling events. In keeping with this thesis, the review of signal transduction mechanisms in this section is from the perspective of collagen-induced activation up to the point of arachidonic acid release.

1.4.1 G-Proteins

GTP-binding proteins, or G-proteins, mediate the interaction of cell surface receptors and intracellular second messenger generating enzymes. Specifically the G-

proteins relay external stimuli from receptors with seven transmembrane domains to intracellular signalling pathways.

The G-proteins are heterotrimeric guanine nucleotide regulatory binding proteins consisting of an α, β and γ subunit. The γ-subunit (Gγ) binds tightly to the β-subunit (Gβ) to form a hydrophobic, isophrenylated heterodimer that helps anchor the complex to the cell membrane. The α-subunit (Gα) completes the heterotrimer and contains the guanine nucleotide-binding site which, in some cases, is a substrate for ADP-ribosylation by bacterial toxins, e.g. pertussis and cholera toxins (Manning & Brass, 1991; Brass *et al.*, 1993, Brass *et al.*, 1997).

There are multiple variants of each subunit, creating numerous combinations, each with unique activation and signal propagation properties. In platelets, at least nine different G-protein subunits (G_s, G_i, G_{ia}, $G_{q\alpha}$, $G_{11\alpha}$, $G_{12\alpha}$, $G_{13\alpha}$, $G_{z\alpha}$ and $G_{s\alpha}$) have been identified (Manning & Brass, 1991; Brass *et al.*, 1993, Brass *et al.*, 1997). Under basal conditions, G-proteins exist as heterotrimer with GDP bound to the α-chain. Receptor activation promotes the release of GDP and the binding of GTP from the cytosol and the dissociation of GTP-Gα from the Gβγ complex. Both the Gα and the Gβγ subunits can now interact with their effectors and propagate the signal. Endogenous GTPase activity within the G_α subunit hydrolyses GTP to GDP, terminates G-protein ability to regulate effector activity and finally the complex reassociates to form a heterotrimer (Brass *et al.*, 1997).

1.4.2 Phospholipase C

The phospholipase C (PLC) superfamily of enzymes hydrolyses the inositol phospholipids PI-4,5-bisphosphate (PIP_2), PI-4-phosphate (PIP) and PI. Substrate hydrolysis proceeds in two sequential reactions: cleavage of the phospholipids into DG and inositol 1,2 cyclic phosphates, followed by the conversion of the inositol 1,2 cyclic phosphates into acyclic inositol phosphates (Katan, 1998). Most important to signalling is the hydrolysis of PIP_2 to yield IP_3 and DG.

Within this enzyme superfamily are a group of bacterial enzymes (not applicable to this thesis) and three families of eukaryotic isozymes, PLCβ, PLCγ and PLCδ. Eukaryotic PLC's show a substrate preference of PIP_2>PIP>PI. Multiple isoforms of each

isozyme exist in a variety of tissues, which are stimulated by a variety of distinct mechanisms. Numerous isozymes of PLC have been identified in platelets, including PLCβ1, PLCβ2, PLCβ3,PLCβ4, PLCγ1, PLCγ2, PLCδ1, PLCδ2, PLCδ3 and PLCδ4 (Banno *et al.*, 1995). In platelets, PLC hydrolyses all three inositol phospholipids, although PIP and PIP_2 are the preferred substrates. Selectivity for PIP_2 over PI decreases in the order PLCβ1>PLCδ1>PLCγ1 (Rhee and Choi, 1992; Katan, 1998).

While the overall homology between PLC isoforms is low, there are two highly conserved catalytic domains designated X and Y respectively. These domains are straddled by an N-terminal pleckstrin homology (PH) domain on the X side and a C2 homology domain on the Y side. The PH domains may be involved in interactions with the βγ subunits of G-proteins and PKC. The C2 domains are believed to be binding sites for Ca^{2+} as they are homologous to the Ca^{2+}-binding domains in PKC and cytosolic $cPLA_2$ ($cPLA_2$), and are required for full Ca^{2+} dependence of PLC (Rhee and Choi, 1992; Cockcroft and Thomas, 1992).

1.4.2.1 PLCδ:

Four distinct PLCδ isozymes have been identified in mammalian cells (PLCδ1 – PLCδ4). The δ-isomer contains a short sequence of 50-70 amino acids separating the X and Y domains. In addition to the PH, catalytic and C2 domains, the δ-isomer contains four EF-hand motifs between the PH and the X domains that are associated with chelating Ca^{2+}. The activity of PLCδ1 is stimulated by an increase in Ca^{2+} concentration in the absence of another activator. Deletion of the Ca^{2+}-binding sites in the C2 domain of PLCδ1 has no effect on enzyme activity, leaving the role of Ca^{2+} in phospholipid binding unclear (Katan, 1998).

1.4.2.2 PLCβ:

The β-isomer also contains a short sequence of 50-70 amino acids separating the X and Y domains and an extended carboxyl terminus of approximately 450 amino acids after the Y domain. The C-terminal on PLCβ contains regions required for membrane

association and stimulation by $G\alpha_q$ and may be involved in the enhancement of GTPase activity of $G\alpha_q$ (Rhee and Choi, 1992; Cockcroft and Thomas, 1992).

Stimulation of PLCβ occurs through receptors coupled to heterotrimeric G-proteins and is caused by two distinct mechanisms, one through the α-subunit of the pertussis toxin insensitive Gq family and another through the βγ subunits of the pertussis toxin-sensitive G-proteins. The actions of α and βγ subunits likely occur at separate sites on the PLCβ enzyme since studies have shown α_q can further stimulate PLCβ3 in the presence of saturating βγ.

Agonists such as TxA_2 and vasopressin stimulate PLCβ1 through activation of $G\alpha_q$ or $G\alpha_{11}$. $G\alpha_{16}$ was found to be most effective in stimulating PLCβ2. In addition, calpain cleavage of the C-terminus of platelets PLCβ increases βγ-stimulated activity (Rhee and Choi, 1992; Banno *et al.*, 1995; Singer *et al.*, 1997; Katan, 1998).

In resting platelets, 90%, 87% and 100% of PLCβ2, PLCβ3a and PLCβ3b, respectively is found in the cytosol, whereas the membrane skeleton containes 9% of PLCβ2 and 10% of PLCβ3a. Thrombin stimulation causes translocation of all isozymes to the cytoskeleton, but regulation of PLCβ3 and PLCβ2 is different for the two isozymes (Banno *et al.*, 1996). Extensive studies by Banno and colleagues have demonstrated that platelet activation by PLCβ2 occurs prior PLCβ3 activation, likely as a direct result of G-protein linked receptor occupation, whereas PLCβ3 activation is dependent on fibrinogen binding, which occurs downstream of a tyrosine kinase mediated event (Banno *et al.*, 1995; Banno *et al.*, 1996).

1.4.2.3 PLCγ

PLCγ1 (145 kDa) and PLCγ2 (135 kDa) have long regions of approximately 400 amino acids between the X and Y domains, containing *src* homology (SH) domains (SH2 and SH3) that mediate interaction of this protein with phosphorylated growth factor receptors and other signalling mediators. The SH2 domain targets the enzyme to tyrosine phosphorylation sequences present in other proteins while the SH3 domain targets the enzyme to cytoskeletal components and is implicated in localizing PLCγ1 to the cytoskeleton. These domains are found in a large number of unrelated proteins such as

GTPase activating protein, PI 3-kinase, tyrosine phosphatases, and kinases (Banno *et al.*, 1992; Cockcroft and Thomas, 1992; Rhee and Choi, 1992; Singer *et al.*, 1997; Katan, 1998).

In resting platelets majority (70-90%) of PLCγ2 is in the cytosol and the remainder associated with the membrane skeleton (Tate and Rittenhouse, 1993; Blake *et al.*, 1994; Banno *et al.*, 1996). Following receptor stimulation, PLCγ translocates from the cytosol to the membrane, binds to the activated receptor and is rapidly phosphorylated on tyrosine residues by Syk. Phosphorylation of PLCγ increases its binding to phospholipids (Rhee and Choi, 1992; Singer *et al.*, 1997; Watson and Gibbins, 1998). In general, PLCγ's increase Ca^{2+} more slowly and for a longer duration than do PLCβ's, suggesting the formation of IP_3 is slower and more prolonged (Clapham, 1995).

In platelets, PLCγ1 associates with the GTPase activating protein of $p21^{ras}$, which is tyrosine phosphorylated in response to thrombin stimulation. However, in indomethacin-treated platelets collagen, FcγRII cross-linking or thrombin stimulation does not phosphorylate PLCγ1, indicating dependence on arachidonic acid for phosphorylation (Tate and Rittenhouse, 1993; Blake *et al.*, 1994; Rhee and Choi, 1992; Singer *et al.*, 1997). Conversely, collagen or cross-linking of FcγRII induces tyrosine phosphorylation of PLCγ2, but not PLCγ1 (Blake *et al.*, 1994) demonstrating different regulatory mechanisms for PLCγ isozymes in platelets.

PLCγ2 is most important to collagen-induced activation of platelets. At different Ca^{2+} concentrations, PLCγ2 activity shows variation in phospholipids preference. At ranges of 0.1-1 mM, PI-hydrolysing activity is maximal, but at 10 μM, PIP_2-hydrolysing activity is maximal (Banno *et al.*, 1990). Stimulation of platelets with low doses of collagen or by cross-linked FcγRIIa causes the tyrosine phosphorylation of PLCγ2. Phosphorylation occurs upstream of PKC activity and Ca^{2+} mobilization (Daniel *et al.*, 1994; Blake *et al.*, 1994).

1.4.2.4 PLC Inhibitors

1-[6-[[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl-]amino]hexyl]-1H-pyrrole 2,5-dione (U-73122) selectively inhibits PLC-dependent processes in human platelets and

neutrophils (Smith *et al.*, 1990; Bleasdale *et al.*, 1990). In platelets, U73122 inhibits aggregation, increased intracellular Ca^{2+} concentrations and [^{3}H]-IP_3 production in response to thrombin and U46619 (Bleasdale *et al.*, 1990). In neutrophils, U73122 also inhibits increases in intracellular Ca^{2+}, IP_3 production and DG production induced by N-formyl-methionyl-leucyl-phenylaline (Smith *et al.*, 1990) as well as DG production induced by phorbol esters (Bleasdale *et al.*, 1990). Based on studies of acetylcholine receptor sequestration in SK-N-SH neuroblastoma cells, the proposed mechanism of action of U73122 lies at the level of Gp regulation of PLC (Thompson, 1991).

However, other investigators have found additional effects of U73122 on non-PLC dependent processes. Vickers (1993) demonstrated that U73122 affects the equilibrium between membrane PIs in both resting and thrombin-stimulated platelets, likely through inhibiting inositol phosphate and phosphatidylinositol 4-phosphate kinase activity. Furthermore, U73122 affects Ca^{2+} homeostasis in rat neutrophils (Wang, 1996) and mouse fibroblasts (Grierson and Meldolesi, 1995), possibly through inhibition of the Ca^{2+} ATPase.

1.4.3 Intracellular Ca^{2+}

Intracellular Ca^{2+} is a key mediator of the activation-response coupling in platelets. Not only is Ca^{2+} released in response to a number of stimuli, but many processes also require the presence of threshold amounts of Ca^{2+} to proceed. Agonist-induced elevations in Ca^{2+} occur as a consequence of both IP_3-mediated mobilization and influx from the external milieu across the plasma membrane.

1.4.3.1 Regulation

In resting platelets, the cytosolic Ca^{2+} concentration is approximately 100 ηM, which can increase to 0.1-3 mM upon activation. The bulk of non-cytosolic Ca^{2+} is sequestered in dense granules (a non-mobilisable store) and the DTS and the remainder in mitochondria, α-granules, and the inner surface of the plasma membrane (Zucker and Nachmias, 1985; Salzman and Ware, 1989; Cavallini *et al.*, 1995). This is a multi-step process involving both mobilization of Ca^{2+} from internal stores and the influx of Ca^{2+} from the external milieu.

The plasma membrane concentration gradient drives a constant passive leak of extracellular Ca^{2+} into the platelet. The leak is primarily countered by a Ca^{2+} extruding P-type ATPase in the plasma membrane (PMAC) (Salzman and Ware, 1989; Clapham, 1995). At near micromolar concentrations Ca^{2+} is also actively removed from the cytoplasm by an Na^{+}/Ca^{2+} exchange in the plasma membrane (Salzman and Ware, 1989). Two other P-type ATPase Ca^{2+} pumps, smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA) 2b (100 kDa) and SERCA 3 (98 kDa) operate at higher cytosolic Ca^{2+} concentrations (Salzman and Ware, 1989; Cavallini *et al.*, 1995; Haynes, 1993; Clapham, 1995).

Some Ca^{2+} influx also arises through store-mediated Ca^{2+} entry (SMCE), a capacitative mechanism that is regulated by the filling state of intracellular Ca^{2+} stores (Murphy *et al.*, 1996; Rosado and Sage, 2001; Rosado *et al.*, 2001). A direct (conformational) coupling model, in which there is a physical interaction between the endoplasmic reticulum and the plasma membrane, is proposed as the mechanism for SMCE. Such a mechanism may involve IP_3 receptors, Trp1 channels and/or the actin cytoskeleton (Rosado and Sage, 2000; Rosado and Sage, 2001; Rosado *et al.*, 2001).

1.4.3.2 IP_3 Receptor:

In platelets, engagement of IP_3 receptors stimulates the release of Ca^{2+} from the DTS into the cytosol (Cavallini *et al.*, 1995; Clapham, 1995). Platelets contain multiple isoforms of IP_3 receptors: IP_3RI in the intracellular membrane, IP_3RIII in the plasma membrane and IP_3RII in both membranes (Berridge, 1993; Rosado and Sage, 2000; Rosado and Sage, 2001; Rosado *et al.*, 2001). Human platelets also contain transient receptor potential (Trp) which can form non-selective cation channels. In store-depleted platelets, only IP_3RII was found to be co-precipitated with Trp1 (Rosado and Sage, 2000; Rosado and Sage, 2001; Rosado *et al.*, 2001).

1.4.3.3 Function

Ca^{2+} is involved in numerous signalling and facilitative events in platelets. Agonist-induced increase in cytosolic Ca^{2+} simulates phosphorylation of a number of proteins such as myosin light chain (by myosin light chain kinase), $cPLA_2$, phosphorylase

b kinase and cyclic nucleotide phosphodiesterase. In addition, Ca^{2+} potentiates or activates the fusion of intracellular membranes, disassembly of microtubules, ATPase activity of actomyosin and activation of PKC and calpain (Salzman and Ware, 1989).

1.4.4 Diacylglycerol

DG is composed of two fatty acids on a glycerol backbone. Since cells contain a multitude of different fatty acids, there are numerous possible combinations contributing to the composition of any give DG. Therefore, DGs are generally categorised according to the class of fatty acid found at various positions on the glycerol backbone. In platelets the *sn*-2 position is often arachidonic acid (Wakelam, 1998).

1.4.4.1 Metabolism:

There are negligible levels of DG in the membranes of normal, resting mammalian cells. The production of DG differs depending on whether DG is functioning as a biosynthetic intermediary or a second messenger. As part of the biosynthetic pathway, DG is generally derived from phosphatidic acid (PA) by the action of phosphatidic acid phosphatase (PAP) (Wakelam, 1998).

The production of DG as part of a signalling pathway occurs by the PLC-catalyzed hydrolysis of PIP_2 to DG and IP_3 (see 1.4.2) (Exton, 1994). In thrombin-stimulated platelets, DG accumulates in a biphasic pattern - an early transient phase and a late sustained phase (Bishop *et al.*, 1992). The majority of DG accumulation occurs during the sustained phase and occurs prior to maximal PKC translocation, consistent in its stimulatory role. DG is assumed to cause changes in the physical properties of the membrane microdomains and accounts for the activation of PLA_2 by DG in "*in vitro*" assays (Wakelam, 1998). Of significance to the signalling pathway, DGs bind to the cysteine-rich regions of conventional and novel PKCs to activate the enzyme in co-operation with PS and Ca^{2+} (see 1.4.5).

Alternatively, the enzyme diacylglycerol lipase may catalyze the hydrolysis of DG, yielding 2-arachidonylmonoglycerol and free fatty acid. Subsequently, free arachidonate plus glycerol is released by the action of a monoacylglycerol lipase

(Tohmatsu *et al.*, 1987; Oglesby and Gorman, 1984; Werner *et al.*, 1991). Alternatively diacylglycerol kinase can metabolize DG to PA (Tohmatsu *et al.*, 1987).

Some DG is produced by hydrolysis of PC in response to agonist stimulation of signalling pathways other than PLC, although these pathways have not been well characterized in platelets. For example, the primary lipid product of phospholipase D (PLD) activity is PA, which is rapidly converted to DG since PAP activity appears to be solely regulated by substrate concentration (Nozawa *et al.*, 1991; Exton, 1994; Wakelam, 1998). In mammalian cells PLD's principal substrate is PC (Cockcroft, 1991). The species of DG arising from hydrolysis of PC differ from those arising from PI hydrolysis by PLC both in fatty acid composition and time course of formation (Martinson, 1995).

In platelets PLD appears to be stimulated by PKC in a manner that is independent of PLC and Ca^{2+} and involves a G-protein or small GTP-binding protein. Inhibition of PKC with Rö31-8220 inhibits both PMA and thrombin induced PLD activity in the presence of RGDS, and in platelets from type I Glanzmann's thrombasthenia, demonstrating a role for PKC activation of PLC independent αIIb/β3 (Martinson *et al.*, 1995). In general, PLD is thought to play a secondary role in platelet aggregation (Chiang, 1994).

1.4.4.2 Inhibitors

RHC 80267 is a DG lipase inhibitor that inhibits arachidonic acid release and the formation of both TxB_2 (the inactive stable metabolite of TxA_2) and lipoxygenase products in response to high doses of collagen. At low doses of collagen, RHC 80267 inhibits platelet aggregation and secretion. RHC 80267 also blocked aggregation, secretion and TxB_2 generation in response to arachidonic acid, consistent with an additional inhibitory action on cyclooxygenase. This is supported by the lack of effect of RHC 80267 on platelet aggregation and secretion in response to prostaglandin H_2 (PGH_2), the immediate product of cyclooxygenase and precursor of TxA_2. Further studies using collagen and the stable TxA_2 mimetic U46619 demonstrate that identical concentrations of RHC 80267 are required to inhibit DG lipase and cyclooxygenase. RHC 80267 also inhibits the formation of DG and PA and release of arachidonic acid, suggesting an action on PLC (Oglesby and Gorman, 1984; Amin *et al.*, 1986).

R 59 022 is a DG kinase inhibitor that primarily inhibits DG kinase-II and to a lesser extent DG kinase-III, but has little effect on DG kinase-I (Nozawa *et al.*, 1991). R 59 022 potentiates the accumulation of DG and consequently the release of serotonin induced by thrombin, but attenuates the increase of Ca^{2+}. R 59 022 also enhances serotonin secretion stimulated by OAG, suggesting increased PKC activity due to increased DG concentrations. The attenuated rise in cytosolic Ca^{2+} was possibly as a result of decreased PA formation which is known to have ionophoretic action (Tohmatsu *et al.*, 1987).

1.4.5 Protein Kinase C

PKC is a family of serine/threonine protein kinases that are potently activated by DG. As discussed above, DG is normally only present as a result of PLC activity and therefore *in vivo*, PKC activity is downstream of PLC activity (Baldassare *et al.*, 1992; Blobe *et al.*, 1995, Nishizuka, 1984). The isozymes of PKC can be functionally divided into three groups:

1. Conventional, including PKC α, βI, βII, and γ, requiring Ca^{2+}, DG, phospholipids or phorbol esters for activation;
2. Novel, including PKC δ, ξ, η, θ, and μ (PKD), requiring DG, phospholipids or phorbol esters, but no Ca^{2+} for activation;
3. Atypical PKC's ζ and λ/ι, which do not require Ca^{2+} or respond to DG or phorbol esters in vitro or in vivo.

Platelets contain predominantly Ca^{2+}-dependent PKC isozymes, PKC-α, βI, βII, as well as the Ca^{2+}-independent PKC isozyme, PKC-δ, with small amounts of PKC-ξ, ζ and η (Baldassare *et al.*, 1992; Blobe *et al.*, 1995).

1.4.5.1 Structure:

Each PKC isozyme is composed of a pseudosubstrate (autoinhibitor) domain and four C domains (C1-C4). The pseudosubstrate domain controls the activity of PKC. This domain contains a consensus sequence of substrates for the kinase but no phophorylatable residues and is missing in PKCμ (Hofmann, 1997).

The C1 regulatory domain is believed to be essential for binding DG and phorbol esters. C1 contains tandem repeats of cyctein-rich motifs that are highly conserved in the conventional and novel PKC's, but are partially missing in the atypical PKC's, hence their insensitivity to DG and phorbol esters. The C2 domain is involved in Ca^{2+} binding and is absent in the novel PKCs. The C3 domain contains the ATP-binding site that is highly conserved in all human PKC isoforms. The C4 catalytic domains contain the substrate binding site and phosphoryl transfer region (Hofmann, 1997). There is a hinged region between C2 and C3. In the inactive conformation, C3 and C4 are in juxtaposition with C2 and C1 respectively. Upon activation, the protein opens and the catalytic domain (C4) becomes accessible to the substrate (Nishizuka, 1984 & 1986; Walker and Watson, 1993; Hofmann, 1997).

1.4.5.2 Activators:

Conventional and novel PKCs require Ca^{2+} and phospholipid, particularly PS, for activation (Nishizuka, 1984; Nishizuka 1986; Baldassare *et al.*, 1992; Blobe *et al.*, 1995; Wakelam, 1998). Activation occurs by a two-step process: first Ca^{2+} and PS recruit inactive PKC from the cytosol to the membrane where it remains inactive; and second PKC is activated upon interaction with DG in the membrane. DG serves to increase the affinity of PKC for Ca^{2+} and PS so that physiological levels are able to active PKC. Other phospholipids also affect PKC activity. For example, PE lowers the threshold requirement for Ca^{2+} for PKC activation but PC and sphingomyelin have inhibitory effects on activation (Nishizuka, 1984; Blobe *et al.*, 1995).

Synthetic DGs, such as OAG (1-oleoyl-2-acetyl-glycerol) activate PKC. Evidence suggests that OAG intercalates into the phospholipid bilayer and directly activates PKC without interaction with any cell surface receptors (Kaibuchi *et al.,* 1983).

A number of synthetic agents, such as the phorbol esters, phorbol-12 myristate-13-acetate (PMA) and phorbol-12, 13-dibutyrate (PDBu), are synthetic compounds that have been used extensively to stimulate PKC and study its cellular function. PMA stimulates PKC activity by increasing its affinity for Ca^{2+} thereby activating the enzyme without increases in intracellular Ca^{2+}. On the other hand, PDBu requires Ca^{2+} and phospholipids to bind to PKC for activity (Nishizuka, 1984; Nishizuka 1986; Baldassare

et al., 1992; Blobe *et al.*, 1995). While phorbol esters are capable of inducing cellular changes on their own, their effects are greatly potentiated by Ca^{2+} (Adunyah and Dean, 1986). Phorbol esters are relatively selective for PKC although some non-PKC, phorbol ester sensitive receptors, such as η-chimaerin, have been identified (Adunyah and Dean, 1986). Atypical PKCs are insensitive to DGs and phorbol esters and have been reported to be regulated by a variety of lipids including PIP_3, PA and ceramides (Wakelam, 1998).

1.4.5.3 Function

In resting platelets 16%, 14%, 40% and 5% of PKC-α, PKC-β, PKC-δ and PKC-ζ, respectively, is membrane bound (Baldassare *et al.*, 1992). Platelet stimulation by different agonists produces different effects on the PKC isoform profiles. Stimulation with thrombin causes a rapid translocation of PKC-α, PKC-β and PKC-ζ without any effect on the distribution of PKC-δ (Moussazadeh and Haimovich, 1998). In contrast, PMA causes translocation of PKC-α, PKC-β and PKC-ζ to the membrane, with PKC-α being the slowest (Crabos *et al.*, 1992).

PKC stimulation by phorbol esters or OAG leads to aggregation in the absence of arachidonic acid or TxA_2 production (Kaibuchi *et al.*, 1983; Jerushalmy *et al.*, 1988; MacIntyre *et al.*, 1985a). Conversely, inhibition of PKC decreases, but does not abolish, aggregation by U46619 or ADP in aspirinated platelets, indicating that PKC activation promotes, but is not essential, for aggregation (Pulcinelli *et al.*, 1995). Phorbol ester-induced aggregation occurs without shape change, indicating that increased intracellular Ca^{2+} is not necessary for PKC induced aggregation (Font *et al.*, 1992; Walker and Watson, 1993; Pulcinelli *et al.*, 1995).

Activation of PKC is essential for serotonin secretion (Nishizuka, 1986). Stimulation of PKC by any agonist invariably leads to the phosphorylation of pleckstrin, a 47 kDa protein, which has been widely accepted as a marker for PKC activity (McNicol and Gerrard, 1997). Given the apparent close relationship between pleckstrin phosphorylation and secretion, popular opinion links the two events (Habib *et al.*, 1999). This is supported by histological studies showing that PMA changed the number of intramembranous particles suggested that PKC activation alters platelet membranes making them prone to cohesion in the presence of lower concentrations of Ca^{2+}, and thus

facilitating secretion (Jerushalmy *et al.*, 1988; Walker and Watson, 1993). However, low concentrations of OAG phosphorylate pleckstrin without significant serotonin secretion suggesting a dichotomy between pleckstrin phosphorylation and secretion (Kaibuchi *et al.* 1983). Recent work with myristoylated alanine-rich C kinase substrate (MARKS) has also been implicated in secretion (Elzagallaai *et al.*, 2000) and likely accounts for this difference.

Another prominent protein that is phosphorylated by PKC is the light chain of myosin (MLC). PKC phosphorylation of MLC occurs at different sites than Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) and appears to be Ca^{2+}-dependent (Kaibuchi *et al.*, 1983; Walker and Watson, 1993).

1.4.5.4 Inhibitors:

A wide variety of compounds have been reported to inhibit PKC. However, relatively few show significant selectivity for PKC over other kinases such as calmodulin-dependent kinases, cAMP-dependent kinases or tyrosine kinases. For example, staurosporine has been used extensively as a PKC inhibitor, although it also inhibits tyrosine kinases (Fallon, 1990).

Rö31-8220 is a selective inhibitor of PKC, inhibiting pleckstrin phosphorylation and secretion by numerous agents (Pulcinelli *et al.*, 1995). *In vitro*, Rö31-8220 inhibits PKCα, PKCβ, PKCγ and PKCε (Toullec *et al.*, 1991; Wilkinson *et al.*, 1993). Calphostin C inhibits phorbol ester binding to the regulatory domain on PKC, but does not compete with Ca^{2+} or phospholipid. Calphostin C reportedly inhibits PKC 1000 times more than other protein kinases such as cAMP-dependent kinase and tryosine specific kinase (Tao *et al.*, 1992).

Hofmann (1996) collated an extensive list of drugs that also interact with the catalytic domains (C3 and C4 domains) or regulatory domains (C1 and C2 domains) of PKC.

1.4.5.5 PKC and Ca^{2+}

PKC has an intricate relationship with intracellular Ca^{2+}. As outlined above PKC works synergistically with Ca^{2+} to effect a full range of cellular responses. However in

some situations PKC appears to regulate Ca^{2+} homeostasis in the platelet. For example, in platelets treated with the Ca^{2+} ionophore ionomycin, the rate of Ca^{2+} decay increases following stimulation with PMA, suggesting a PKC-mediated role in the operation of a plasma-membrane associated Ca^{2+}-ATPase (Pollock *et al.*, 1987; Cavallini and Alexandre, 1994). Several studies have shown that PMA increases Ca^{2+} sequestration into internal stores (Adunyah and Dean; 1986; Tao *et al.*, 1992; Nishio *et al.*, 1994), although this may occur in the absence of PKC activation (Adunyah and Dean; 1986). It has been widely shown that PMA-induced aggregation is enhanced by co-incubation with Ca^{2+} ionophores such as A23187 (Font *et al.*, 1992).

Although there is a synergy between PKC and Ca^{2+} in the platelet activation process, there are a number of paradoxical reports in which stimulation of PKC results in an inhibition of platelet function. Pre-treatment with phorbol esters inhibited the formation of PA (an indicator of phosphoinositide metabolism) and the increase in Ca^{2+} induced by platelet activating factor (PAF), vasopressin, A23187 and thrombin suggesting a feedback regulatory role for PKC (MacIntyre *et al.*, 1985a; Font *et al.*, 1992). Similarly the inhibition of PKC by Rö31-8220 enhances arachidonic acid release in collagen stimulated platelets, suggesting that PKC may have a negative feed back role in arachidonic acid formation (Krishnamurthi *et al.,* 1987; Lockhart *et al.,* 2001).

1.4.6 Tyrosine Kinases

Several tyrosine kinases, including the Src family (Src, Fyn, Yes and Hck), Lyn, FAK, Tyk, JAK1, JAK2 and Syk have been identified in platelets (Shattil *et al.,* 1998; McNicol, 2005). Src family tyrosine kinases are constitutively tyrosine phosphorylated in resting platelets. Src and Fyn both associate with PI-3 kinase and Yes, Fyn and Lyn all associate with the GTPase Activating Protein (GAP) of the low molecular mass GTP binding protein $p21^{ras}$.

1.4.7 Mitogen-Activated Protein (MAP) Kinases

In addition to PKC (see section 1.3.4), platelets contain multiple serine/ threonine protein kinases, of which much attention has focused on the Mitogen-Activated Protein Kinase (MAPK) family. This is a super family of proline-directed, serine-threonine

kinases all lie within protein kinase cascades consisting of at least three sequentially activated enzymes. In general terms, each MAPK is activated by a MAPK kinase (MAPKK) through common mechanisms involving phosphorylation of regulatory threonine and tyrosine residues within the activation lip. Thus MAPKKs fall within a relatively rare class of protein kinases with dual specificity toward Ser/Thr and Tyr residues on exogenous substrates. MAPKKs in turn are activated by phosphorylation at Ser/Thr residues by MAPKK kinase (MAPKKKs), which in turn are activated by signals from receptors on the cell surface. This can occur through receptor tyrosine kinase associated or G-protein coupled receptors, although different cascades are evoked in each case (Lewis *et al.*, 1998 ; Cobb, 1999; Cowan and Storey, 2003).

MAPKs are classified according to three major signalling pathways (Borsch-Haubold *et al.*, 1996; Lewis *et al.*, 1998 ; Cobb, 1999; Sundaresan and Farndale, 2002): extracellular signal regulating kinases (ERKs);stress activated protein kinases (SAPKs), or c-jun N-terminal kinases (JNK); $p38^{MAPK}$.

1.4.7.1 ERK

Of the ERK family, ERK1 ($p44^{MAPK}$) and ERK2 ($p42^{MAPK}$) have been studied the most, although other, less well characterized members, such as ERK3, ERK4, ERK5, ERK6 and ERK7, have been identified. In mammalian cells, the cascades involving ERK1/2 have been linked to regulation of cell growth and differentiation (Schaeffer and Weber, 1999; Cowan and Storey, 2003).

ERK1/2 are specifically recognized and phosphorylated at two sites, a tyrosine and a threonine, by MAPKKs 1 and 2 (Cobb, 1999; Borsch-Haubold *et al.*, 1996; Lewis *et al.*, 1998). In platelets, phosphorylation of both sites is required for high ERK activity (Makashima *et al.*, 1994; Bugaud *et al.*, 1999). Activation of the ERK cascade can be initiated by tyrosine kinase or G-protein linked receptors.

Stimulation of receptor tyrosine kinases facilitates the binding of adaptor molecules containing SH2, or other phosphotyrosine binding domains, to the receptor. The SH3 domains of the adaptor molecules such as Grb2 and Shc, link the receptor to the guanine nucleotide exchange protein son of sevenless (Sos). Sos enhances GTP binding to membrane-bound GTPase, Ras, which in turn binds to members of the Raf family.

Rafs are not associated with the membrane. MAPKKs bind to Rafs and ERKs bind to the N-terminal of MAPK (Leo & Schraven, 2000; Lopez-Ilasaca, 1998; Cobb, 1999).

Ligand binding to G-protein receptors results in the dissociation of Gα and Gβγ-subunits, and to the stimulation of PLC (Lewis *et al.*, 1998). Gβγ and PLC each activate a different sequence of intracellular signals. Gβγ leads to tyrosine phosphorylation of Shc and progression through the cascade outlined above. The tyrosine kinases Src and Syk are implicated in this process. Sensitivity to wortmannin also suggests a role for PI3K downstream of Gβγ and upstream of Ras (Lewis *et al.*, 1998). Gα stimulates the activation of PLC resulting in the generation of DG and IP_3. Elevation of intracellular Ca^{2+} by IP_3 and other mechanisms can stimulate MAPK (Lewis *et al.*, 1998).

The role of ERK in platelets is ambiguous. ERK1/2 associate with the contractile cytoskeleton and have been implicated in store-mediated Ca^{2+} entry, independent of both PKC and the actin cytoskeleton. Inhibition of ERK activation inhibits PKC-induced aggregation, has no effect on pleckstrin phosphorylation, $cPLA_2$ phosphorylation, aggregation by thrombin, collagen, arachidonic acid, U46619 or PMA. Inhibition of PKC activation inhibited ERK phosphorylation (Borsch-Haubold *et al.*, 1996; Sundaresan and Farndale, 2002; McNicol *et al.*, 2003).

1.4.7.2 JNK

There are three types of JNKs: JNK1, JNK2 and JNK3. JNK1/2 are gene products of alternate splicing and are expressed in many tissues, but JNK3 is found only in the brain. JNK/SAPKs are phosphorylated by MAPKK4 and MAPKK7 (Borsch-Haubold *et al.*, 1996; Lewis *et al.*, 1998; Sundaresan and Farndale, 2002). JNKs function mainly in environmental stress responses such as heat shock, osmotic stress, inflammation, ischemia, and ultraviolet exposure (Schaeffer and Weber, 1999; Cowan and Storey, 2003). JNK1 has been identified in platelets, but its function is unknown (Makashima *et al.*, 1994; Bugaud *et al.*, 1999).

1.4.7.3 $p38^{MAPK}$

The p38 subfamily has been categorized as stress-activated protein kinases and contains five isoforms (α, β, γ, δ and p38-2), all of which have been detected in platelets.

p38 MAPKs are activated by MAPKK3 and MAPKK6 (Borsch-Haubold *et al.*, 1996; Sundaresan and Farndale, 2002). MAPKK3 selectively phosphorylates $p38^{MAPK}$. MAPKK6 is closely related to MAPKK3 (80% homology) and also phosphorylates $p38^{MAPK}$, although its basal activity is approximately 300-fold higher than that of MAPKK3 (Lewis *et al.*, 1998).

Activation of $p38^{MAPK}$ leads to activation of MAPK-activated protein kinases 2 and 3, which in turn phosphorylates small heat shock protein 27 (HSP27) involved in actin remodelling. This suggests a role for $p38^{MAPK}$ in cytoskeletal reorganization (Schaeffer and Weber, 1999; Cowan and Storey, 2003).

1.4.7.4 MAPK Activation in Platelets

In platelets, stimulation with collagen causes rapid, but transient, phosphorylation of $p38^{MAPK}$. Reports of the effect of collagen-induced $p38^{MAPK}$ activation on $cPLA_2$ activity in platelets have been conflicting. High concentrations of collagen activate $p38^{MAPK}$, primarily the α isoform, and subsequent phosphorylation of $cPLA_2$ (Borsch-Haubold *et al.*, 1997). However, arachidonic acid release in response to low concentrations of collagen does not involve the $p38^{MAPK}$ (McNicol and Jackson, 2003). Similarly, when cyclooxygenase is blocked, inhibition of $p38^{MAPK}$ prevented phosphorylation of $cPLA_2$ in collagen stimulated platelets (Borsch-Haubold *et al.*, 1997).

Thrombin phosphorylates all isoforms of $p38^{MAPK}$ (McNicol *et al.*, 2003), in addition to $p38^{MAPK}$ substrates Mnk1 and PRAK1. ERK1/2 is also phosphorylated, but distal to PKC activity. In thrombin-stimulated platelets, $p38^{MAPK}$ phosphorylation only partially activates $cPLA_2$ indicating another kinase is involved in $cPLA_2$ phosphorylation. In thrombin-stimulated platelets $cPLA_2$ is insensitive to ERK blockade with PD 98059 and completely blocked by the $p38^{MAPK}$ inhibitor SB20580 (Lewis *et al.*, 1998). Inhibition of phosphorylation of $cPLA_2$ by PD 98059, a MAPK inhibitor, could be detected in FcγRIIA cross-linked platelets, suggesting that different stimuli might employ the ERK 1/2 and the $p38^{MAPK}$ pathways to different extents to regulate $cPLA_2$ (Borsch-Haubold *et al.*, 1997).

1.4.7.5 MAPK Inhibitors

Several compounds have been reported to inhibit the MAPK pathways. SB203580 is an inhibitor of the $p38^{MAPK}$ pathway, whereas PD 98059 and U0126 are both MAPK inhibitors. PD 98059 acts by binding to the inactivated form of MAPK, preventing the phosphorylation through the activity of c-Raf (Borsch-Haubold *et al.*, 1996; McNicol & Jackson, 2003). Both PD 98059 and SB203580 have also been shown to inhibit cyclooxygenase. Okadaic acid (OA) and fostriecin are inhibitors of PP2A (Borsch-Haubold *et al.*, 1997).

1.4.8 Phospholipase A_2

The phospholipase A_2 (PLA_2) family is a group of enzymes with varying sensitivity to Ca^{2+}, each of which hydrolyses phospholipids at the sn-2 position. This leads to the production of fatty acids, primarily arachidonic acid, and of lysophospholipids (Gijon and Leslie, 1999). In platelets, these enzymes are classified in two general categories: secretory ($sPLA_2$) and cytosolic ($cPLA_2$). The secretory form is sensitive to higher (mM) concentrations of intracellular Ca^{2+} than the cytosolic form (ηM-μM) (Leslie, 1997; Gijon and Leslie, 1999). Although Ca^{2+}-insensitive forms of PLA_2 have been detected in other cell types, to date they have not been identified in platelets (Mayer and Marshall, 1993).

1.4.8.1 Secretory PLA_2

$sPLA_2$ is a 14-kDa enzyme that requires mM levels of Ca^{2+} for enzymatic activity (Murthy *et al.*, 1995). It is bound to the luminal surface of secretory granules and secreted upon platelet activation, exerting its effects on platelet extracellular substrates (Faili *et al.*, 1994; Nakashima *et al.*, 1997). Unlike $cPLA_2$, $sPLA_2$ does not demonstrate sn-2 acyl substrate specificity. Snitko *et al.* (1997) demonstrated that PA was a preferred substrate over PC, PE, PS and phosphatidylglycerol (PG). $sPLA_2$ activity generates lysophosphatidic acid, and in platelets it may be the major source of lysophosphatidic acid (Snitko *et al.*, 1997). Its mechanism involves two kinetically distinct steps: adsorption of PLA_2 to the interface and subsequent substrate binding to the active site (Mayer and Marshall, 1993).

In platelets agonists such as thrombin and U46619 stimulate sPLA2 activation. The activation of $sPLA_2$ also parallels the activation of PLC by these agonists although $sPLA_2$ activity it is not dependent on PLC since inhibition of PLC by neomycin inhibited inositol phosphate formation without affecting $sPLA_2$ (Faili *et al.*, 1994).

1.4.8.2 Cytosolic PLA_2:

$cPLA_2$ is an 85 kDa enzyme that is sensitive to μM concentrations of intracellular Ca^{2+} and demonstrates a substrate selectivity for *sn*-2 arachidonate containing phospholipids. The N-terminal, Ca^{2+}-dependent, phospholipid-binding domain shares homology with the C2 domains in the conventional isoforms of PKC, PLCγ1, $p120_{GAP}$ and a number of other enzymes (Leslie, 1997).

Activation of $cPLA_2$ releases sn-2 arachidonic acid followed by the sn-1 fatty acid of DG. PC is the predominant substrate, with hydrolysis of arachidonyl residues from the sn-2 position of PC and PE both proceeding more readily than the hydrolysis of linoleoyl, oleate or palmitate residues from the sn-2 position of either phospholipid. $cPLA_2$ is estimated to release 1-2 ηmoles arachidonic acid/min/10^9 platelets in thrombin stimulated platelets (Kramer *et al.*, 1993; Leslie, 1997).

Ca^{2+} is necessary for $cPLA_2$ binding to membrane or phospholipid vesicles but is not required for its catalytic activity. At resting Ca^{2+} (~100 ηM), $cPLA_2$ is soluble in the platelet cytosol while increasing Ca^{2+} to 0.3-1.0 μM triggers binding of $cPLA_2$ to platelet membranes (Mayer and Marshall, 1993). The fragment of $cPLA_2$ containing the Ca^{2+} binding region binds to membranes at the same Ca^{2+} concentration as the intact enzyme. C-terminal fragments that lack the C2 domain, do not bind to the membrane, although they retain their catalytic activity, suggesting a separation of membrane translocation and catalytic activity (Kramer *et al.*, 1993; Leslie, 1997; Nakashima *et al.*, 1997). However, some agonists (e.g. epinephrine) activate $cPLA_2$ without increasing intracellular Ca^{2+}. Mechanisms such has the possible involvement of Na^+/H^+ exchanges have been proposed for initial $cPLA_2$ activation in response to these agonists (Seiler *et al.*, 1995). A link between G-protein activity and $cPLA_2$ stimulation has also been proposed (Nakashima *et al.*, 1997).

The activation of the catalytic activity of $cPLA_2$ is thought to occur through phosphorylation. Some studies have suggested that the phosphorylation and activation of $cPLA_2$ occurs by a member, or members, of the mitogen-activated protein (MAP) kinase family of enzymes (Kramer *et al.*, 1993). MAP kinases have been shown to phosphorylate $cPLA_2$ on serine residues, thereby increasing activity (Nemenoff *et al.*, 1993). Studies in human platelets show that thrombin and SFLLRN, a thrombin receptor agonist peptide, induced different activation profiles for $cPLA_2$ (Kramer *et al.*, 1995). Compared to thrombin, SFLLRN induces smaller increases in arachidonic acid release, more transient elevation of Ca^{2+}, incomplete phosphorylation of $cPLA_2$, and does not stimulate the MAP kinases ERK1 nor ERK2 (Kramer *et al.*, 1995). High doses of collagen stimulate the phosphorylation of cPLA2, which is unaffected by Rö31-8220 (Borsch-Haubold *et al.*, 1995; Leslie, 1997). Interestingly Rö31-8220 inhibited collagen-induced MAPK activation indicating MAPK is regulated down stream of PKC in collagen stimulated platelets (Lockhart and McNicol, 1999). Stimulation of PKC by PDBu induces pleckstrin phosphorylation, MAPK activation and $cPLA_2$ activation each of which are inhibited by pre-treatment with Rö31-8220, placing $cPLA_2$ activation downstream of PKC (Borsch-Haubold *et al.*, 1995). This demonstrates that $cPLA_2$ can be activated through different mechanisms and to varying extents, and that the phosphorylation caused by the ERK1 or ERK2 does not appear to be essential (Kramer *et al.*, 1993; Kramer *et al.*, 1995; Leslie, 1997; Nakashima *et al.*, 1997).

The $p38^{MAPK}$ form of MAP kinase is activated in response to thrombin, collagen (low doses), U46619, and Ca^{2+} ionophores (Kramer *et al.*, 1995, Saklatvala *et al.* 1996). Two studies have concluded that, although thrombin causes the proline-directed phosphorylation of $cPLA_2$ by $p38^{MAPK}$, this phosphorylation is not required for arachidonic acid mobilization (Kramer *et al.* 1996, Leslie, 1997). In contrast it has been suggested that $p38^{MAPK}$ is important in the regulation of $cPLA_2$ in collagen-stimulated platelets (Börsch-Haubold *et al.*, 1997).

The role of other kinases, such as PKC and PKA, on $cPLA_2$ activity has also been examined. PKC can phosphorylate $cPLA_2$ i*n vitro*, but may not lead to increased activity. Various studies have found activation of PKC to have no affect on $cPLA_2$ activity

(Watson *et al.*, 1984) or to stimulate MAPK and $cPLA_2$ activation (Borsch-Haubold *et al.*, 1995; Leslie, 1997).

1.4.8.3 $cPLA_2$ Inhibitors

Few selective inhibitors of $cPLA_2$ have been identified. Arachidonyl trifluoromethyl ketone ($AACOCF_3$) is an inhibitor that has been shown to inhibit recombinant human $cPLA_2$ in a mixed micelle assay and PLA_2 activity isolated from the cytosolic fraction of platelet preparations. $AACOCF_3$ inhibits thrombin-induced arachidonic acid release in platelets prepared in the presence of indomethacin, although aggregation remains unaffected (Street *et al.*, 1993; Bartoli *et al.*, 1994; Riendeau *et al.*, 1994). Furthermore, $AACOCF_3$ pre-treatment does not affect collagen-induced arachidonic acid liberation, but does inhibit collagen (low concentrations)- and thrombin-generated TxA_2 production and collagen-induced PA production (McNicol and Nickolaychuk, 1995). These results not only suggest that $AACOCF_3$ may also affect cyclooxygenase but also that collagen-induced arachidonic acid release may not involve $cPLA_2$ (McNicol and Nickolaychuk, 1995).

Mepacrine, bromophenacylbromide and OOPC (oleoyloxyethylphosphocholine) are other putative $cPLA_2$ inhibitors (Akiba *et al.*, 1993; Seiler *et al.*, 1995), however their selectivity and effectiveness have been questioned.

1.4.9 Cyclooxygenase

Cyclooxygenase (COX), also known as prostaglandin endoperoxidase, converts arachidonic acid to unstable cyclic peroxides (PGH_2 and PGG_2) from which prostaglandins (PGE_2, PGD_2 and $PGF_{2\alpha}$), prostacyclin (PGI_2) and TxA_2 are derived (Patrignani *et al.*, 1994; Smith *et al.*, 1997). Two isoforms, COX-1 and COX-2, have been characterized and the existence of a third variant, COX-3 is under dispute.

COX-1 is constitutively expressed in most tissues and is localized in the endoplasmic reticulum and nuclear envelope. This enzyme is responsible for the physiological production of prostaglandins and appears to use arachidonic acid produced by $cPLA_2$ (Smith *et al.*, 1997). Only COX-1 is present in platelets, (Kurumbail *et al.*, 1996; Smith *et al.*, 1997; Geis, 1999).

COX-2 is induced by cytokines, mitogens and endotoxins in inflammatory cells and, while present in the endoplasmic reticulum and nuclear envelope, it is more prevalent in the nuclear envelope. This enzyme is associated with the elevated production of prostaglandins during inflammation. COX-3 has been hypothesized based on the fact than acetaminophen does not have anti-coagulant activity, has weak anti-inflammatory activity and is a weak inhibitor of COX-1 and COX-2 in vitro. Acetominophen also reduces levels of prostaglandin metabolites in urine suggesting some cyclooxygenase activity, but does not reduce synthesis of prostaglandins by blood platelets or by the stomach mucosa (Botting, 2000).

COX inhibitors are broadly classified into four types (Kurumbail *et al.*, 1996; Smith *et al.*, 1998):

1. Aspirin is an irreversible inhibitor which inactivates both COX-1 and COX-2 by acetylating an active-site serine and interfering with proper binding of arachidonic acid;
2. Ibuprofen and mefenamate are reversible, competitive inhibitors of COX-1 and COX-2;
3. Indomethacin and flurbiprofen are slow, time-dependent inhibitors of COX-1 and COX-2
4. Celecoxib and rofecoxib are slow, time-dependent inhibitors selective for COX-2 and have anti-inflammatory activity with minimal gastric or platelet-related side effects.

1.4.10 Low Molecular Weight GTP binding proteins

In addition to various forms of Gα (section 1.4.1), platelets have a number of low molecular weight proteins (21 to 28 kDa), which can bind guanine nucleotides. Most of the low molecular weight GTP-binding proteins are of the *ras* superfamily, e.g. ras (rap1A, rap1B, rap2B, ralA), rho (rhoA, rac1, G25K), rab (rab3A, c25KG, ram), and arf. The sequence of the ras-related proteins is most highly conserved in the domains thought to be most directly involved in the binding and hydrolysis of GTP, the same regions which show the greatest homology with Gα. These proteins are present in either the active, GTP-bound from or the inactive, GDP-bound form. The ras proteins are essential

components of receptor-meditated signal transduction pathways, while members of the rab and raf subfamilies are through to regulate the transport of vesicles between intracellular compartments. The inherent slow GTPase activity of low molecular weight GTP binding proteins converts them from the active to the inactive form. GAPs enhance the rate of hydrolysis, particularly $p120^{ras}$ GAP in platelets (Manning & Brass, 1991; Ridley and Hall, 1992).

1.5 THESIS OBJECTIVES

This thesis focused on the mechanism of the release of arachidonic acid from human platelets in response to low concentrations of collagen. The specific objectives were to evaluate the contribution of the following enzyme pathways depicted in the figure below (Figure 1), using pharmacological inhibitors:

1. PLC, which generates DG;
2. PKC which is activated by DG and in turn phosphorylates $cPLA_2$ (McNicol & Shibou, 1998);
3. $cPLA_2$ which liberates arachidonic acid from membrane phospholipids;
4. DG lipase (DGL), which liberates arachidonic acid from DG.

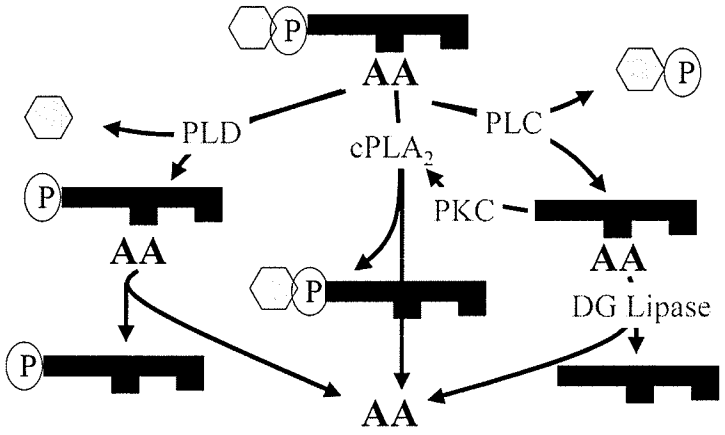


Figure 1 Potential mechanisms for arachidonic acid release from phospholipids with arachidonic acid in the *sn*-2 position.

2 MATERIALS AND METHODS

2.1 MATERIALS

The Spherisorb-hexyl 5 µm, 250x4 mm HPLC column was obtained from CSC Inc., Montreal, PQ and the Spherisorb CN 5 µm, 150 mm x 4.6 mm guard columns from Alltech Associates Inc., Deerfield, IL.

U73122 and Rö31-8220 were obtained from Calbiochem (La Jolla, CA.), prepared as stock solutions in DMSO, and stored at –20 °C until use. U46619 was obtained from Cayman Chemicals (Ann Arbour, MI), collagen from Helena Laboratories (Beaumont, TX), and PMA from Sigma (St. Louis, MO). [^{3}H]-serotonin and [^{3}H]-arachidonic acid was obtained from Amersham, Oakville, ON. Arachidonic acid and heptadecanoic acid, used as HPLC standards, were obtained from Cayman Chemicals (Ann Arbour, MI).

Fura-2 was obtained from Molecular Probes (Eugene, OR). Bromophenylacyl bromide from Calbiochem, (La Jolla, CA), and 18-crown-6 from Aldrich Chemicals (Milwaukee, MI) were both prepared in acetonitrile. Gluteraldehyde and paraformaldehyde were of electron microscopy quality. All other materials were of the highest grade available.

2.2 BUFFERS

2.2.1 HEPES-Tyrodes Buffer

134 mM NaCl	12 mM NaHCO3
2.9 mM KCl	0.34 mM Na_2HPO_4
1 mM $MgCl_2$	10 mM HEPES
5 mM dextrose	0.3% bovine serum albumin (BSA)
pH 7.4	

2.2.2 Acid, citrate, dextrose anticoagulant (ACD)

3.8 mM citric acid
7.5 mM trisodium citrate
125 mM dextrose

2.2.3 White's Saline Solutions

5% White's solution A:

2.4 M NaCl
0.1 M KCl
46 mM $MgSO_4$
64 mM $Ca(NO_3)_2.4H_2O$
pH 7.4

5% White's solution B:

0.13 mM $NaHCO_3$
8.4 mM $Na_2HPO_4.7\ H_2O$
3.8 mM anhydrous KH_2PO_4
0.01% phenol red
pH 7.4

2.2.4 Borate Buffered Saline (BBS)

0.62% w/v H_3BO_3
0.95% w/v NaH_2BO_3
0.44% w/v NaCl

2.2.5 HEPES Buffer for Fura 2 studies

145 mM NaCl 5 mM KCl
1 mM $MgSO_4$
10 mM HEPES
10 mM dextrose
pH 7.4

2.2.6 Glutaradehyde solutions

0.1% glutaradehyde:

0.5 ml 5% White's solution A
0.5 ml 5% White's solution A
0.125 ml 8% glutaraldehyde
8.875 ml distilled, deionised water

3% glutaradehyde:

0.25 ml 5% White's solution A
0.25 ml 5% White's solution A
1.875 ml 8% glutaraldehyde
2.62 ml distilled, deionised water

2.3 METHODS

2.3.1 Platelet Isolation.

Human platelets were isolated from the blood of healthy volunteers who had given written informed consent. For aggregation, secretion, fibrinogen receptor expression and electron microscopy studies, volunteers were free of aspirin or any other medication that may interfere with platelet function for at least 10 days prior to the experiment. Arachidonic acid release studies did not require that volunteers be free of non-steroidal anti-inflammatories.

Blood was obtained by venipuncture and collected in non-vaccumized syringes containing 1.9 ml of the anti-coagulant ACD per 8.1 ml of blood. Platelet rich plasma (PRP) was obtained by centrifugation at 800g for five minutes. PRP was centrifuged at 2000g for 10 minutes to obtain isolated platelets that were resuspended in HEPES-Tyrodes buffer (McNicol, 1996). Calcium chloride (1 mM final) was added to all platelet suspensions just prior to experimental manipulations.

In all subsequent functional studies, concentrations of agents (agonists and inhibitors) were prepared in 0.9% saline so that the volumes added were 1.0%, or less, of the final reaction volume therefore preventing dilution effects. In those situations where DMSO was the vehicle, stock concentrations were prepared so that the volumes added were 0.5% or less of the final reaction volume so that adverse vehicle effects were minimised. In all cases vehicle controls of agonist or inhibitor were carried out using a volume of vehicle equivalent to the largest volume added.

2.3.2 Platelet Aggregation

Aliquots (0.4 ml) of resuspended platelets were dispensed into cuvettes and stirred in an aggregometer at 800 rpm, 37 °C in the presence of inhibitor or vehicle control for two minutes prior to addition of agonist. Aggregation was monitored photometrically continuously by alterations in light transmission. A decrease in light transmission indicated platelet shape change and an increase reflected a decrease in solution turbidity as a result of the formation of platelet aggregates (McNicol, 1996). Aggregation was

recorded on a chart recorder in which baseline was set at 0 cm and maximum aggregation was set to a chart deflection of 10 cm. Unstimulated platelets were used to set baseline, while buffer was used to reflect maximin transmission.

Aggregation was measured in response to stimulation by collagen (0.25-1 μg/ml), U46619 (0.3-1 μM), and PMA (30 ηM).

2.3.3 Dense Granule Secretion

Dense granule secretion was measured as [^{3}H]-serotonin released in response to agonist stimulation. PRP was incubated at 37°C with 0.5-1.0 μCi[^{3}H]-serotonin/ml PRP for 30 minutes at 37°C. ACD (1 ml per 9 ml PRP) was added and platelets were isolated and resuspended in HEPES-Tyrode buffer.

Aggregation was carried out as described above (see 2.3.2). At the end of the aggregation period (4 minutes for collagen, 3 minutes for U46619 and 7 minutes for PMA), release was terminated by the addition of 0.4 ml of 0.1% gluteraldehyde. The entire solution was transferred to a 2 ml plastic microcentrifuge tube and the platelets were pelleted by centrifugation at 10,000g for 10 minutes. An aliquot (0.4 ml) of supernatant was withdrawn for scintillation counting and the remaining supernatant was discarded. The pellet was digested in 0.1 ml of 88% formic acid at 37°C for one hour, transferred to a plastic 2 ml scintillation tube and the unreleased [^{3}H]-serotonin quantified by liquid scintillation counting. [^{3}H]-serotonin release was calculated (Equation 1) as the total [^{3}H]-serotonin in the supernatant (ie. twice that measured in the 0.4 ml aliquot) and expressed as a percent of total [^{3}H]-serotonin (supernatant plus pellet) (McNicol, 1996).

$$\%\ release = \frac{[^3H]\text{ - serotonin supernatent}}{[^3H]\text{ - serotonin supernatent} + [^3H]\text{ - serotonin pellet}} \times 100$$

$$= \frac{2 \times [^3H]\text{ - serotonin liquid scintillation}}{2 \times [^3H]\text{ - serotonin liquid scintillation} + [^3H]\text{ - serotonin pellet}} \times 100$$

Equation 1: Percent Serotonin Release

2.3.4 Fibrinogen Binding.

Fibrinogen binding was analyzed by flow cytometry using fibrinogen labelled with the fluorescent probe FITC (Shattil *et al.*, 1987; Faraday *et al.*, 1994). All handling of the fluorescent probe and labelled samples was carried out under daylight conditions or under illumination with an incandescent light source to prevent decay of the probe by exposure to fluorescent lighting.

FITC- labelled fibrinogen was prepared by incubating 45 µl of 6.1 mg/ml FITC-Celite in BBS with 25 µl of 40 mg/ml fibrinogen in 0.9% saline for 40 minutes at room temperature. The sample was microcentrifuged for 2 minutes and the supernatant applied to a PD-10 (Sephadex G-25M) column that had been pre-conditioned by consecutive washing with 30 mls each of water, BBS with 0.4% BSA and BBS. The labelled fibrinogen was then separated from unlabelled fibrinogen and excess FITC-Celite by eluting with BBS: the FITC-labelled fibrinogen being collected as the first yellow band to elute from the column. The column was further washed with more BBS until the second yellow band was eluted (which was discarded) and the column stored at -4°C for future use.

The protein concentration was calculated from the absorbance (Abs) measured at 280 and 495 nm as follows:

$$Fibrinogen\ -\ FITC\ Concentrat\ ion\ (mg/ml) = \frac{Asb\,280 - (0.35\ x\ Abs\,495)}{1.55}$$

Equation 2: Protein Concentration

Similarly, the fibrinogen/protein molar ratio was calculated as follows:

$$Fibrinogen/Protein\ Molar\ Ratio\ (mg/ml) = \frac{6.76\ x\ Asb495}{Abs280 - (0.35x\ Abs495)}$$

Equation 3: Fibrinogen/Protein Molar Ratio

Aliquots (0.2 ml) of plasma-free platelet suspensions in HEPES-Tyrodes buffer were incubated with purified FITC-labelled fibrinogen (to yield 18 µg/ml final), 1mM $CaCl_2$ and varying concentrations of inhibitor, or vehicle control, for 1 minute at 37°C. The platelets were then stimulated with 200 ηM PMA for 10 minutes and the reaction

was stopped by transferring 0.04 ml of the treated platelets to 0.36 ml of 0.1% paraformaldehyde in BBS.

Fibrinogen receptor expression was detected by flow cytometry, using an EPICS model 753 flow cytometer, equipped with an argon laser (500 mW, 488 nm). Forward and 90° light scatter measurements were used to establish gates for intact viable platelets. Single parameter, 255-channel, log integral green fluorescent histograms were obtained, and the negative and positive fluorescent populations were resolved.

2.3.5 Electron Microscopy

Aliquots (0.4 ml) of plasma-free platelet suspensions in HEPES-Tyrodes buffer were incubated with inhibitor or vehicle control (DMSO) for 1 minute prior to stimulation with 30 ηM PMA for 10 minutes at 37°C. The platelet activation was stopped and fixing begun by the adding 0.4 ml of 0.1% gluteraldehyde. After at least 15 minutes, the platelets were pelleted by centrifugation, the supernatant removed and 0.4 ml of 3.0% gluteraldehyde added and the samples were allowed to fix for 1-2 hours. The platelets were again pelleted, the supernatant removed and the pellets incubated with 0.4 ml of 1.0% osmium tetroxide solution for 90 minutes at 4°C. After washing twice with distilled water, the pellets were gently disrupted and stained in 3.0% aqueous uranyl acetate overnight. The samples were dehydrated by successive washes in 70% and 90% ethanol for 15 minutes each and two additional 15-minute washes in 100% ethanol. Incubating the pellets in propylene oxide twice for 15 minutes finished the fixing process. The samples were then embedded in 1:1 ratio of propylene oxide and Epon/Araldite resin mixture (17.6% Araldite 502, 22% Epon 812, 58.6% Hardener DDSA and 1.8% accelerator DMP 30) for 1 hour. The propylene oxide/resin mixture was replaced with pure resin and allowed to sit for 4 hours. Finally, the pellets were embedded in fresh resin and polymerised overnight at 60 °C (Israels and Gerrard, 1996). The blocks were then sectioned and mounted.

Four to six micrographs of each treatment were scanned and 16 to 25 platelets digitised using Uthscsa Image Tool software (version 1.21; University of Texas Health Sciences Centre, San Antonio, TX). For each platelet the number of vacuoles counted, and area encompassed by vacuoles and the total platelet area was measured. The number

of vacuoles and the percentage of total platelet area encompassed by vacuoles were used in the statistical analysis.

2.3.6 [^{3}H] Arachidonic Acid Measurements.

Isolated platelets were resuspended in 2 ml of the platelet poor plasma generated in the platelet isolation procedure, and incubated with 10-15 μCi [^{3}H]-arachidonic acid and 100 μM of the dual cyclooxygenase/lipoxygenase inhibitor BWC355 for 1 hour at 37 °C. Following the incubation, 1 ml of ACD was added, the platelets isolated again and resuspended in HEPES-Tyrodes buffer containing fatty acid free BSA. Platelets were incubated at 37 °C until used.

Aliquots (0.4 ml) of suspended platelets, containing 1 mM $CaCl_2$, were gently rocked with inhibitor, or vehicle control, at room temperature for two minutes prior to the addition of agonist, or vehicle control, for a further 10 minutes.

In some studies, the free fatty acids were immediately extracted from the samples either by the method of Bligh & Dryer (see 2.3.8.1) or by a modified Dole's method (see 2.3.8.2). In other studies, a permeabilizing agent (50 μM digitonin or 20 μg/ml saponin) was added for five minutes, the samples centrifuged for 10 minutes to remove the membrane fraction of the platelet, then the free fatty acids extracted from the supernatant by the modified Dole's method (see 2.3.8.2).

Extracted samples were dried under nitrogen, resuspended in 50 ml of chloroform/methanol (1:1) and the applied onto heat-activated silica gel 60 TLC plates which had been previously dried at 200°C for 1 hour and allowed to cool. In addition, 5 μg of arachidonic acid was applied to separate lanes to serve as a reference. The fatty acids were separated using a mobile phase of chloroform/methanol/acetic acid/water (90:8:1:0.8, by vol.). The TLC plate was exposed to iodine and arachidonic acid was identified by comparison with the reference. The plate was then subjected to radiographic scanning and both the counts corresponding to arachidonic acid and total counts for the lane were recorded.

2.3.7 Unlabelled Arachidonic Acid Extraction

Aliquots (0.4 ml) of suspended platelets, containing 1 mM CaCl2, were gently rocked at room temperature in the presence of inhibitor, or vehicle control, for two minutes prior to addition of agonist. Platelets were stimulated with 2-10 μg/ml collagen or 1 μM U46619 for 5 minutes. In some studies samples were incubated with 10-15 μg/ml saponin for 5-10 minutes to permeabilize the platelets. In all cases the free fatty acids were extracted by the modified Dole's method (see 2.3.8.2).

2.3.8 Free Fatty Acid Extractions

2.3.8.1 Bligh and Dyer Extraction

The sample (0.4 ml) was added to 2 ml of chloroform/methanol/10M HCl (55:50:4, by vol.) and thoroughly vortexed. Chloroform and water (0.625 ml each) were added, the sample vortexed again and the phases allowed to separate. If necessary, centrifugation was employed to reduce the emulsion that tended to form at the phase interface. The lower layer, containing the fatty acids, was removed to a separate tube and dried under nitrogen.

2.3.8.2 Modified Dole's Extraction

The sample was transferred to 5 volumes of Dole's mixture (isopropanol/ heptane/ phosphoric acid (2M): 40/10/1). The tube was gently vortexed for approximately 5 to 10 seconds. Approximately 5 minutes after vortexing, 2 volumes of heptane and 3 volumes of water were added. The sample was vortexed again for 5 to 10 seconds. The top layer was removed to another tube and dried under nitrogen at 25 °C. The samples were stored at –20°C if the derivatization was not done the same day.

In those samples prepared for HPLC analysis, heptadecanoic acid (1×10^{-6}M based on the theoretical approximate volume of supernatant) was added to each tube containing Dole's mixture.

2.3.9 Derivatization.

The derivatization of arachidonic acid was based on the method of Puttmann *et al.* (1993). Bromophenylacyl bromide (50 mM in acetonitrile) was used as the chromaphore and 18-crown-6 (5 mM in acetonitrile) as the catalyst. Chromaphore-catalyst mixture (3 µl), KHCO3 (1 mg) and acetonitrile (500 µl) were added to reaction tubes containing extracted samples. The tubes were sealed with Teflon tape, capped securely, gently vortexed and heated at 80-85 °C for 45 minutes. After cooling, the acetonitrile was evaporated and the derivatized sample was resuspended in 100 µl of acetonitrile. The samples were stored at -20°C until separated by HPLC.

2.3.9.1 Arachidonic Measurement by HPLC.

HPLC was performed on a Water system consisting of a Model 441 detector, M-45 pump and U6K injector.

Aliquots of samples (typically 25 µl) were injected on to a Spherisorb-hexyl 5 µm, 250x4 mm column with a Spherisorb CN 5 µm, 150 mm x 4.6 mm guard column. The mobile phase was acetonitrile/water (77/23 by volume) at a flow rate of 1.3 ml/min. Detection was UV at 254 nm and Waters Baseline 810 program was used to collect and analyse data. A standard curve covering a range of arachidonic acid concentrations was prepared and run in parallel for each experiment.

2.3.10 Statistical Analysis.

Data are reported as mean ±S.E. Raw data or reciprocal percent data were analysed by Analysis of Variance techniques. Where appropriate, the LSD-test or Tukey's HSD-test was used to test for significance differences at $p<0.05$.

3 RESULTS

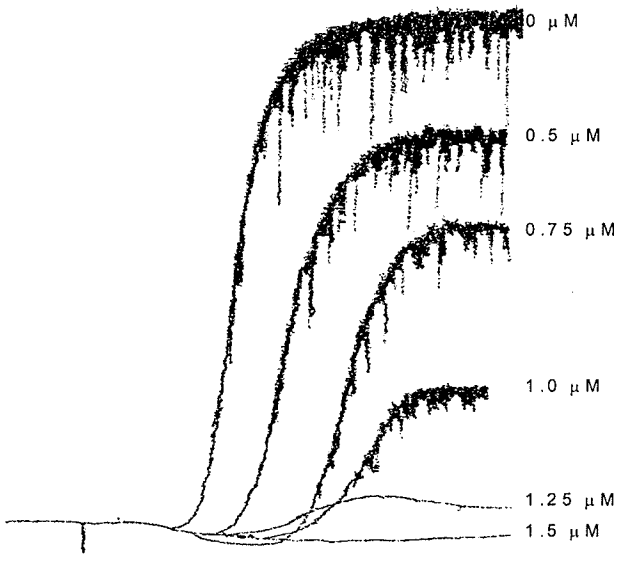
3.1 The role of PLC and PKC in platelet aggregation

Aggregation was measured as light transmission through platelet suspensions. A decrease in light transmission indicated a change in platelet shape from discoid to spherical, while an increase in light transmission reflected a decrease in solution turbidity as a result of the formation of platelet aggregates. In each series of measurements, the maximum dose of inhibitors did not have any effect on light transmission in unstimulated samples, indicating the inhibitors themselves did not stimulate shape change, nor did they induce aggregation.

3.1.1 The effect of PLC and PKC inhibitors on collagen-induced aggregation

The effect of the PLC inhibitor, U73122, was examined on aggregation in response to one dose of collagen, 1.0 μg/ml. Collagen-induced a significant increase in platelet aggregation that was not seen in unstimulated platelets. Pre-incubation with U73122 inhibited aggregation in a dose-dependent manner (Figure 2).

Figure 2 Effects of U73122 on collagen-induced platelet aggregation.



Washed human platelets were incubated with 0.25-1.5 μM U73122 or DMSO for 2 minutes prior to addition of 1 μg/ml collagen. The effect of 3.0 μM U73122 (not shown) on aggregation was the same as 1.5 μM U73122. Aggregation was monitored as a change in light transmission measured in mm chart deflection.

Aggregation was not significantly affected by the lowest dose of U73122 tested (0.25 μM), but was significantly inhibited by 0.5-3.0 μM U73122 (see Table 2). Aggregation was abolished by 0.75 μM or greater of U73122, since light transmission was not statistically different from unstimulated platelets. Consistent with the observations of Bleasdale *et al.* (1990), addition of 3 μM U73122 had no effect on light transmission measured in unstimulated platelets confirming U73122 was without effect on basal PLC activity.

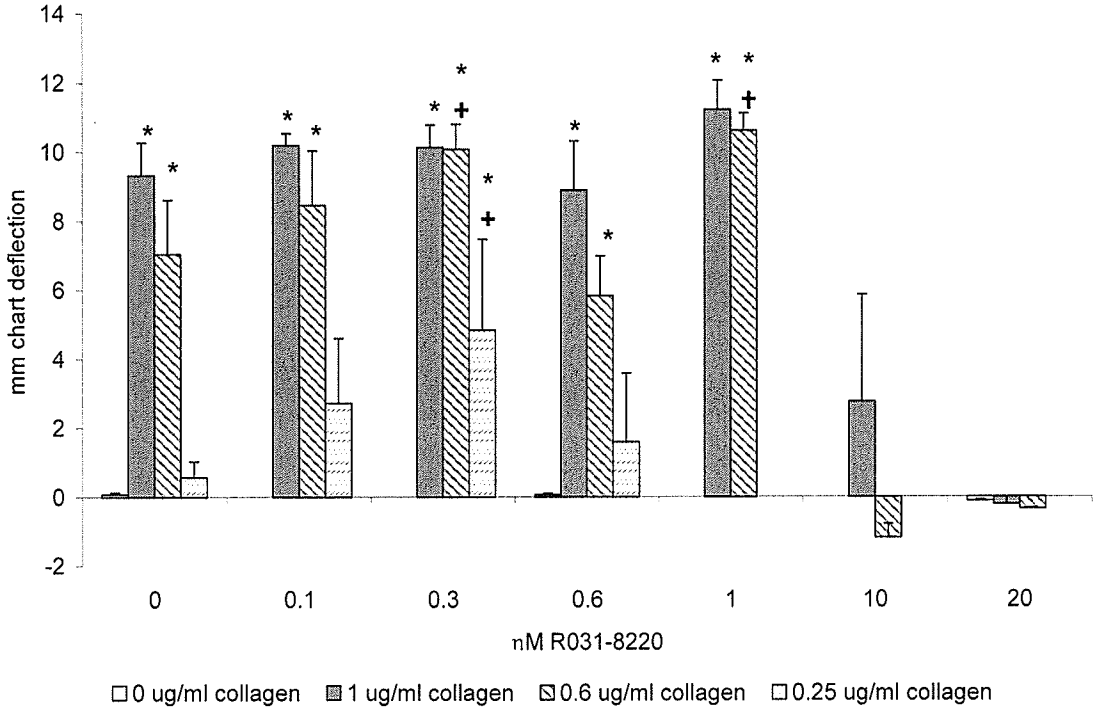
Table 2 Effects of U73122 on collagen-induced platelet aggregation

U73122 (μM)	Collagen (μg/ml)	Aggregation (AU±SE)
0	0	0.06±0.01*
3	0	0.07±0.02*
0	1	10.13±2.92†
0.25	1	9.09±2.87†
0.5	1	3.19±0.85*†
0.75	1	-0.27±0.09*
1	1	-0.10±0.03*
3	1	-0.43±0.13*

Washed human platelets were incubated with 0.25-1.5 μM U73122 or DMSO for 2 minutes prior to addition of 1 μg//ml collagen. Aggregation was monitored as a change in light transmission. Results are mean values (±SE) of six to ten samples. (*) $p<0.05$ vs. collagen alone; † vs. unstimulated.

The effect of the PKC inhibitor, Rö31-8220, was examined on aggregation in response to 1.0, 0.6 and 0.25 μg/ml of collagen (Figure 3). While the two highest doses of collagen (1.0 and 0.6 μg/ml) induced a significant increase in platelet aggregation, the lowest dose of collagen, 0.25 μg/ml, did not. There was a trend for aggregation stimulated by all doses of collagen to increase in the presence of lower concentrations of Rö31-8220 (0.1 and 0.3 μM), where as 10 and 20 ηM Rö31-8220 essentially abolished aggregation.

Figure 3 Effects of Rö31-8220 on collagen-induced platelet aggregation.



Washed human platelets were incubated with 0.1-20.0 μM Rö31-8220 or DMSO for 2 minutes prior to addition of 0.0-1.0 μg/ml collagen. Aggregation was monitored as a change in light transmission. Results are mean values (±SE) of six to ten samples. (*) $p<0.05$ vs. unstimulated; † vs. collagen in the absence of inhibitor for each respective dose of collagen.

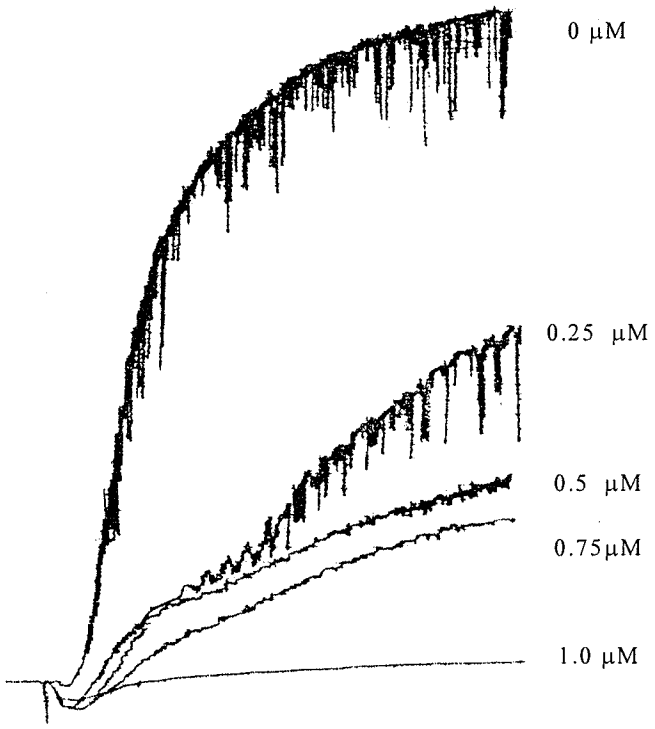
Major observations from Figure 3 are as follows:

1. 1.0 and 0.6 μg/ml collagen caused significant platelet aggregation.
2. Aggregation induced by 0.25 μg/ml collagen was not significantly different from unstimulated platelets.
3. Aggregation by 0.25 μg/ml collagen was significantly increased by 0.3 ηM Rö31-8220
4. Aggregation by 0.6 μg/ml collagen was significantly increased by 0.3 and 1.0 ηM Rö31-8220
5. 10.0 ηM and 20.0 ηM Rö31-8220 abolished aggregation by 0.6 and 1.0 μg/ml collagen.

3.2 Effects of PLC and PKC inhibitors on thromboxane-induced aggregation

Thromboxane A_2 has a short half-life and consequently thromboxane-induced platelet aggregation was induced by the stable endoperoxide analogue, U46619. The effects of PLC or PKC inhibition were examined on aggregation induced by three doses of U46619 (1.0, 0.6 and 0.3 μM) (Figure 4).

Figure 4 Effects of U73122 on U46619-induced platelet aggregation



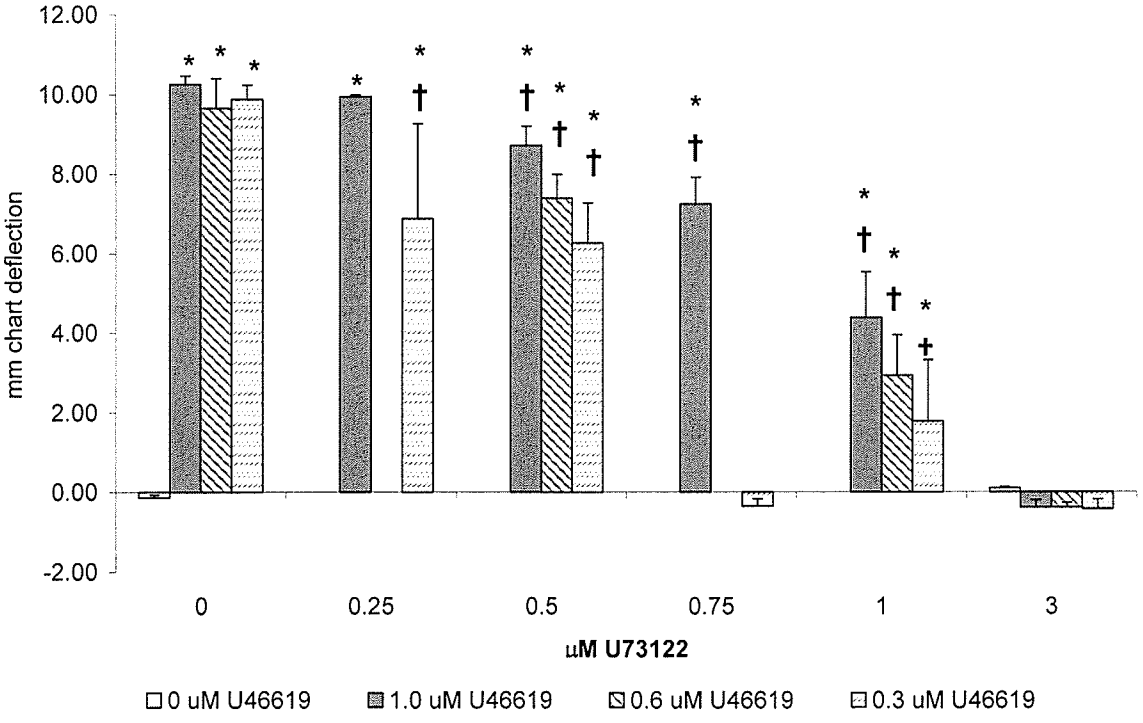
Washed human platelets were incubated with 0.25-1.0 μM U73122 or DMSO for 2 minutes prior to addition of 1 μM U46619. Aggregation was monitored as a change in light transmission measured in mm chart deflection.

U46619 induced a significant increase in platelet aggregation at all concentrations tested (0.3, 0.6 & 1.0 μM). Inhibition of PLC by U73122 caused a dose dependent inhibition of aggregation. While the initial magnitude of aggregation was similar for all three concentrations of U46619, U73122 had greater inhibitory effects on the lower concentrations of U46619 (Figure 5).

Data for each concentration of collagen-inhibitor series are presented in Figure 5. Major observations are as follows:

1. All concentrations of U73122 (0.25-3.0 μM) significantly inhibited aggregation by 0.3 and 0.6 μM U46619.
2. 0.5-3.0 μM U73122 significantly inhibited aggregation by 1.0 μM U46619.
3. Aggregation in response to 0.3 μM U46619 was more variable, therefore aggregation was abolished by 0.75 and 3.0 μM U73122, by only significantly inhibited by 0.25, 0.5 and 1.0 μM U73122.
4. The maximum concentration of U73122 (3.0 μM) had no effect on unstimulated platelets, but universally abolished aggregation in response to U46619.

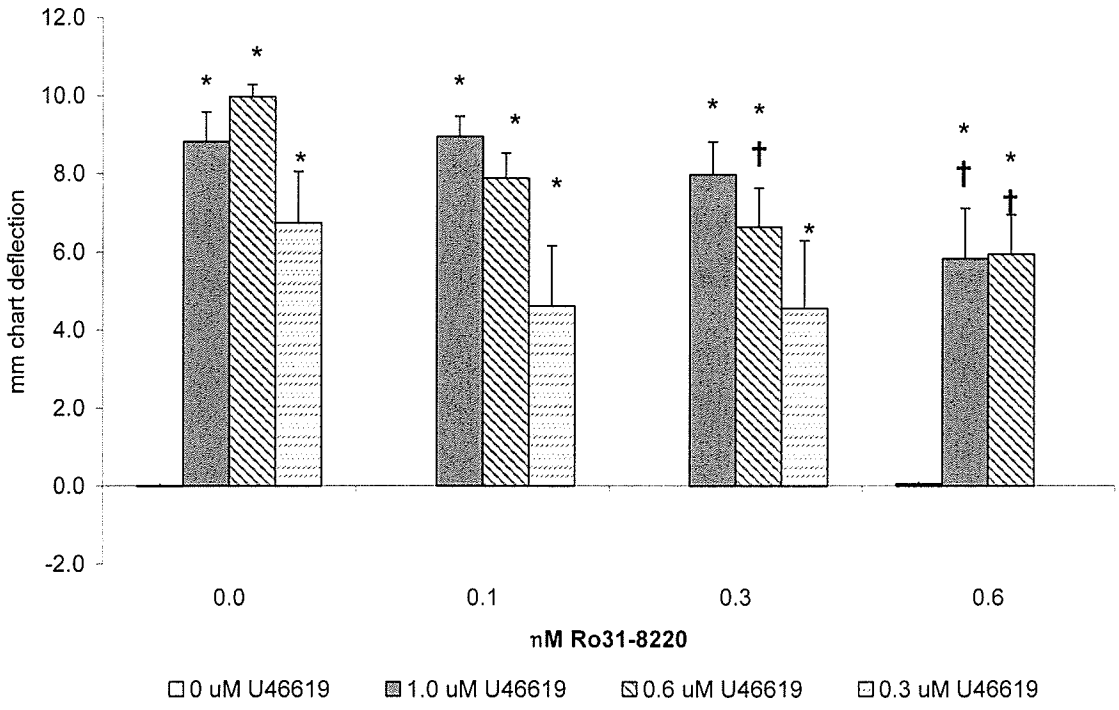
Figure 5 Effects of U73122 on U46619-induced aggregation



Washed human platelets were incubated with 0.25-10.0 μM U73122 or DMSO for 2 minutes prior to addition of U46619 as indicated above. Aggregation was monitored as a change in light transmission. Results are mean values (±SE) of six to ten samples. * $p<0.05$ vs. unstimulated; † vs. U46619 in the absence of inhibitor for each respective dose of U46619.

As with the previous series of studies, U46619 induced a significant increase in platelet aggregation at all concentrations tested (0.3, 0.6 & 1.0 μM). Inhibition of U46619-induced aggregation by the PKC inhibitor, Rö31-8220 (0.1-0.6 μM), was minimal compared to that of PLC inhibition, and only slightly dose related (Figure 6). None of the doses of Rö31-8220 tested abolished aggregation, regardless of the concentration of U46619 used. Addition of the maximum concentration of Rö31-8220 (0.6 μM) had no effect on unstimulated platelets, but did inhibit aggregation in response to 0.6 and 1.0 μM U46619 (Figure 6) With the exception of the effect of 0.3 μM Rö31-8220 on 0.6 μM U46619-induced aggregation, the lower doses of Rö31-8220 (0.1 and 0.3 μM) did not affect U46619-induced aggregation.

Figure 6 Effects of Rö31-8220 on U46619-induced aggregation

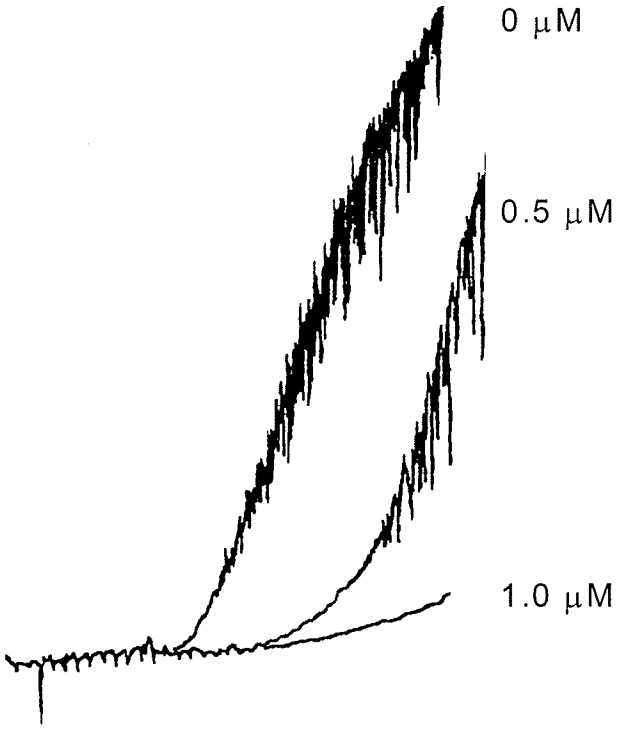


Washed human platelets were incubated with 0.1-0.6 ηM Rö31-8220 or DMSO for 2 minutes prior to addition of U46619 as indicated above. Aggregation was monitored as a change in light transmission. Results are mean values (±SE) of six samples. * $p<0.05$ vs. unstimulated ; † vs. U46619 in the absence of inhibitor for each respective dose of U46619.

3.3 Effects of PLC inhibitors on PMA-induced aggregation

The phorbol ester PMA has been widely used to mimic the effect of the endogenous formation of DAG to stimulate PKC. Consequently PMA was used to induce PKC-mediated platelet aggregation. PMA (30.0 ηM) induced a significant increase in platelet aggregation over unstimulated platelets (Figure 7). The magnitude of aggregation was less than that seen with either collagen or U46619 and the time to reach maximum aggregation was longer, approximately 7 minutes versus 4 and 3 minutes for collagen and U46619 respectively.

Figure 7 Effects of U73122 on PMA-induced platelet aggregation



Washed human platelets were incubated with 0.25-1.0 μM U73122 or DMSO for 2 minutes prior to addition of 15 ηM PMA. Aggregation was monitored as a change in light transmission measured in mm chart deflection.

The PLC inhibitor U73122 significantly inhibited aggregation at all concentrations (1.0-3.0 μM) tested. However, aggregation was only abolished by the highest concentration of U73122 (10.0 μM) tested (see Table 3).

Table 3 Effects of U73122 on PMA-induced aggregation

U73122 (μM)	PMA (ηM)	Aggregation (AU±SE)
0	0	0.12 ±0.04
0	30	7.1± 2.26 *
1.0	30	1.68±0.68 *†
3.0	30	1.61±0.54 *†
5.0	30	1.17± 0.48 *†
10.0	30	0.85±0.30 †

Washed human platelets were incubated with 3.0-10.0 μM U73122 or DMSO for 2 minutes prior to addition of 30 ηM PMA. Aggregation was monitored as a change in light transmission. Results are mean values (±SE) of 10 samples. * $p<0.05$ vs. unstimulated; † vs. PMA alone.

The effect of Rö31-8220 inhibition of PKC-mediated platelet aggregation is well documented in the literature (Murphy and Westwick, 1992; Pulcinnelli *et al.*, 1995) and was not repeated in this study.

Dense granule release was measured as [^{3}H]-serotonin released in response to agonist stimulation. The maximum dose of inhibitor used in each experimental series did not have any affect on basal [^{3}H]-serotonin in unstimulated platelets.

3.4 The role of PLC and PKC in platelet dense granule secretion.

3.4.1 Effects of PLC and PKC inhibitors on collagen-induced dense granule secretion.

The effects of PLC inhibition were addressed by pre-incubating the platelets with the PLC inhibitor U73122 (0.25-3.0 μM). Collagen (1.0 μg/ml) induced a significant increase in [^{3}H]-serotonin release compared to unstimulated platelets (see Table 4). This increase was significantly inhibited by 0.25 and 0.5μM, and totally abolished by 0.75-3.0 μM, U73122 (see Table 4). The IC_{50} of 0.61 was similar to that for PLC inhibited aggregation.

Table 4 Effects of U73122 on collagen-induced [^{3}H]-serotonin secretion

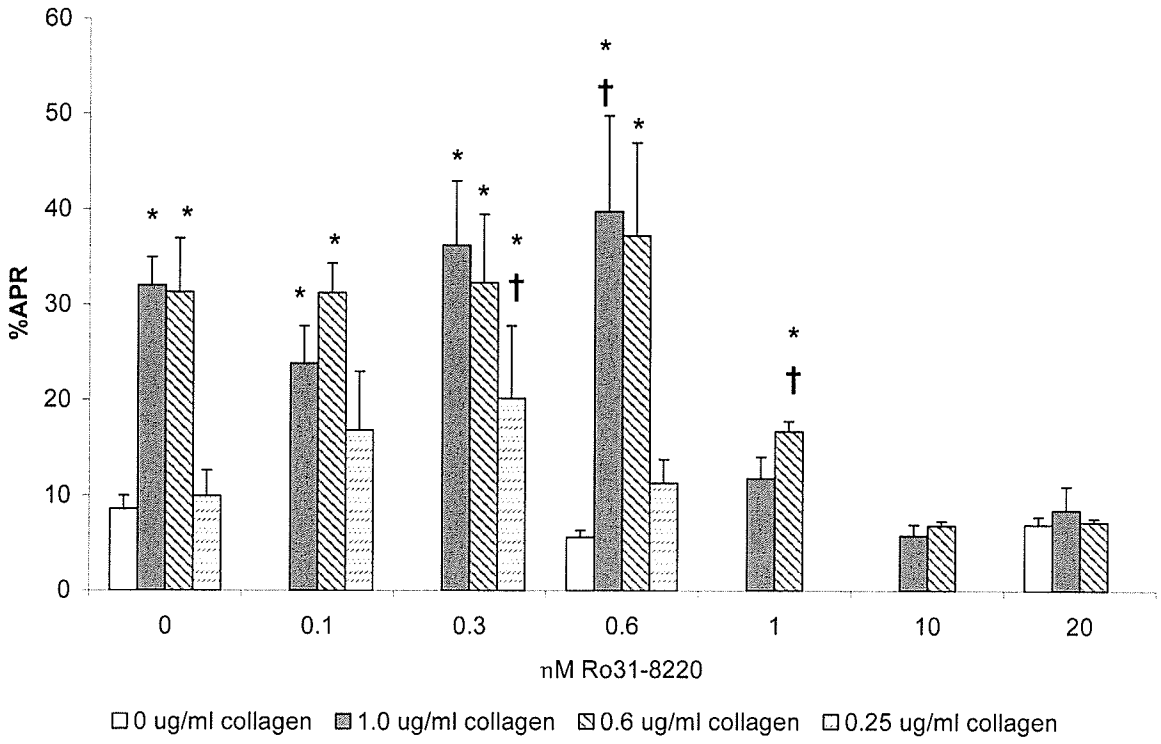
U73122 (μM)	Collagen (μg/ml)	%APR
0	0	10.77±0.83
3.0	0	10.19±1.21
0	1	45.21±3.26 *
0.25	1	33.01±6.06 *†
0.5	1	19.26±1.81 *†
0.75	1	14.03±2.21
1.0	1	11.76±1.25
3.0	1	13.22±1.95

Pre-labelled platelets, without cyclooxygenase inhibition, were pre-incubated for 2 minutes with U73122 at the concentrations indicated or with DMSO vehicle control before addition of 1 μg/ml collagen. Secretion was terminated 4 minutes after addition of collagen. Results are mean values (±SE) of ten samples. * $p<0.05$ vs. unstimulated and † vs. collagen in the absensce of inhibitor.

The effects of PKC inhibition were addressed by pre-incubating the platelets with the PKC inhibitor Rö31-8220. Collagen (1.0 μg/ml) significantly increased [^{3}H]-serotonin release, compared to unstimulated platelets (Figure 8). Secretion induced by 0.6 mg/ml collagen was slightly lower, but 0.25 μg/ml collagen alone did not induce any detectable secretion since the levels were not statistically different from unstimulated platelets. There was a trend for PKC inhibition to have a biphasic effect on collagen-induced [^{3}H]-serotonin (Figure 8) where mid-doses of Rö31-8220 significantly increased secretion relative to levels in the absence of inhibitor. Major observations were:

1. 0.3 μM Rö31-8220 significantly decreased 0.25 μg/ml collagen-induced [^{3}H]serotonin where collagen alone had not stimulated any detectable release
2. 0.6 μM Rö31-8220 significantly increased 1.0 μg/ml collagen-induced [^{3}H]-serotonin relative to collagen alone.
3. 1.0 μM Rö31-8220 significantly decreased 0.6 μg/ml collagen-induced [^{3}H]-serotonin relative to collagen alone.
4. 10 and 20 μM Rö31-8220 abolished [^{3}H]-serotonin induced by 1.0 and 0.6 μg/ml collagen, since secretion was equivalent to unstimulated levels.

Figure 8 Effects of Rö31-8220 on collagen-induced [^{3}H]-serotonin secretion



Pre-labelled platelets, without cyclooxygenase inhibition, were pre-incubated for 2 minutes with Rö31-8220 at the concentrations indicated or with DMSO vehicle control before addition of 1 μg/ml collagen. Secretion was terminated 4 minutes after addition of collagen. Results are mean values (±SE) of six samples. * $p<0.05$ vs. unstimulated; † vs. collagen in the absensce of inhibitor for each respective dose of collagen..

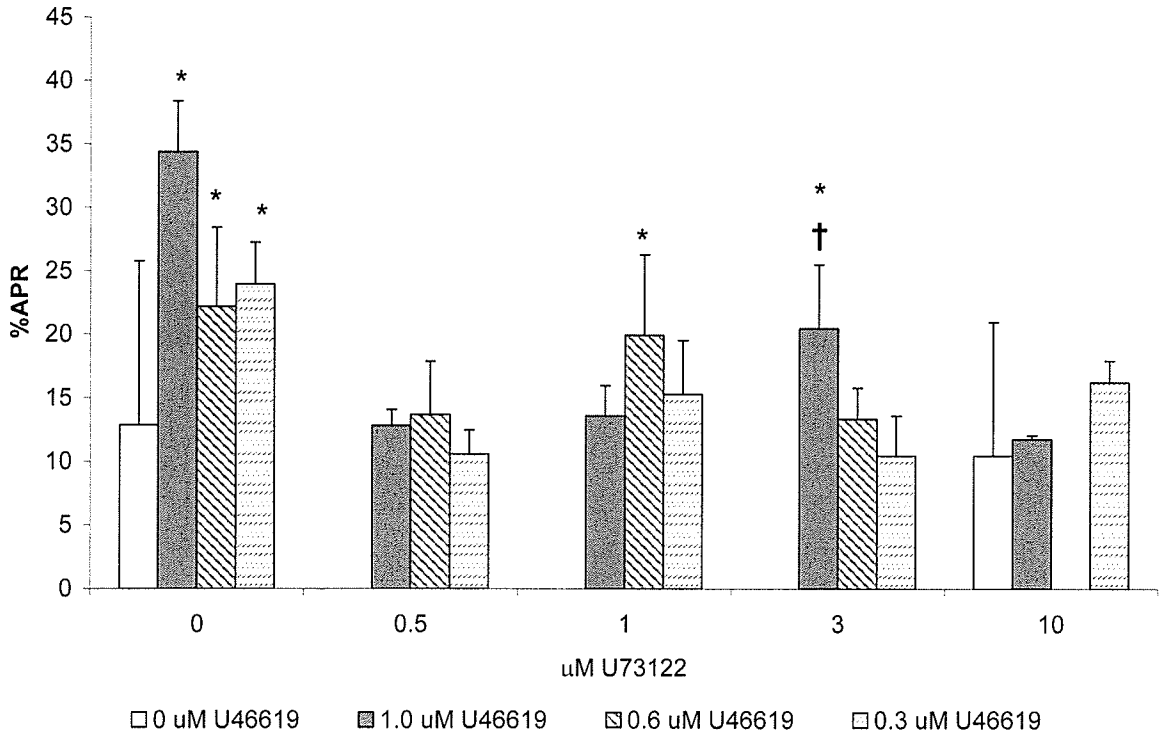
3.4.2 Effect of PLC and PKC inhibitors on thromboxane-induced dense granule secretion.

The effects of PLC inhibition were addressed by pre-incubating the platelets with the PLC inhibitor U73122. The thromboxane mimetic, U46619 (0.3-1.0 μM) induced a significant increase in dense granule secretion measured by [^{3}H]-serotonin secretion to a similar extent over all doses tested.

Major observations (Figure 9) from this experimental series were:

1. 0.5-3.0μM U73122 abolished secretion induced by 0.3-0.6 μM U46619.
2. 0.5 and 1.0μM U73122 abolished secretion induced by 1.0 μM U46619, but 3.0μM U73122 only significantly inhibited secretion induced by 1.0 μM U46619.

Figure 9 Effects of U73122 on U46619 induced [^{3}H]-serotonin secretion



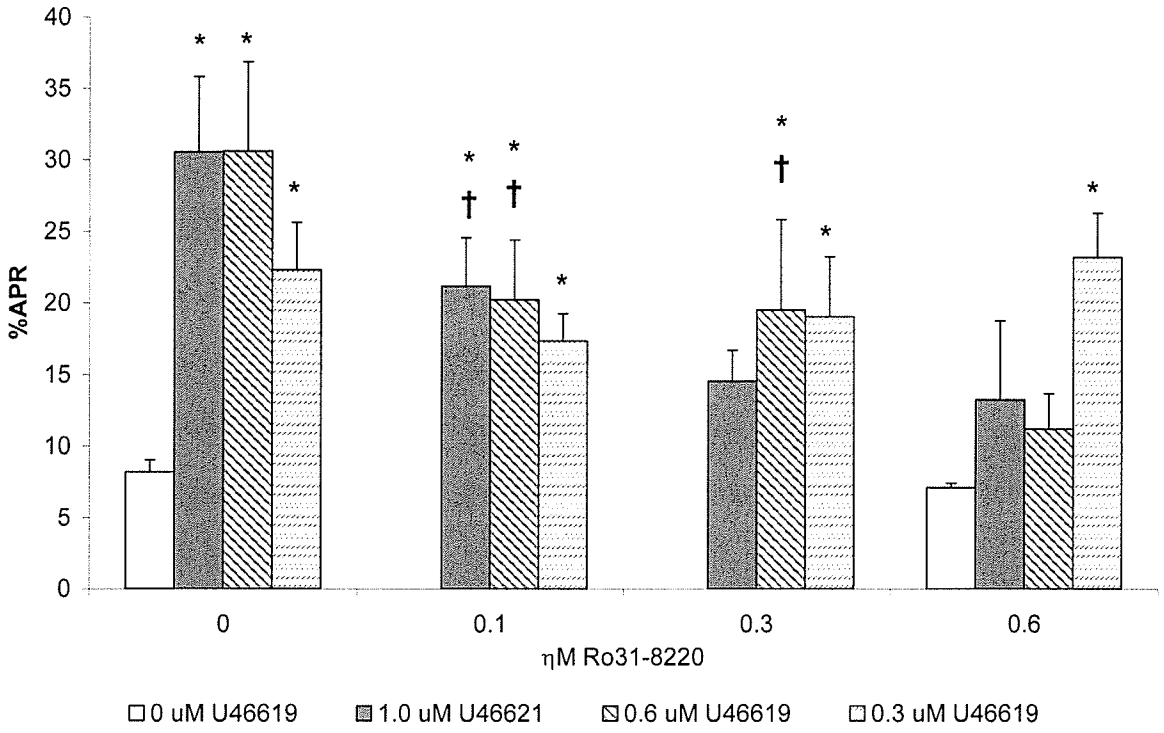
Pre-labelled platelets were pre-incubated for 2 minutes with at the concentrations indicated or with DMSO vehicle control before addition of U46619. Secretion was terminated 3 minutes after addition of U46619. Results are mean values (±SE) of four to ten samples. * $p<0.05$ vs. unstimulated † vs. U46619 in the absensce of inhibitor for each respective dose of U46619.

The effects of PKC inhibition were addressed by pre-incubating the platelets with the PKC inhibitor Rö31-8220. U46619 (0.3-1.0 μM) induced a significant increase in dense granule secretion, as measured by [^{3}H]-serotonin secretion, over unstimulated platelets (Figure 10). The highest dose of Rö31-8220, 0.6 μM had no effect on basal secretion levels. At the higher concentration of U46619 (0.6-1.0 μM), Rö31-8220 inhibited secretion in a dose dependent manner. The lower the concentration of U46619, the less relative effect PKC inhibition had on the increase in secretion, e.g. 0.6 μM Rö31-8220 did not reduce secretion to unstimulated levels, despite a lower overall lower secretion in the absence of Rö31-8220.

Major observations (Figure 10) from this experimental series were:

1. 0.6 and 1.0 μM U46619 each stimulated a significant increase in secretion, which was inhibited by all doses of Rö31-8220 tested (0.1-0.6 μM).
2. Secretion induced by 1.0 μM U46619 was abolished by 0.3 μM.
3. Secretion induced by 0.6 μM U46619 was abolished by 0.6 μM.
4. The lowest of concentration U46619 (0.6 μM) significantly increased secretion over unstimulated levels, which was unaffected by any dose of Rö31-8220 tested.

Figure 10 Effects of Rö31-8220 on U46619-induced [^{3}H]-serotonin secretion



Pre-labelled platelets were pre-incubated for 2 minutes with at the concentrations indicated or with DMSO vehicle control before addition of U46619. Secretion was terminated 4 minutes after addition of U46619. Results are mean values (±SE) of four to ten samples. * $p<0.05$ vs. unstimulated and † vs. U46619 in the absensce of inhibitor for each respective dose of U46619.

3.4.3 Effect of PLC Inhibiors on PMA-Induced Dense Granule Secretion.

[^{3}H]-serotonin secretion in response to 30ηM PMA (see Table 5) was minimal, 15.6±5.9% vs. 10.1±3.6% in unstimulated control, and unaffected by PLC inhibition by preincubation with up to 10 μM U73122.

Table 5 Effects of U73122 on PMA-induced [^{3}H]-serotonin secretion

U73122 (μM)	PMA (ηM)	%APR
0	0	10.09 ± 3.57
0	30	15.55 ± 5.92
0.25	30	13.24 ± 9.36
0.5	30	12.73 ± 9.00
1.0	30	12.64 ± 6.32
3.0	30	12.65 ± 4.78
5.0	30	12.49 ± 6.25
10.0	30	13.44 ± 6.72

Pre-labelled platelets were pre-incubated for 2 minutes with U73122 at the concentrations indicated or with DMSO vehicle control before addition of 30 ηM PMA. Secretion was terminated 7 minutes after addition of PMA. Results are mean values (±SE) of eight samples.

3.5 The Effect of U73122 on PMA-induced Fibrinogen Binding

The effect of U73122 on PMA stimulation of fibrinogen binding was evaluated by flow cytometery (see Table 6). PMA (200ηM) stimulated a significant increase in fibrinogen binding (40.73 ±1.74) compared to unstimulated platelets (2.56 ± 0.87) and was significantly inhibited in a dose dependent manner, but not abolished, by pre-treatment with U73122 (1-10μM).

Table 6 Effects of U73122 on PMA-induced fibrinogen binding

U73122 (μM)	PMA (ηM)	Mean±SEM
0	0	2.56±0.87
0	200	40.73±1.74*
1.0	200	23.96±1.62*†
3.0	200	18.91±3.36*†
5.0	200	19.17±3.12*†

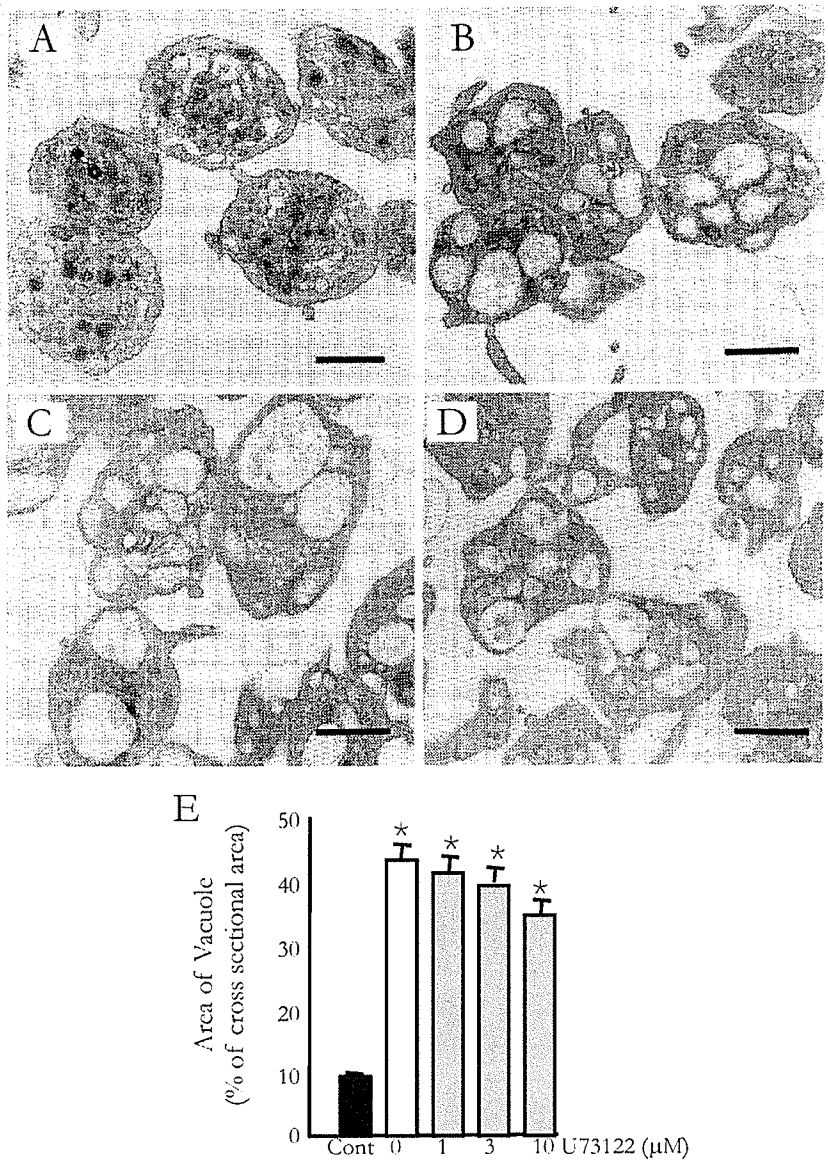
Platelets were pre-incubated with FITC labelled fibrinogen and U73122 the concentrations indicated or with DMSO vehicle control for 1 minute before addition of PMA. Binding was terminated 10 minutes after addition of PMA. Results are mean values (±SE) of six samples. * $p<0.05$ vs. unstimulated and †$p<0.05$ vs. 0 μM U73122.

3.6 The Effect of U73122 on PMA-induced Morphological Changes

PKC activation by PMA is known to cause the formation of intracellular vacuoles (Gerrard *et al.*, 1985). The effect of U73122 on the area of platelet occupied by vacuoles was evaluated in PMA-stimulated platelets (see Figure 11). Unstimulated (vehicle control) platelets were found to have 9.3±1.1% of their cross-sectional area occupied by vacuoles, whereas PMA (30ηM)-stimulated platelets had 43.3±2.7% of their area occupied by vacuoles. Pre-treatment with 1μM U73122 (41.5±2.7%), 3μM U73122 (39.4±2.5%) or 10μM U73122 (34.7±2.6%) had no significant effect on the area occupied by vacuoles.

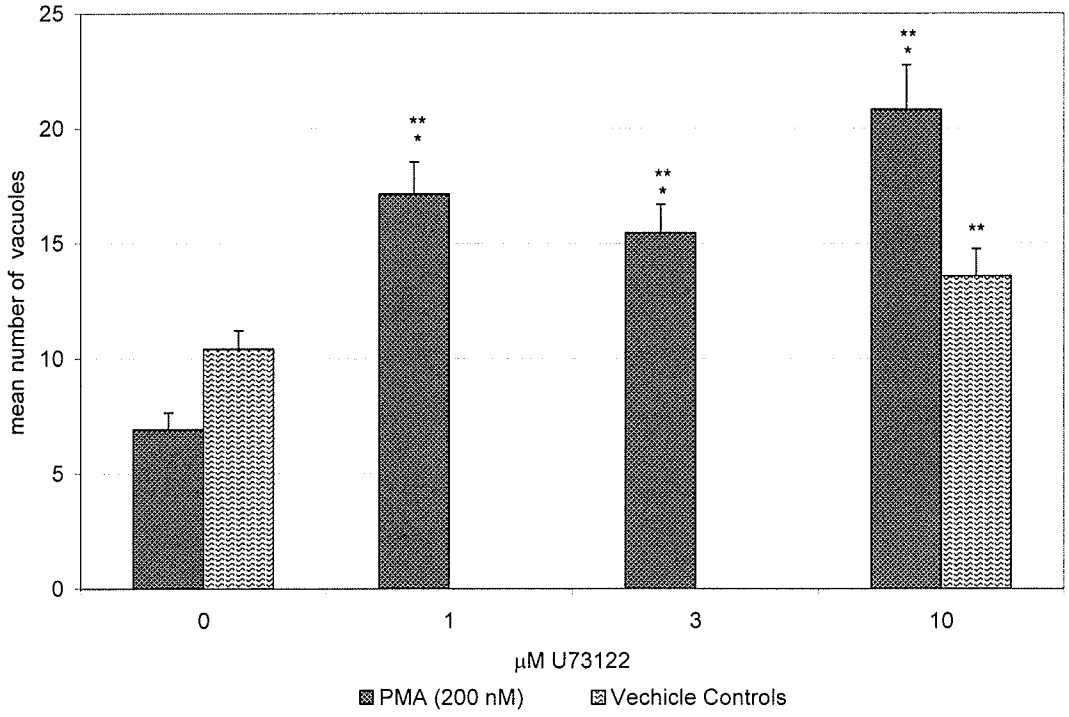
Interestingly, pre-treatment with U73122 did have a significant effect on the number of vacuoles formed (Figure 12). Pre-treatment of unstimulated platelet with 10μM U73122 caused a significant increase in the average number of vacuoles per platelet from 6.9 ± 0.7 to 13.6 ± 1.2. Platelet stimulation with PMA did not affect the average number of vacuoles from 10.4 ± 0.8 vs 6.9 ± 0.7 when compared to unstimulated platelets. Increasing doses of U73122 caused a significant increase in the average number of vacuoles per platelet in PMA stimulated platelets when compared to both the unstimulated platelets and PMA-stimulated platelets which had not been pre-treated with U73122. In comparison to U73122 pre-treated-unstimulated platelets, only PMA-stimulated platelets that had been pre-treated with 10μM U73122 showed a significant increase in the average number of vacuoles per platelet.

Figure 11 Effects of U73122 on the cross-sectional area of PMA-induced vacuole formation



Platelets were pre-incubated U73122 for 2 minutes with at the concentrations indicated or with DMSO vehicle control before addition of 200 ηM PMA. Activation was terminated 10 minutes after addition of PMA. The platelets were fixed, embedded and imaged by electronmicroscopy. Micrographs depict typical examples of: A –control platelets; B – Stimulated, inhibited platelets; C – stimulated platelets with 1 μM U73122; and D – stimulated platelets with 10 μM U73122. Vacuole area (E) expressed as percent of cross-sectional area occupied by vacuoles ± SE. Results calculated from 4-6 micrographs from two experiments. * $p<0.05$ vs. unstimulated.

Figure 12 Effects of U73122 on PMA-induced vacuole formation



Platelets were pre-incubated U73122 for 2 minutes with at the concentrations indicated or with DMSO vehicle control before addition of 200 ηM PMA. Activation was terminated 10 minutes after addition of PMA. The platelets were fixed, embedded and imaged by electronmicroscopy. Vacuole area expressed as percent of cross-sectional area occupied by vacuoles ± SE. Vacuole number is expressed as average number of vacuoles per platelet ± SE. Results calculated from 6 micrographs from two experiments. * $p<0.05$ vs. unstimulated and ** vs. PMA in the absensce of inhibitor..

3.6.1 Platelet Arachidonic Acid Production

Platelets in experiments involving arachidonic acid recovery were pre-treated with cyclooxygenase inhibitors (100 μM acetylsalicylic acid or 10 mM BW755C) to prevent formation of thromboxane A_2. This was done to prevent both the underestimation of arachidonic acid release by the stimulating agonist and the confounding effects of thromboxane A_2.

3.6.1.1 Comparison of the time course for intact and permeabilized [^{3}H]-arachidonic acid release

Initial experiments using the Bligh and Dyer extraction found permeabilization with 50 μM digitonin did not increase the unstimulated absolute [^{3}H]-arachidonic acid counts recovered as free arachidonic acid, relative to intact platelets, nor was there an effect of incubation time with the permeabilizer (Table 7). In addition, stimulation with collagen did not increase levels of [^{3}H]-arachidonic acid release for either intact platelets or those permeabilized with digitonin prior to extraction. However, when results were expressed as a percent of the total counts (Table 7) collagen-induced a significant increase in [^{3}H]-arachidonic acid release in the whole platelets. The samples permeabilized with digitonin continued to show no difference between stimulated vs. unstimulated levels of [^{3}H]-arachidonic acid release.

Table 7 Time course of collagen-induced [^{3}H]- arachidonic acid release in intact and digitonin-permeabilized platelets

	Time (Minutes)	Control CPM (Mean±SEM)	Collagen CPM (Mean±SEM)
Whole extract	10	144.3±49.9	138.53 ±13.30
Digitonin	1	N.D.	133.9 ±53.90
	5	121.1±50.1	136.8 ±21.6
		% Total CPM (Mean±SEM)	% Total CPM (Mean±SEM)
Whole extract	10	14.6 ±5.5%	27.15 ±1.1*
Digitonin	1	N.D.	46.95±10.05
	5	46.7±1.4	44.75±6.15

Pre-labelled platelets were simulated with 10 μg/ml collagen or with DMSO vehicle control for the times indicated, then permeabilized with 50 μM digitonin. Results are expressed as the average of the absolute counts in each sample ± SE from four samples. *$p<0.05$ vs. corresponding time/permeabilizer unstimulated control.

As a consequence of these inconclusive results, the procedure was repeated using a modification of the Dole's extraction and an alternative permeabilizing agent, 20 μM saponin, for comparison (Table 8). This extraction method showed a dramatic increase in the extraction of free arachidonic acid on both stimulated and unstimulated whole platelet samples.

Table 8 Time course of collagen-induced [^{3}H]- arachidonic acid release in intact and permeabilized platelets using Dole's Extraction

	Time (Minutes)	Control CPM (Mean±SEM)	Collagen CPM (Mean±SEM)
Whole extract	10	31.40 ±2.25	88.53 ±19.09*
Saponin	1	47.95 ±6.73	91.25 ±15.28
	5	47.15 ±3.52	104.45 ±7.07*
	10	45.35 ±2.08	102.50 ±15.17*
Digitonin	1	45.85 ±6.48	59.75 ±13.99
	5	40.70 ±3.40	62.25 ±9.13
	10	44.60 ±2.07	74.25 ±11.46

Pre-labelled platelets were simulated with 10 μg/ml collagen or with DMSO vehicle control for the times indicated. Results are expressed as the average of the absolute counts in each sample ± SE from six samples. *p<0.05 vs. corresponding time/permeabilizer unstimulated control.

The use of either saponin or digitonin as a permeabilizing agent improved extraction efficiency of free arachidonic acid in unstimulated platelets. When the results were expressed as a percentage of the total counts in each extract (Table 9), stimulation with collagen-induced a significant increase in free arachidonic acid in the saponin treated platelets. In saponin treated platelets, percent total counts significantly increased after 5 and 10 minutes of collagen stimulation. However, in digitonin treated platelets, percent total counts did not change from unstimulated.

Table 9 Time course of collagen-induced [^{3}H]- arachidonic acid release in intact and permeabilized platelets using Dole's Extraction

	Time (Minutes)	Control % Total CPM (Mean±SEM)	Collagen % Total CPM (Mean±SEM)
Whole extract	10	11.50±1.96	31.80±7.03*
Saponin	1	29.43±2.56	47.28±2.35*
	5	27.85±1.58	50.55±0.29*
	10	25.60±3.16	53.13±4.19*
Digitonin	1	35.30±2.06	37.90±3.53
	5	30.13±2.17	39.78±2.99
	10	35.85±1.23	44.38±1.88

Pre-labelled platelets were simulated with 10 μg/ml collagen or with DMSO vehicle control for the times indicated. Results are expressed as the average of the % total counts in each sample ± SE from six samples. *p<0.05 vs. corresponding time/permeabilizer unstimulated control.

3.6.1.2 Development of HPLC assay

3.6.1.2.1 Comparison of intact and permeabilized platelets in HPLC arachidonic acid release

A comparison of HPLC results did not find a significant increase in extracted free arachidonic acid in whole or permeabilized platelets stimulated with 10 μg/ml collagen for 10 minutes (Table 10).

Table 10 Comparison of cell permeabilization techniques on arachidonic acid recovery, as measured by HPLC

	Arachidonic acid (Mean ±SE μM)	
	Control	Collagen
Whole extracts	0.04±0.03	0.31‡
Saponin	1.06±0.25	0.40±0.07
Digitonin	0.25±0.11	0.61±0.09

Platelets were stimulated with 10 μg/ml collagen for 10 minutes before addition of permeabilizer, 50 μM digitonin or 20μg/ml saponin. The sample was incubated for a further 10 minutes, centrifuged and the recovered platelets extracted derivatized and measured by HPLC. Results are expressed as mean ± SE from four samples. ‡ no SE available.

An examination of the type of permeabilization technique found the relative extraction efficiencies to be different from those found with labelled platelets (Table 11). Saponin showed a significant difference where the collagen platelets released significantly less arachidonic acid than the unstimulated platelet. Basal arachidonic acid in unstimulated platelets was equivalent in intact and digitonin-permeabilized platelets. Therefore, digitonin was chosen as a permeabilizer for the remaining experiments.

Table 11 Effects of cell permeabilization techniques on arachidonic acid recovery, as measured by HPLC

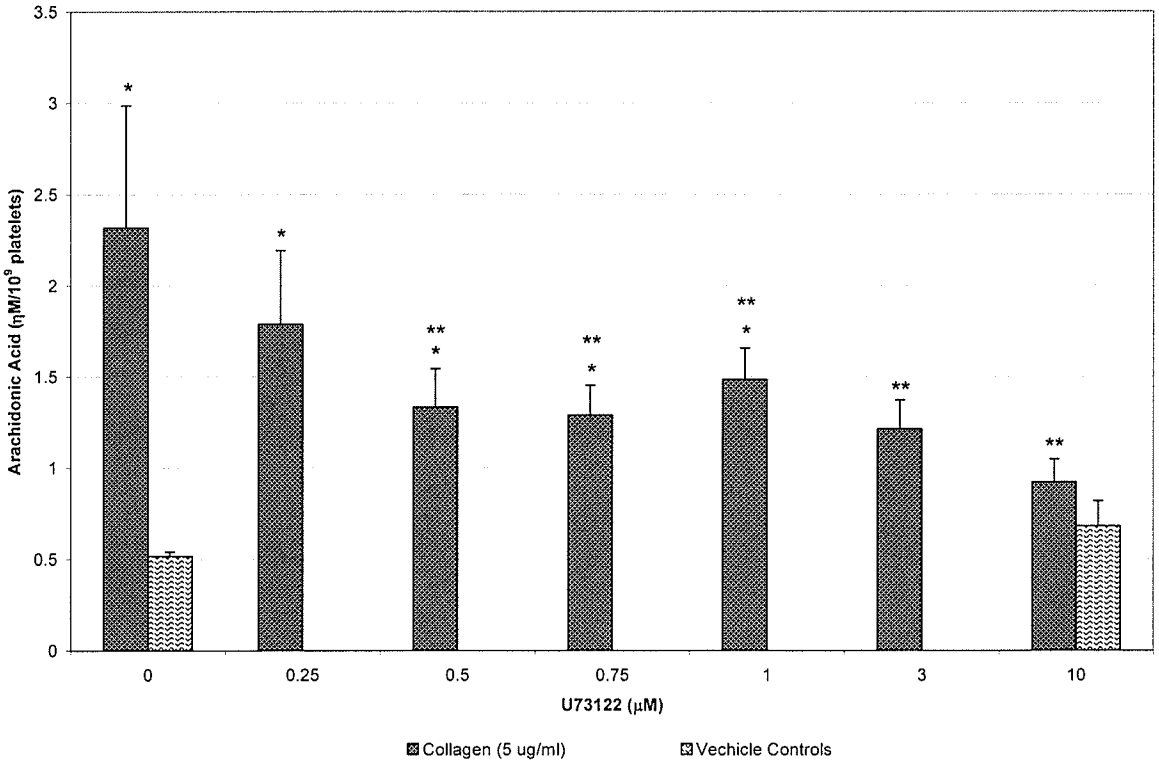
	Time (Minutes)	Arachidonic acid (μM; Mean ±SE)	
		Control	Collagen
Saponin	1	0.33±0.22*	0.51±0.02
	5	0.11±0.02*	0.24±0.11*
	10	1.06±0.25	0.40±0.07*
Digitonin	1	0.14±0.03*	0.72±0.01
	5	0.21±0.07*	0.67±0.07
	10	0.25±0.11*	0.61±0.09

Platelets were stimulated with 10 μg/ml collagen for 5 minutes before addition of permeabilizer, 50 μM digitonin or 20μg/ml saponin. The sample was further incubated as indicated, centrifuged and the recovered platelets extracted, derivatized and measured by HPLC. Results are expressed as mean ± SE from two experiments. * $p<0.05$ vs. unstimulated 10-minute saponin treated.

3.6.1.2.2 Effects of the PLC inhibitor U73122 on collagen-induced arachidonic release

Stimulation of platelets with 10 μg/ml collagen caused a significant increase in the amount of arachidonic acid produced (Figure 13). Addition of U73122 had no effect on arachidonic acid levels in unstimulated platelets. The lowest dose of U73122 (0.25 μM) did not significantly reduce arachidonic acid release. All other doses of U73122 (0.5 - 10.0 μM) significantly inhibited arachidonic acid release when compared to collagen-stimulated, uninhibited platelets. Furthermore, doses higher than 1.0 μM, abolished arachidonic acid release, reducing it to levels equivalent in unstimulated platelets. These results closely parallel the inhibitory effects of U73122 on aggregation and secretion induced by low doses of collagen (1 μg/ml) in that U73122 virtually abolishes the effects of collagen at very low doses.

Figure 13 Effects of U73122 on collagen-induced arachidonic acid release

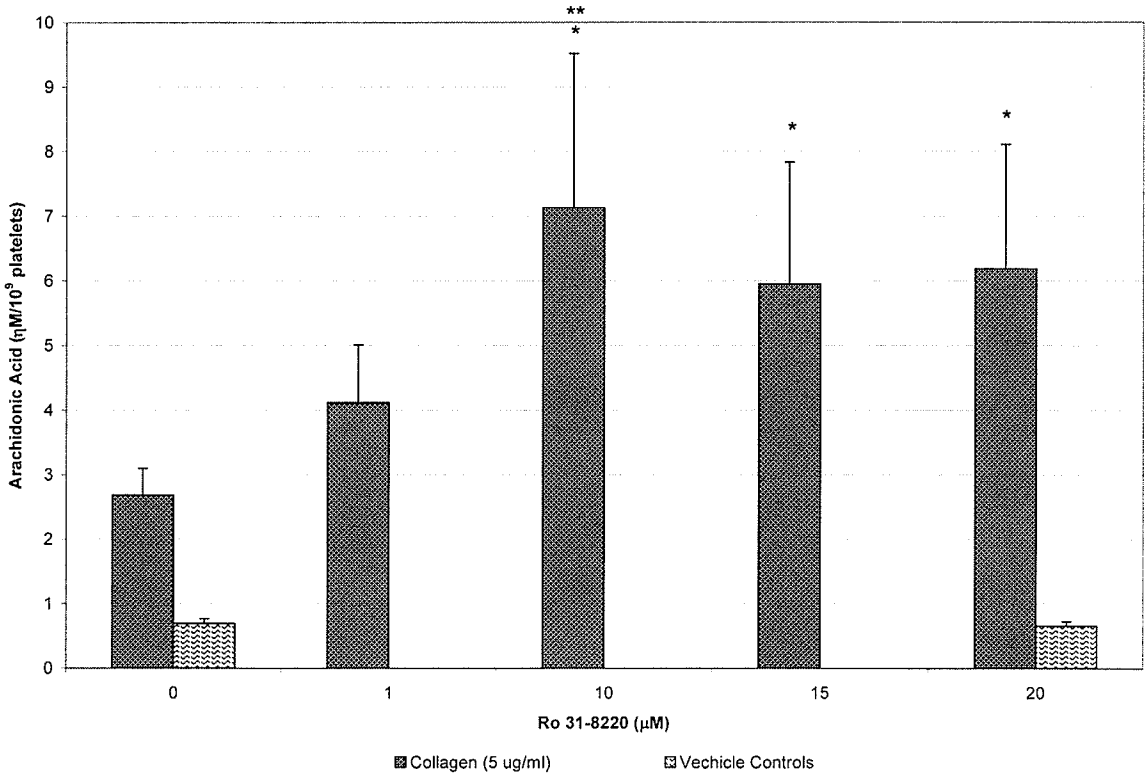


Platelets were pre-incubated for 2 minutes with U73122 at concentrations indicated before stimulation with 10 μg/ml collagen for 5 minutes. The samples were permeabilized, extracted, derivatized and measured by HPLC. Results are expressed as mean ± SE six samples. * $p<0.05$ vs. unstimulated and ** $p<0.05$ vs. collagen stimulated.

3.6.1.2.3 Effects of the PKC inhibitor Rö31-8220 on collagen-induced arachidonic release

Stimulation of platelets with 10 μg/ml collagen caused a significant increase in arachidonic acid (see Figure 14). Addition of the highest dose of Rö31-8220 (20 μM) had no effect on basal arachidonic acid levels. However, in the presence of 10 μg/ml collagen, addition of 1-20 μM Rö31-8220 caused an increase in arachidonic acid release. The increase in arachidonic acid release peaked at 10 μM Rö31-8220, which was significantly higher than in the absence of Rö31-8220.

Figure 14 Effects of Rö31-8220 on collagen-induced arachidonic acid release

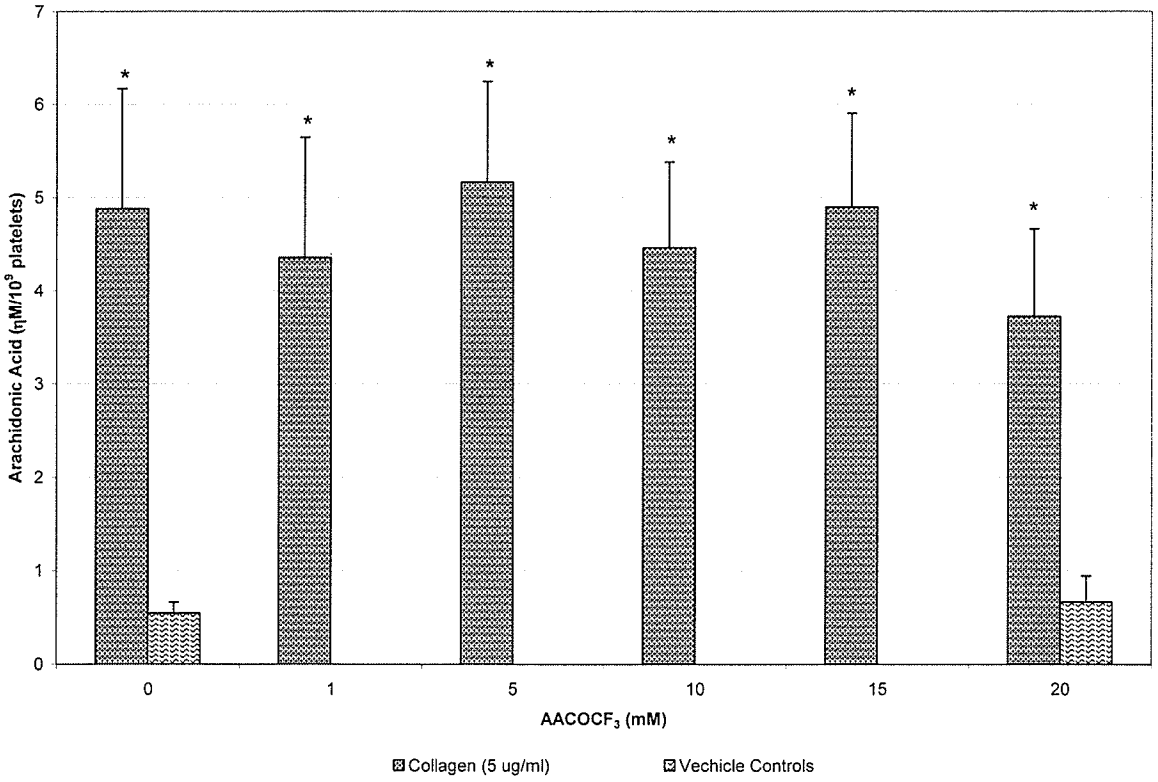


Platelets were pre-incubated for 2 minutes with Rö31-8220 at concentrations indicated before stimulation with 5 μg/ml collagen for 5 minutes. The samples were permeabilized, extracted, derivatized and measured by HPLC. Results are expressed as mean ± SE from four samples. * $p<0.05$ vs. unstimulated and ** $p<0.05$ vs. collagen stimulated.

3.6.1.2.4 Effects of the cPLA$_2$ inhibitor AACOCF$_3$ on collagen-induced arachidonic release

Stimulation of platelets with 10 μg/ml collagen caused a significant increase in the amount of arachidonic acid produced (see Figure 15). Addition of 1.0 to 20.0 μM $AACOCF_3$ had no effect on collagen-induced arachidonic acid release, nor did 20.0 μM $AACOCF_3$ affect arachidonic acid levels in unstimulated platelets.

Figure 15 Effects of $AACOCF_3$ on collagen-induced arachidonic acid release

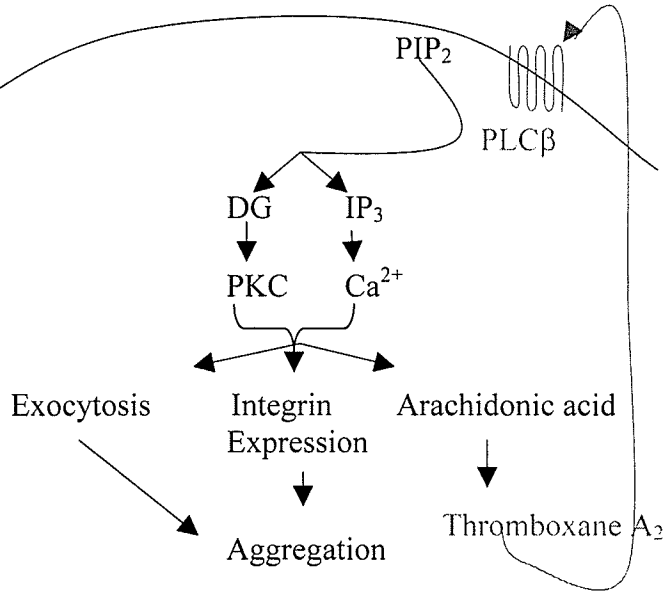


Platelets were pre-incubated for 2 minutes with $AACOCF_3$ at concentrations indicated before stimulation with 5 μg/ml collagen for 5 minutes. The samples were permeabilized, extracted, derivertized and measured by HPLC. Results are expressed as mean ± SE from eight samples. * $p<0.05$ vs. unstimulated .

4 DISCUSSION

A variety of agonists, acting on specific extracellular receptors are known to stimulate the range of platelet functional responses including shape change, secretion of dense and alpha granule contents, expression of the procoagulant surface, synthesis and release of TxA_2, and the conformation change in adhesive receptors, notably the αIIb/β3 integrin receptor, culminating in the platelet aggregation. The intracellular mechanisms underlying the activation of human platelets have been the focus of numerous studies and several wide-ranging reviews (Siess, 1989; Nozawa *et al.*, 1991; McNicol 2005). Pivotal to the actions of agonists such as thrombin, platelet activating factor, vasopressin and TxA_2 is the activation of GTP-binding protein-mediated PLCβ with consequent hydrolysis of PIP_2 and production of DG and IP_3. DG has been shown to stimulate PKC and IP_3 mobilises intracellular Ca^{2+}, which act synergistically to induce the full range of functional responses (Wakelam, 1998). Figure 10 delineates this pathway for TxA_2.

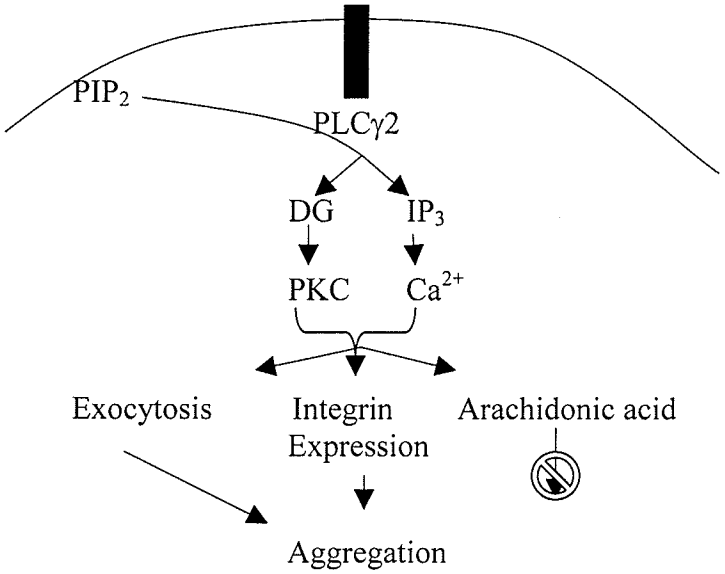
Figure 16 PLCβ-mediated platelet signalling



In contrast to the above agonists, the PLCβ-mediated phosphoinositide pathway is not invoked in response to collagen. Several groups have provided evidence that has partially elucidated the biochemical mechanisms associated with platelet activation in

response to high concentrations (≥20 μg/ml) of collagen, involving the tyrosine phosphorylation, and activation, of PLCγ2. A "two site, two step" model has been proposed which involves the coordination of collagen binding to several receptors in concert with their complexing with an immunoreceptor tyrosine-based activation motif (ITAM) activation (Watson and Gibbins, 1998; Watson, 1999). Central to the process is the activation of p59fyn (Ezumi *et al.*, 1998; Briddon & Watson, 1999) leading to the activation of the Fc-receptor γ-chain (Gibbins *et al.*, 1997; Tsuji *et al.*, 1997). The Fc-receptor γ-chain is responsible for recruiting several other signalling molecules, notably p72syk (Yanaga *et al.*, 1995; Ichinohe *et al.*, 1997; Asselin *et al.*, 1997), and PLCγ2 (Blake *et al.*, 1994; Daniel *et al.*, 1994). The subsequent phosphorylation and activation of PLCγ2 yields DG and IP_3, which are responsible for the range of platelet functional responses such as exocytosis and aggregation. These responses are insensitive to cyclooxygenase inhibition and are therefore TxA_2 independent (see Figure 17).

Figure 17 PLCγ2-mediated platelet signalling



At lower concentrations (1-10μg/ml), similar to *in vivo* exposure (Seymour *et al.*, 1984; Gerrard *et al.*, 1989), collagen also induces biochemical responses such as IP_3

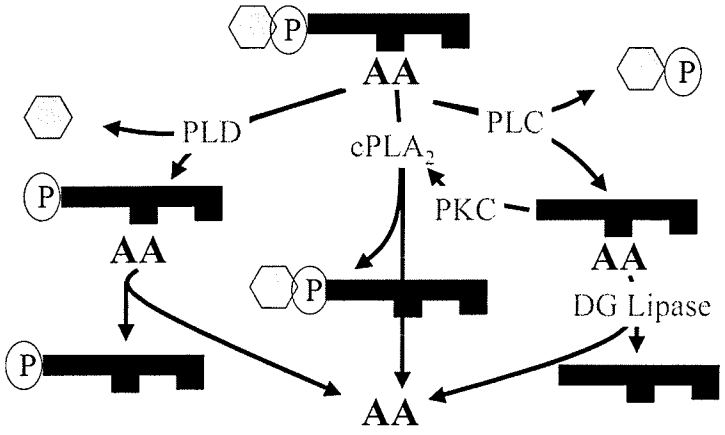
formation, increases in intracellular Ca^{2+} and DG generation, and full platelet aggregation. However under these conditions both the biochemical and functional responses are acutely sensitive to cyclooxygenase inhibitors, and are therefore TxA_2-mediated (Rittenhouse-Simmons & Allen, 1982; Sano *et al.*, 1983; Watson *et al.*, 1985; Narita *et al.*, 1985; Pollock *et al.*, 1986; Vedelago & Mahadevappa, 1988). For example cyclooxygenase inhibitors, such as indomethacin, block both IP_3 formation and Ca^{2+} increases in platelets stimulated with collagen (Nakano *et al.*, 1989). Interestingly SQ29 548, a TxA_2/PGH_2 receptor antagonist also attenuates collagen-induced IP_3 formation (Barry *et al.*, 1997), suggesting a critical role for TxA_2 in the process. Consequently the early signalling events, in particular the mechanism of arachidonic acid liberation, in response to low collagen concentrations are of great interest.

The receptor involved in platelet responses to low concentrations of collagen is unknown. Interestingly in platelets that lack the $\alpha 2$ subunit of $\alpha_2\beta_1$, or when monoclonal antibody 6F1 is used to block $\alpha_2\beta_1$, aggregation is absent at low concentrations of collagen but present at high concentrations (Watson and Gibbins, 1998). This suggests that $\alpha_2\beta_1$ serves as a high affinity receptor for collagen which is critical for responses to low, but not high, collagen concentrations.

Therefore there are clear concentration-dependent differences in the platelet response to collagen. This relates both to the receptors engaged, signalling pathways evoked and sensitivity to cyclooxygenase inhibition. In light of these differences, the results presented here must be viewed in the context of other studies conducted with low concentrations of collagen to make appropriate comparisons. This thesis focused on the mechanism of arachidonic acid release. The objective of the thesis was to evaluate the contribution of the enzyme pathways illustrated in Figure 18 in the release of free arachidonic acid. Specifically, these pathways were:

1. PLC, which generates DG;
2. PKC which would be activated by DG and in turn phosphorylates $cPLA_2$ (McNicol & Shibou, 1998);
3. $cPLA_2$ which liberates arachidonic acid from membrane phospholipids;
4. DG lipase (DGL), which liberates arachidonic acid from DG.

Figure 18 Potential mechanisms for arachidonic acid release from phospholipids with arachidonic acid in the *sn*-2 position.



To pharmacologically evaluate each of these potential enzymatic pathways, the effect of inhibitors of PLC, $cPLA_2$ and PKC on arachidonic acid release was examined. DG lipase was not directly evaluated in this thesis. The inhibitors used were U73122 (PLC), $AACOCF_3$ (PLA_2), and Rö31-8220 (PKC). Substantial evidence exists that PLD is not a major source of arachidonic acid release in platelets (Chiang, 1994), therefore this pathway was not investigated. A three-pronged approach was used to evaluate this hypothesis.

1. Evaluation of the specificity of U73122 in the inhibition of PLC in platelets;
2. Development of a direct assay of arachidonic acid release from platelets;
3. Measurement of the effect of the above inhibitors on arachidonic acid release in collagen-stimulated platelets.

4.1 U73122 SPECIFICITY IN PLATELETS

As a PLC inhibitor, U73122 has been shown to inhibit a number of events in platelets and polymorphonuclear neutrophils. Consistent with PLCβ inhibition, U73122 inhibits the formation of DG and [^{3}H]-IP_3 and increases in intracellular Ca^{2+} in thrombin stimulated platelets and in N-formyl-methionyl-leucyl-phenylaline stimulated neutrophils (Bleasdale *et al.*, 1990, Smith *et al.*, 1990). Down-stream events such as pleckstrin

phosphorylation, phosphatidic acid production and [^{3}H]-serotonin release induced by U46619 are also inhibited by U73122 (Lockhart and McNicol, 1999). Based on studies of acetylcholine receptor sequestration in SK-N-SH neuroblastoma cells, the proposed mechanism of action of U73122 lies at the level of Gp regulation of PLC (Thompson, 1991), which would be consistent with inhibition of PLCβ.

The results presented here show that U73122 also inhibited platelet aggregation (Table 2) and [^{3}H]-serotonin release (Table 4) in response to collagen. In the absence of cyclooxygenase inhibition, collagen activation stimulates both PLCγ2 and PLCβ. Therefore, these results cannot differentiate between the isoforms of PLC being affected (Tate and Rittenhouse, 1993; Daniel *et al.*, 1994; Blake *et al.*, 1994).

As expected, U73122 had no effect on PKC stimulation by PMA as measured by pleckstrin phosphorylation (Lockhart and McNicol, 1999), [^{3}H]-serotonin release (Table 5) or intracellular vacuole formation (Figure 11), all of which are largely dependent on PKC activity (Walker and Watson, 1993). Under the same conditions, U73122 inhibited U46619-induced pleckstrin phosphorylation and the selective PKC inhibitor, Rö31-8220, inhibited both PMA and U46619-induced pleckstrin phosphorylation (Lockhart and McNicol, 1999). This demonstrated that inhibition of PLC activity by U73122 could result in the secondary inhibition of PKC activity but could not *per se* inhibit PKC activity. Furthermore, as Rö31-8220 decreased both U46619- and PMA-induced pleckstrin phosphorylation (Lockhart and McNicol, 1991), PKC was being inhibited in platelets.

U73122 also inhibited PMA-induced, thus PKC-mediated, platelet thromboxane release (Lockhart and McNicol, 1999), fibrinogen receptor expression (Table 6) and consequently aggregation (Table 3). Occupation of αIIb/β3, the primary fibrinogen receptor on platelets, stimulates membrane lipid hydrolysis and phosphoinositide metabolism (Du and Ginsberg, 1997). Since PKC is not known to simulate membrane lipid hydrolysis or phosphoinositide metabolism and yet results in the formation of TxA_2, this outside-in signalling may initiate TxA_2 production. Therefore, inhibition of the fibrinogen receptor may account for the decreased PKC-mediated TxA_2 release. Furthermore inhibition of fibrinogen receptor expression may also account for other, apparently non-PLC mediated, inhibitory effects of U73122.

These observations are consistent with previous studies using U73122 where non-PLC dependent effects have been reported. Vickers (1993) found that U73122 affected the equlibria between membrane phosphoinositides in resting and thrombin stimulated platelets, likely through inhibiting inositol phosphate and phosphatidylinositol 4-phosphate kinase activity. Ohata and colleagues (1997) reported that U73122 inhibited the lysoPA sensitised, mechanical stress-induced Ca^{2+} responses in cultured smooth muscle cells. In rat neutrophils and mouse fibroblasts, Ca^{2+} homeostasis was affected by U73122, possibly through inhibition of the Ca^{2+} ATPase (Grierson and Meldolesi, 1995; Wang, 1996). Interestingly Ca^{2+} channel activity has been associated with αIIb/β3 (Adunyah and Dean, 1986) consistent with the possibility the non-PLC-related effects of U73122 are secondary to an action on the fibrinogen receptor αIIb/β3.

In summary, U73122 had inhibitory effects on PLC activity in platelets. The additional inhibitory effects on Ca^{2+}-mediated platelet activation are in accordance to those found in other cell types. However, the inhibitory effects on PKC stimulation by PMA, events that are independent of PLC and Ca^{2+}, are novel. Since U73122 did not affect PMA-induced pleckstrin phosphorylation, it is unlikely that U73122 inhibits PKC activity, but does affect downstream events in PKC induced aggregation. These results suggest that the non-PLC-associated mechanism of action of U73122 is a late event likely related to the fibrinogen receptor.

4.2 ARACHIDONIC ACID ASSAY DEVELOPMENT

Traditionally, arachidonic acid release has been measured using radiolabelled arachidonic acid and/or TLC separation (Passi *et al.,* 1981; Mahadevappa and Holub, 1987; Purdon *et al.,* 1987; Vedelago and Mahadevappa, 1988). However, evidence suggests that labelled arachidonic acid is not uniformly incorporated into phospholipids in platelets and other cells (Chilton and Murphy, 1986; Ramesha and Taylor; 1991). For example, in neutrophils most radiolabelled arachidonic acid is incorporated into diacyl pools of phospholipids (Chilton and Murphy, 1986). As different agonists activate different intracellular mediators, it is likely that this leads to the release of arachidonic acid by specific enzymes acting on selective intracellular phospholipid pools. Consequently the differential arachidonic acid labelling profile almost certainly

introduces artefacts resulting in misleading conclusions. Furthermore, the results of labelled experiments are generally expressed as counts or percentages of control, rather than actual quantities, making comparisons between studies difficult.

Detection of arachidonic acid release from platelets has two components. Firstly the arachidonic acid must be extracted from the platelets since, in its unmetabolized form, it is not secreted nor otherwise released into the extracellular medium. A variety of methods have been used to extract arachidonic acid from platelets, most notably those of Bligh and Dyer (1959) and of Dole and Meinertz (1960), or modifications thereof. Secondly, the arachidonic acid must be detected and quantified. Several approaches have been taken including, as outlined above, scintillation counting of radiolabeled fractions, ELISA assay of a stable metabolite (TxB_2, the stable metabolite of TxA_2), high pressure liquid chromatography (HPLC) and gas chromatography (GC) measurements.

In the studies presented here, the effect of permeabilization on extraction method and time course of arachidonic acid release was initially assessed in [^{3}H]-arachidonic acid radiolabelled platelets. The Bligh and Dyer method did not extract [^{3}H]-arachidonic acid from intact or digitonin permeabilized platelets following collagen stimulation for 10 minutes (Table 7), nor was there an increase in basal [^{3}H]-arachidonic acid in unstimulated platelets as reported by other investigators (Ramesha and Taylor; 1991). Consequently this method was abandoned.

The results of experiments using the Dole and Mertz (1960) extraction method were more consistent with previous literature reports. Puttmann and colleagues (1993) reported that the Dole and Mertz extraction was the most efficient in terms of time, extent of sample manipulation and precision in extracting fatty acids from human sera. Permeabilization of the platelets increased the absolute amount of [^{3}H]-arachidonic acid extracted in both stimulated and unstimulated platelets relative to intact platelets. As expected, basal radioactivity in unstimulated platelets remained constant from 1 to 10 minutes of incubation regardless of technique used (Tables 8 & 9), demonstrating that permeabilization per se did not activate the platelets and also that the preparation was stable over time. There was a significant increase in free [^{3}H]-arachidonic acid in response to collagen in both the intact and permeabilized platelets. Comparison of the time course for permeabilized platelets showed a slight, but non-significant increase from

the 1 to 5 minute incubation times (Tables 8 & 9), demonstrating that when cyclooxygenase is blocked, collagen-induced arachidonic acid rapidly reaches equilibrium. However, there was no significant difference between the 5 and 10-minute incubation times for either permeabilizer. These results correspond with the findings of Dragan and Ellis (1990) who found that arachidonic acid metabolite levels were stable 5 minutes after addition of [^{3}H]-arachidonic acid to unstimulated platelets.

A number of HPLC fatty acid measurement methods reported in the literature, using ultraviolet (UV), fluorescent or luminescence detection (Durst *et al.*, 1975; Miller *et al.,* 1978; Passi *et al.*, 1981; Dragan and Ellis, 1990; Wolff *et al.*, 1991; Akira *et al.,* 1993; Kramer *et al.*, 1993; Abushufa *et al.*, 1994; Fujita *et al.,* 1994). Each of these methods is hampered by technical difficulties. The HPLC method of separating and quantifying arachidonic acid developed by Puttmann and colleagues (Puttmann *et al.*, 1993) was chosen for this study for a number of reasons; firstly it was faster than other HPLC methods (Durst *et al.*, 1975; Miller *et al.,* 1978; Ramesha and Taylor; 1991); secondly it was sensitive in the picomolar range (Durst *et al.*, 1975; Puttmann *et al.*; 1993); and thirdly it used available equipment.

Since most fatty acids do not absorb in the UV region, derivatization techniques are used to increase the detection limits of the reaction. Previously this has been shown to result in an ester with high molar UV absorptivity, (Durst *et al.*, 1975) and high quantitative yields (>97%) without interfering by products, eliminating the necessity of further purification. Furthermore, the derivatization method was found to be selective for carboxylic groups and unreactive with ester or amide bonds, increasing the sensitivity of the method for fatty acids (Puttmann *et al.*, 1993) and eliminating the need for additional purification steps. Extracts from whole platelet had many peaks, making identification and quantification of arachidonic acid more difficult. Therefore, the extraction method was modified by permeabilizing the platelet membrane to improve extraction efficiency and improve the signal to noise ratio. Other investigators have used disruption of cell membranes for similar purposes (Abushufa *et al.*, 1994; Akira *et al.*, 1993). In the current study HPLC measurements showed an increase in arachidonic acid recovery with the use of a permeabilizing agent samples in both the unstimulated and simulated platelets (Table 10), but the proportion remained the same compared to the whole platelet samples.

However, HPLC measurements of arachidonic acid showed a different sensitivity to the permeabilzing agent than the radiolabelled measurements. Both the intact and digitonin treated samples showed consistent increases in arachidonic acid release in response to collagen, showing it to be the more reliable permeabilizer.

A comparison of HPLC results in intact and permeabilized platelets was undertaken. There was no significant difference between the extracted free arachidonic acid in intact and permeabilized platelets stimulated with 10 μg/ml collagen over a 10 minute period. Nor was there a significant increase with time. However, when compared to unstimulated platelets, permeabilized platelets show a significant increase in free arachidonic acid, whereas intact platelets did not . This indicates the variability associated with extraction from intact platelets.

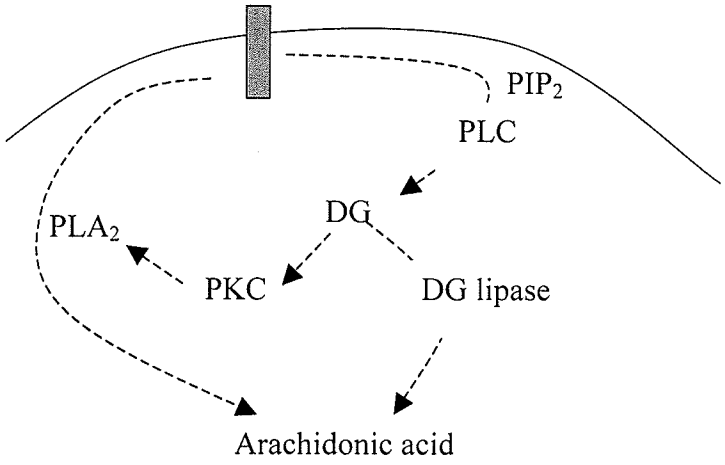
4.3 COLLAGEN-INDUCED PLATELET ACTIVATION

It has previously been demonstrated that inhibition of low-concentration collagen-induced aggregation by cyclooxygenase inhibitors abolished TxA_2 synthesis (Thomas and Holub, 1991; Lockhart *et al.*, 2001). The precise mechanism of arachidonic acid release by low concentration collagen, is unclear, although it is clearly central to the platelet activation process. There are several major differences in messenger generation with low-dose collagen, when compared to higher doses. Most critically, when cyclooxygenase is blocked, both IP_3 formation and cytosolic Ca^{2+} increases are blocked (Pollock *et al.*, 1986; Nakano *et al.*, 1989; Poole and Watson, 1995; Roberts *et al.*, 2004), whereas DG is still produced (Werner *et al.*, 1991). This is consistent with the rlease of arachidonic acid occurring at basal cytosolic Ca^{2+} levels. The receptor involved in platelet responses to low concentrations of collagen is unknown, although it is not likely GPVI-FcγRIIA since activation of this complex evokes responses not seen with low-dose collagen. DG lipase has been shown to play a minor role (Vedelago & Mahadevappa, 1988), however most interest has focused on $cPLA_2$ (Kramer *et al.,* 1993a,b; McNicol & Nickolaychuk, 1995; Börsch-Haubold *et al.*, 1995, 1997, 1998). PLD does not appear to be involved in arachidonic acid release in platelets (Chiang, 1994).

In the present studies collagen concentrations used to simulate platelets were sufficient to elicited full aggregation, but still low enough to be inhibited by

cyclooxygenase inhibitors such as BW755C (a dual cyclooxygenase/lipoxygenase inhibitor), acetylsalicylic acid and indomethacin. Recovery of arachidonic acid from collagen-stimulated platelets was similar to that reported by other investigators (Piche and Mahadevappa; Fujita *et al.*, 1994). Under these conditions collagen-induced aggregation was inhibited, in a concentration-dependent manner by U73122, Rö31-8220 and $AACOCF_3$, inhibitors of PLC, PKC and $cPLA_2$ respectively. Direct measurements of arachidonic acid release, in cyclooxygenase-inhibited platelets, were carried out in the presence of these inhibitors to further evaluate the role of PLC, PKC and $cPLA_2$ in collagen-induced platelet activation.

Figure 19 Potential pathways for arachidonic acid release in response to low-dose collagen.



4.3.1 PLA_2

The $cPLA_2$ inhibitor $AACOCF_3$ has previously been shown to block arachidonic acid release from platelets stimulated by thrombin (Bartoli *et al.*, 1994; McNicol & Nickolaychuk, 1995) or A23187 (Riendeau *et al.*, 1994). $AACOCF_3$ blocked collagen-induced aggregation (Lockhart *et al*, 2001) with no effect on arachidonic acid release as measured by HPLC (Figure 14), confirming the previous observations using

radiolabeling techniques (McNicol & Nickolaychuk, 1995). These effects of $AACOCF_3$ were accompanied by the inhibition of TxA_2 synthesis (Lockhart *et al.*, 2001), previously ascribed to an action on cyclooxygenase or thromboxane synthetase (Riendeau *et al.*, 1994; McNicol & Nickolaychuk, 1995). The lack of effect of $AACOCF_3$ is also compatible with the fact that $cPLA_2$ is a calcium-dependent enzyme (Leslie, 1997) and intracellular calcium in not increased in low-dose collagen stimulation (Pollock *et al.*, 1986; Poole and Watson, 1995). Therefore, $cPLA_2$ would not be expected to be involved in arachidonic acid liberation in response to low concentrations of collagen as further supported by the present data.

4.3.2 PKC

The PKC inhibitor Rö31-8220 blocked collagen-induced aggregation in a dose-dependent manner (Figure 3), but arachidonic acid release (Figure 14) and TxA_2 production (Lockhart *et al.*, 2001) were increased. Rö31-8220 aslo had a biphasic effect on collagen-induced secretion, increasing it at lower concentration and inhibiting it at higer concentrations (Figure 8). These data are consistent with the dual role that PKC plays in platelet activation. Stimulation of PKC by phorbol esters both activates platelets and acts in a negative feedback inhibitory manner (Hargreaves *et al.*, 1996). PKC plays a negative feedback role in a number of biochemical processes, including arachidonic acid release (Krishnamurthi *et al.* 1987). If this negative feedback role is attenuated by Rö31-8220 then the result would be an increase in arachidonic acid release, and its subsequent conversion to TxA_2. Nucciarelli and colleagues also reported PKC inhibition enhanced arachidonic acid release in G-protein stimulated platelets (Nucciarelli *et al.*, 1999). However the enhanced TxA_2 synthesis is accompanied by an inhibition of aggregation suggesting that the effects of inhibition of PKC also lie downstream of TxA_2. This is consistent with the platelet TxA_2 receptor being linked to PLCβ (and Choi, 1992; Banno *et al*, 1995; Singer *et al*, 1997; Katan, 1998) with the subsequent DG synthesis and PKC activation (Figure 10). Inhibition of TxA_2-induced PKC activation by Rö31-8220 would result in inhibition of aggregation; even under conditions there is increased TxA_2 production. The data presented here suggest that the negative feed back role for PKC in

low-dose collagen occurs prior to thromboxane, since Rö31-8220 only inhibited activation by U46619 (Figures 6 & 10).

4.3.3 PLC

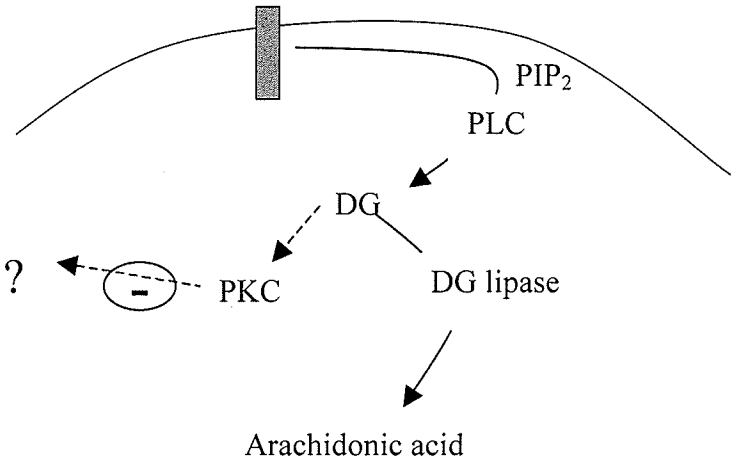
The inhibition of collagen-induced aggregation by U73122 could be explained by an effect on PLCγ (Heemskerk *et al.*, 1997) prior to TxA_2, an effect on PLCβ (Bleasdale *et al.*, 1990) distal to TxA_2 release, or an effect independent of PLC potentially as a result of blocking fibrinogen binding (Lockhart & McNicol, 1999). However, in acetylsalicylic acid pre-treated platelets, U73122 also inhibited low-dose collagen-induced arachidonic acid release (Figure 13), almost certainly localizing its effects to inhibition of PLCγ. Consistent with this, U73122 has been shown to inhibit collagen-induced tyrosine phosphorylation of PLCγ2 (Lockhart *et al.*, 2001). Taken together these data therefore support a role for PLCγ in the early platelet response to both low and high concentrationsof collagen, although it does not distinguish the PLCγ isoform involved in low-dose collagen stimulation.

Interestingly, under similar conditions, U73122 also inhibited collagen-induced p38MAPK phosphorylation, suggesting that p38MAPK lies downstream of PLCγ, consistent with a role for p38MAPK in collagen-induced activation (McNicol and Jackson, 2003). Initial studies implicated p38MAPK in $cPLA_2$ phosphorylation at higher collagen concentrations (Börsch-Haubold *et al.*, 1997), but subsequent reports have indicated that p38MAPK-induced phosphorylation does not induce phospholipase activity (Kramer *et al.*, 1996) despite the presence of phosphorylation domains on $cPLA_2$ (Börsch-Haubold *et al.*, 1998). Previous studies using Rö31-8220 clearly dissociates p38MAPK from arachidonic acid release (Lockhart *et al.*, 2001), suggesting that p38MAPK plays a role in the aggregation response to low concentrations of collagen but a relatively minor, if any, role in arachidonic acid release (Saklatvala *et al.*, 1996; Lockhart *et al.*, 2001).

4.3.4 Summary

Taken together the data suggest that collagen-induced arachidonic acid release occurs distal to PLCγ2 but not as a consequence of $cPLA_2$. The data further suggests that a DG lipase is acting on DG synthesised as a consequence of PLCγ2 activity to release arachidonic acid (Figure 20). DG lipase and monoglyceride lipase are both present in platelets (Tohmatsu *et al.*, 1987; Oglesby and Gorman, 1984; Werner *et al.*, 1991). Arachidonic acid release can occur during collagen-induced platelet activation at resting cytosolic Ca^{2+} levels by a combination of PLC and DG and monoglyceride lipases (Moriyama *et al.*, 1994) and a DG lipase inhibitor blocks collagen-induced aggregation and TxA_2 production.

Figure 20 Mechanism of arachidonic acid release in low-dose collagen-stimulated platelets



4.4 CONCLUSION

The data presented in this thesis demonstrate that in platelets:

1. U73122 is a non-selective PLC inhibitor which also acts by attenuating fibrinogen binding, which ultimately results in disruption of outside-in signalling arising from fibrinogen receptor occupation;
2. A combination of permeabilization, fatty acid extraction, derivatization and separation by HPLC can successfully be used to quantify arachidonic acid release from platelets;
3. Arachidonic acid release, and subsequent conversion to TxA_2, is fundamental to full platelet aggregation in response to low concentrations of collagen;
4. PLCγ2 activity is central to the early platelet response to low, as well as high, concentrations of collagen;
5. Arachidonic acid release in response to low concentrations of collagen likely occurs by the consecutive actions of PLCγ2 and DG (and/or monoglyceride) lipase;
6. There is a minor, if any, role for $cPLA_2$ in arachidonic acid release in response to low concentrations of collagen.

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