ROLE OF PHOSPHOINOSITIDE 3-KINASE IN PLATELET RESPONSES TO PLATELET-ACTIVATING FACTOR

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in

THE FACULTY OF GRADUATE STUDIES
(Experimental Medicine Program)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

1997

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Date April 29, 1997
Platelets are necessary for hemostasis but are also involved in a broad range of pathophysiologic processes such as atherosclerosis and thrombosis. Platelet activating factor (PAF), an inflammatory mediator, is an important physiological regulator of platelet function. The major objective of this work was to determine the role of phosphoinositide 3-kinase (PI 3-kinase) in platelet responses to platelet activating factor. We show, for the first time in platelets, that PAF activates PI 3-kinase over a rapid time course that correlates closely with the aggregation response. The potent PI 3-kinase inhibitors, wortmannin and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) were used to probe the dependence of PAF-induced aggregation and dense granule release on PI 3-kinase. Both compounds markedly inhibited PAF-induced aggregation; however, only at low activation states giving reversible aggregation (primary phase) did this correlate with PI 3-kinase inhibition. Secretion, measured as release of $\text{H}^3$-hydroxytryptamine, was inhibited to a maximum of 30% and only at low concentrations of PAF. We suggest that PI 3-kinase activation is important for reversible (primary) aggregation of platelets in response to PAF, perhaps by contributing to the ‘inside-out’ activation of platelet glycoprotein IIbIIIa, the fibrinogen receptor. PI 3-kinase only plays a minor role in PAF induced dense granule release at low activation states. Both responses are dramatically less dependent on PI 3-kinase activity when high concentrations of PAF are used, suggesting greater contribution from other pathways. Tyrosine kinases appear to be important regulators of PI 3-kinase at high PAF levels as stimulation results in a greater than 10 fold increase in PI 3-kinase activity associated with tyrosine-phosphorylated proteins. The p85 regulatory subunit of PI 3-kinase is not tyrosine-phosphorylated. Rather, the enzyme associates rapidly with a major tyrosine-phosphorylated 115 kDa protein, a potential regulator of PI 3-kinase activation in platelets. This protein was not immunoreactive with several antibodies to proteins in this
molecular weight range known to be tyrosine phosphorylated and to associate with PI 3-kinase on cell activation.
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<tr>
<td>GP</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet-activating factor</td>
</tr>
<tr>
<td>TXA₂</td>
<td>thromboxane A₂</td>
</tr>
<tr>
<td>PLA₂</td>
<td>phospholipase A₂</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PI 3-kinase</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol (3,4,5) trisphosphate</td>
</tr>
<tr>
<td>PI (3,4)P₂</td>
<td>phosphatidylinositol (3,4) bisphosphate</td>
</tr>
<tr>
<td>PI (3) P</td>
<td>phosphatidylinositol (3) monophosphate</td>
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<tr>
<td>3-PPI</td>
<td>3-phosphoinositide</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol (1,4,5) trisphosphate</td>
</tr>
<tr>
<td>IP₄</td>
<td>inositol (1,3,4,5) tetrakisphosphate</td>
</tr>
<tr>
<td>G-protein</td>
<td>guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium-dodecyl sulfate</td>
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<td>PAGE</td>
<td>polyacrylamide gel-electrophoresis</td>
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<td>IC</td>
<td>inhibitory concentration</td>
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<td>p85</td>
<td>85 kDa regulatory subunit of PI 3-kinase</td>
</tr>
<tr>
<td>p110</td>
<td>110 kDa catalytic subunit of PI 3-kinase</td>
</tr>
<tr>
<td>SH2/3</td>
<td>src-homology domain 2/3</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
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<td>LY294002</td>
<td>2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one</td>
</tr>
<tr>
<td>GPIIbIIIa</td>
<td>integrin α₉β₃ = platelet fibrinogen receptor</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
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<tr>
<td>p115</td>
<td>115 kDa tyrosine-phosphorylated PI 3-kinase associating protein</td>
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ACKNOWLEDGMENTS

I wish to express my sincere appreciation to the following individuals and organizations whose support and assistance were invaluable for the completion of these studies:

my supervisors: Dr. Vincent Duronio and Dr. Hassan Salari for providing laboratory space and equipment to conduct research as well as a stimulating intellectual environment and valuable guidance.

members of my supervisory committee: Dr. Robert Schellenberg, Dr. Hermann Ziltener and Dr. Michael Gold for attending meetings, providing input and reading drafts of the thesis.

the blood drawing services of the Jack Bell Research Centre Animal Unit

the Haematology unit of Vancouver Hospital and Health Sciences Centre for platelet counting services

Mr. David Fong for excellent technical assistance

and last, but not least, my family and friends for their support and patience over the last four years
INTRODUCTION

A. Platelets

A.1 Platelet Physiology

A.1.1 Thrombopoiesis- The Origin of Platelets

The major site for platelet production is the bone marrow, although other secondary tissue sources such as lung and spleen have been proposed (Herd and Page, 1994). Hematopoietic progenitor cells that give rise to cell colonies containing megakaryocytes when cultured in semi-solid medium are named CFU-Meg. In response to specific signals the committed CFU-Meg progenitor cells proliferate and differentiate into immature megakaryocytes. Unique to the megakaryocytic lineage however, cell division then stops after a number of mitotic divisions and the immature megakaryocytes undergo 3-5 rounds of synchronous nuclear endoreduplication (endomitosis) giving rise to mature polyploid megakaryocytes (Kaushansky, 1995). Platelets are then formed from fragmentation of the megakaryocyte cytoplasm. The higher the ploidy of the mature megakaryocyte, the larger the cell volume and therefore the greater the capacity to generate platelets. It has been suggested that 95% of megakaryocytes competent to generate platelets are ≥ 16 N ploidy (Levine et al., 1982).

Data from rodents with acute thrombocytopenia induced using rabbit anti-mouse serum or exchange transfusion have suggested that the processes of CFU-Meg proliferation and megakaryocyte nuclear endoreduplication/cytoplasmic maturation are regulated independently (Broudy and Kaushansky, 1995). The first may be controlled by a "megakaryocyte proliferation factor(s)" which is constitutively present and the second by a
“megakaryocyte-potentiating factor (s)”, the concentration of which would be regulated by the blood platelet concentration.

Interleukin (IL)-3 has been identified in vitro as the major megakaryocyte proliferation factor as it potently, but non-specifically, stimulates CFU-Meg mitosis (in addition to BFU-E and CFU-GM) and is present in the marrow microenvironment. The c-kit ligand may support IL-3 in this effect as well. IL-3 however, does not induce megakaryocyte maturation. Odell et al. (1961) were the first group to describe a humoral regulator of platelet production in the sera of thrombocytopenic patients capable of stimulating megakaryocyte endoreduplication/maturation, the long sought-after thrombopoietin. Efforts to biochemically purify the candidate protein to homogeneity in amounts sufficient for sequencing analysis from urine, plasma, serum and conditioned culture medium consistently failed due to numerous obstacles. To complicate matters, other factors including IL-6, IL-11 and LIF were identified and cloned and were candidate ‘thrombopoietins’ (Kishimoto, 1989; Du and Williams, 1994; Patterson, 1994). Although these cytokines potentiate the effects of IL-3 on CFU-Meg growth their main influence is on megakaryocyte maturation. However, the fact that these cytokines are not potent stimulators of platelet production in vivo and their levels do not fluctuate inversely with the platelet count generally ruled them out as a potential, distinct lineage-specific thrombopoietin (Ishibashi et al., 1989; Neben et al., 1993).

A new avenue for pursuit of this cytokine was opened when the c-mpl gene which encodes the putative receptor for thrombopoietin was cloned in 1992 (Vigon et al., 1993; Vigon et al., 1992). Soon after, several groups independently cloned the cDNA encoding the ligand of the c-mpl receptor, which was identified as thrombopoietin (Bartley et al., 1994; Lok et al., 1994). Sequence analysis suggested significant homology with erythropoietin and a predicted molecular mass of 36 kDa. The mature protein is heavily glycosylated and in bone marrow thrombopoietin mRNA levels vary inversely with platelet count. In vitro, thrombopoietin is a potent stimulator of CFU-Meg proliferation and
promotes megakaryocyte polyploidy (64N). Subsequent animal studies provided proof that thrombopoietin acts as both a proliferation and maturation factor for megakaryopoiesis in vivo (Lok et al., 1994). c-mpl knock-out mice have only 15% normal platelet counts, reduced number of megakaryocyte in the marrow but with normal representation of other blood cell lineages (Gurney et al., 1994). Thrombopoietin holds great therapeutic promise for the treatment of thrombocytopenia (chemotherapy or radiation-induced, for example).

A.1.2 Platelet Ultrastructure/Anatomy

Platelets exhibit many common features with nucleated cells and indeed the megakaryocytes from which they are derived. However, they also contain organelles and sub-cellular structures that are specially adapted to function in specific platelet responses (Blockmans et al., 1995).

Resting platelets are discoid in shape and have a typical bilamellar plasma membrane. Their cell volume is between 5-7 femtoliter (fL) (depending on species) which is about 14 x times smaller than erythrocytes. A normal platelet count in human blood is 1.3-4.0 x 10^8 per ml. The lifetime of platelets in the blood is about 10 days. There are numerous invaginations of the plasma membrane extending throughout the platelet forming a system of channels called the open surface-connected canalicular system (OSCCS). This serves to tremendously increase the surface area of the plasma membrane allowing for enhanced exchange of proteins, granule contents and metabolites between the platelet cytoplasm and the surrounding plasma. Intrinsic glycoproteins spanning the plasma membrane serve as receptors for numerous excitatory and inhibitory agonists as well as adhesive proteins.

The platelet also contains a cytoskeleton composed mainly of two actin filament-based components (Fox, 1993; Fox, 1994). Indeed, actin constitutes 15-20% of the total
platelet protein content. The cytoplasm is filled with a network of long actin filaments associated with proteins such as α-actinin, tropomyosin and actin-binding protein. These filaments mediate contractile events in activated platelets such as granule centralization, extension of filopodia and clot retraction. Platelets also contain a membrane skeleton composed of short actin filaments that coat the inner surface of the plasma membrane very similar to that of the erythrocyte membrane skeleton. This membrane skeleton is connected to the plasma membrane through association with the cytosolic domains of certain membrane glycoproteins such as integrins. Functions attributed to the membrane skeleton include stabilization of the plasma membrane, regulation of platelet shape and distribution of membrane glycoproteins. In addition to the actin-based components, there is a coil/bundle of microtubules, just below the surface of the membrane extending the circumference of the platelet. These microtubules are composed mainly of tubulin and serve to maintain the discoid shape of the unstimulated platelet (Herd and Page, 1994).

The dense-tubular system, a membranous structure derived from the megakaryocyte endoplasmic/sarcoplasmic reticulum is the major site for calcium sequestration and lies in close proximity to the OSCCS. Organelles found in the platelet include a few mitochondria, glycogen particles, lysosomes, peroxisomes and the most numerous, the platelet-specific storage granules (Rendu et al., 1987). The dense (or δ) granules are rich in pro-aggregatory substances including ATP, ADP, Ca\(^{2+}\), pyrophosphate and serotonin. The more numerous α-granules contain vasoactive substances which have either been synthesized by the megakaryocyte or taken up from the circulation. They also contain numerous adhesive proteins and tissue repair factors; these include fibrinogen, fibronectin, thrombospondin, albumin, PDGF, plasminogen activator inhibitor I, vWF (clotting factor VII), TGF-β, clotting factor V and the platelet specific proteins (β-thromboglobulin and platelet factor 4). Platelets also contain residual mRNA transcripts but are capable of only limited protein synthesis. Spent platelets are cleared from
circulation by macrophages of the reticuloendothelial system in the spleen, liver and bone marrow (half-life= 8-12 days).

A. 1. 3. The Platelet in Primary Hemostasis

Platelets play an essential role in the first phases of the hemostatic process (Packham, 1994). The normal physiological process by which platelets contribute to the formation of a hemostatic plug (thrombus) at a site of vascular injury can be roughly divided into four phases; adhesion, aggregation, secretion, and expression of procoagulant activity. The major features and components of each stage will be discussed in the aforementioned order, which is roughly chronological. It should be emphasized however that these stages are (a) not discrete, (b) occur extremely rapidly (except for induction of pro-coagulant activity) (c) are often inter-dependent and (d) in addition to the platelet require the vasculature (endothelium) and numerous blood components.

Adhesion. When the normally non-thrombogenic endothelial lining of a blood vessel is damaged, platelets make contact with, adhere and spread upon the exposed sub-endothelial matrix. This process may initiate in as little as 50 ms. after vessel damage in regions of high flow-rate (Gear, 1994). The mechanism of adhesion is dependent on the rate of blood flow at the site of injury and does not require active metabolism or ATP. Under conditions of slow blood flow (low shear rates), adhesion is mediated primarily by platelet binding to collagen, fibronectin and laminin in the sub-endothelial matrix. The adhesion receptors involved are thought to be GP (glycoprotein) Ia/IIa, GP Ic/IIa and GP Ic'/IIa respectively. When the flow rate is high such as in arterioles and the microcirculation, these interactions are not sufficient to support adhesion and therefore binding to von Willebrand factor (vWF) is required (Baumgartner et al., 1980). vWF (220 kDa) circulates in the plasma and is present in the subendothelial matrix as multimeric complex ranging in size from 500 kDa to 20,000 kDa. The major binding site on the
platelet for vWF is the GP Ib/IX complex and is thought to be the one primarily responsible for mediating adhesion under these conditions. Circulating platelets do not interact with plasma vWF as it may have a different conformation than vWF in the subendothelial matrix (Baumgartner et al., 1980).

**Aggregation** Adhesion is followed closely by aggregation whereby platelets adhere to each other and employ the layer of adherent platelets as a foundation, forming the initial plug or “white thrombus”. Aggregation, in contrast to adhesion, requires active metabolism, platelet stimulation (activation) by primary agonists such as thrombin, collagen, adenosine diphosphate (ADP), platelet-activating factor (PAF) or thromboxane A₂ (TXA₂), divalent cations (Mg^{2+} or Ca^{2+}) in the medium, specific plasma proteins such as fibrinogen or vWF and the fibrinogen receptor (the GP IIb/IIIa complex). Agonist interaction with specific receptors on the platelet membrane results in the generation of internal signals (second messengers) by effector systems such as phospholipase C. These signals transmit the initial stimulus back to the platelet surface resulting in the activation of the fibrinogen receptor (GPIIb/GPIIIa, integrin α₉β₃) followed by binding and cross-linking of adjacent platelets via dimeric fibrinogen. Signal transduction pathways activated by platelet agonists as well as the functional responses they impinge upon are discussed in a later section. Stimulation of phospholipase A₂ (PLA₂) during adhesion/aggregation results in the generation and excretion of the potent agonist TXA₂ which potentiates the response by binding to specific platelet receptors.

Platelet aggregation can be measured *in vitro* in an aggregometer whereby the change in light transmission is recorded as platelet aggregates form, after addition of agonist to a rapidly stirred suspension of platelets. Platelet aggregation can be divided into three phases. The first, immediately after agonist addition is characterized by a transient decrease in light transmission when platelets change shape from discoids to spheres with filopodia. The subsequent increase in transmittance due to aggregate formation occurs in two waves. The first (or primary phase), a direct result of platelet
stimulation, is reversible and is not associated with (or dependent on) granule secretion. The second (secondary phase) is associated with (or dependent upon) secretion, is irreversible and results in the formation of large aggregates. Platelet aggregation requires high concentrations (mM) of divalent cations such as Ca\(^{2+}\), a soluble adhesive protein such as fibrinogen or vWF and the membrane glycoprotein GPIIbIIIa (integrin α\(_{IIb}β_3\)) also known as the fibrinogen receptor (Calvete, 1995). Even though the fibrinogen receptor is always present on the platelet membrane, prior platelet activation and an unknown intra-platelet signal resulting in a conformational change in the extracellular domain of GPIIbIIIa are required to render this integrin competent to bind fibrinogen and therefore cross-link adjacent platelets (aggregation).

**Secretion** The role of platelet secretion in primary hemostasis is not well defined. One probable function of secretion is to propagate the aggregation response in a kind of ‘feed-forward’ cycle since secretion occurs concurrently with the second wave (irreversible) of aggregation (Bennett and Kolodziej, 1992). ADP released from dense-granules may serve as an autocrine activator as well as activating and recruiting new platelets to the growing thrombus. Subsequent to secretion, many α-granule contents as well as granule specific membrane glycoproteins are incorporated in the platelet plasma membrane and are used as markers to verify α-granule secretion. Substances released by platelets are also important in wound repair and healing.

**The Procoagulant Response** Another important platelet response is the plasma membrane exposure of specific membrane phospholipids which provides a catalytic surface for the assembly of enzyme complexes of the coagulation cascade (Bevers et al., 1993). This leads to a large increase in the rate of thrombin formation and subsequently the rapid generation of an insoluble meshwork of fibrin which is necessary to consolidate the primary hemostatic plug. The platelet is the primary source of procoagulant lipid surfaces in normal hemostasis. Phosphatidylserine is the major negatively-charged phospholipid responsible for the catalytic properties of a lipid surface and as such is the
molecule responsible for the pro-coagulant properties of the platelet. In quiescent platelets, phosphatidylserine is almost exclusively located in the inner (cytoplasmic) leaflet rendering the plasma membrane non-thrombogenic. Upon stimulation (especially with strong agonists such as thrombin or collagen), this asymmetric distribution is lost and the phosphatidylserine content of the outer leaflet increases. This provides binding sites for enzymes of the tenase and prothrombinase complexes and resultant increase in pro-coagulant activity. Experimental observations suggest that the procoagulant response is regulated independently of other responses. For example, full expression of a procoagulant surface usually requires longer than 5 minutes while aggregation and secretion are complete within 2 minutes. Re-distribution of phosphatidylserine to the outer leaflet has been related to the formation of micro-vesicle/particles originating from the plasma membrane (shedding of microvesicles). In other words, there is a tight coupling observed between microvesicle formation and development of procoagulant activity.

_Clot Retraction_ At least _in vitro_, clot formation is followed by clot retraction only if platelets are present. This indicates that platelets interact with the fibrin network within the thrombus. One mechanism put forward is that the ends of the fibrin strands bind to the GPIIbIIIa complex thereby transmitting the force of contraction of the platelet cytoskeleton to the clot (Hantgan et al., 1985; Cohen et al., 1982).

A.1.4 Congenital Disorders of Hemostasis

Heritable defects in hemostasis resulting in bleeding abnormalities were first described by Glanzmann, Von Willebrand, and Bernard and Soulier early in this century (Caen and Rosa, 1995). The establishment of _in vitro_ tests for platelet function showed that abnormalities in Glanzmann's thrombasthenia (GT), Bernard Soulier syndrome (BSS) and von Willebrand disease (vWD) were localized to the platelet membrane for the first two disorders and to a plasma component (vWF) in the latter (Caen and Levy-Toledano,
The observations that specific platelet membrane glycoproteins were absent in GT (Nurden and Caen, 1974) and BSS (Nurden and Caen, 1975) led to the demonstration that the GPIb-IX complex was required for platelet adhesion to the subendothelium (Fauvel et al., 1983) and the GPIIb-IIIa complex was necessary for platelet-platelet cohesion (aggregation). Cloning and sequencing of the genes for these glycoprotein complexes resulted in the identification of numerous mutations leading to quantitative (low protein levels) or qualitative (functional disruption) deficiencies for these receptors in BSS or GT (Phillips et al., 1988; Lopez, 1994). In vWD, the most common of the congenital disorders of hemostasis, there are quantitative or qualitative abnormalities in vWF itself (Sadler, 1994). Although these disorders in adhesion (BSS or vWD) and aggregation (GT) are the best characterized congenital platelet disorders there are clinical examples related to underlying defects in receptor-agonist interaction, secretion (storage pool deficiencies) and platelet procoagulant activity (Scott syndrome) (Rao, 1990).

Abnormal platelet function primarily resulting in excessive bleeding can be acquired by the use of certain drugs or associated with certain medical conditions and is much more prevalent than the inherited disorders. Use of aspirin, NSAID’s, beta-lactam antibiotics and thrombolytic agents can lead to platelet dysfunction. Medical conditions associated with abnormal platelet function include uremia associated with renal failure, cardiac bypass surgery, liver disease and myeloproliferative disorders (Bennett and Kolodziej, 1992). Normal platelet function can often be restored in these circumstances by amelioration of the primary disease.

A.1.5 Adjunct Roles for Platelets in Other Diseases

Platelets have an established role in the pathogenesis of vascular disease, especially atherosclerosis, thrombosis, thromboembolism and stroke (hemorrhagic/thrombotic)
(Ruggeri, 1994; Becker, 1993; Winocour, 1994; Schror, 1995; Brown and Martin, 1994; Marcus and Safier, 1993; Rao and Rao, 1994). The mechanism for formation of hemostatic plugs and arterial thrombi are essentially the same (Packham, 1994). Considerable work is currently underway to develop drugs that will specifically inhibit discrete platelet responses. This is preferable to non-specific drugs that could totally inhibit platelet activation and lead to global disturbances in hemostasis (Becker, 1993; Schror, 1995; Rao and Rao, 1994). In addition to disorders of the cardiovascular system, platelets have been implicated in lung disease, cancer, allergic reactions and inflammatory disease (Herd and Page, 1994; Honn et al., 1992).

A.2 Mechanisms of Stimulus-Response Coupling (Signal Transduction) in Platelets

The protein components of signal transduction pathways by which a platelet stimulus initiates transmission of a signal to ultimately result in a physiological response are necessarily derived from the megakaryocyte precursor. As such, the coupling mechanisms as well as effectors and second messengers activated in platelets upon agonist stimulation are similar to those that operate in nucleated cells (Kroll and Schafer, 1989; Ashby et al., 1990). A major question with respect to platelet stimulus-response coupling (and signal transduction in general) is whether or not activation of a particular pathway is necessary for a functional response to an agonist.

The net ‘activation state’ of a circulating (or ‘ex-vivo’) platelet is determined by the combination of extracellular factors to which it is exposed (Holmsen, 1991). Platelet agonists interact with specific receptors in the plasma membrane and may be classified as excitatory or inhibitory. Examples of excitatory agonists include thrombin, PAF, collagen, ADP and TXA₂. In contrast, NO, adenosine, prostacyclin (PGL₂), PGE₁, PGD₂ potently inhibit platelet activation. An excitatory agonist is considered ‘strong’ if it is able to induce granule secretion in the absence of TXA₂ synthesis or ‘weak’ if secretion requires
the arachidonic acid pathway. Many platelet mediators bind to receptors that are coupled
to intracellular effector systems through guanine-nucleotide binding proteins (G-proteins)
(Colman, 1990). These receptors are members of a superfamily characterized by single
polypeptides with seven transmembrane domains and lacking intrinsic enzyme activity (the
serpentine receptor family). Adhesive ligands such as fibrinogen and vWF bind to
glycoprotein complexes of the integrin family which employ different coupling
mechanisms to their effector systems (described below).

In classical descriptions of stimulus-response coupling in platelets, many authors
have adopted the position that it is ultimately the calcium status (i.e. cytoplasmic Ca$^{2+}$
concentration) of the platelet that determines the type and magnitude of the response
achieved; i.e. the platelet as a Ca$^{2+}$ driven cell (Scrutton, 1993). The observations that
calcium ionophores (A23187) induce platelet responses very similar to those obtained with
strong physiological agonists supports this theory (Massini and Luscher, 1974). A central
mechanism by which excitatory agonists elicit platelet responses is through activation of
phospholipase C (PLC). Conversely, the effects of inhibitory agonists are often mediated
through activation of adenylyl cyclase. Activation of these enzymes results in the
generation of second messengers which lead to changes in cytoplasmic Ca$^{2+}$/pH or in the
activation of protein kinases. Numerous reviews have been published describing the signal
transduction pathways leading to platelet activation and inhibition (Blockmans et al., 1995;
Siess, 1989; Kroll and Schafer, 1989; Ashby et al., 1990). Here, I will attempt to
summarize the predominant pathways thought to mediate platelet responses with emphasis
on phospholipid signalling and protein-tyrosine kinase pathways.

A.2.1 Phospholipase C

The primary event involving activation of a second-messenger producing effector
system after binding of stimulatory platelet agonists to their receptors appears to be
activation of phosphoinositide-specific phospholipase C (PI-PLC) (Heemskerk et al., 1993; Lapetina and Siess, 1983). This results in the hydrolysis of the minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2) to give the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). Although PI-PLC may employ phosphatidylinositol (PI) and PI-4-P as substrates in vitro, this usually requires much higher concentrations of Ca\(^{2+}\) than is normally present in vivo. The immediate biochemical changes invoked by IP3 and DAG (elevation of cytosolic Ca\(^{2+}\) and activation of PKC respectively) have been causally linked to many of the platelet responses described above, especially secretion and aggregation. The most abundant isoforms of PLC detected in platelets are PLC \(\beta_2\), PLC \(\beta_3a\), and PLC \(\gamma_2\). Present in smaller quantities are PLC \(\beta_1\), PLC \(\gamma_1\) and PLC \(\beta_{3b}\) (Banno et al., 1996; Okano et al., 1990; Banno et al., 1990; Nozawa et al., 1993). Mechanisms for activation of these isoforms involve G-proteins \(G_{PLC_{beta}}\) and protein-tyrosine kinases (PLC \(Y_1\)-2) and are similar in platelets as described for other cell types (Okano et al., 1990).

A.2.1.1 IP3/Ca\(^{2+}\) in Platelet Function

The very rapid kinetics of IP3 generation in platelets generally correlate with the time course for increases in [Ca\(^{2+}\)] after agonist stimulation. IP3 functions to release Ca\(^{2+}\) from the dense-tubular system after binding to a specific receptor on this structure. The IP3 signal is rapidly terminated by either dephosphorylation to an inactive compound or phosphorylation to Ins (1,3,4,5)P4 (IP4). IP4 is then rapidly de-phosphorylated to Ins (1, 3, 4)P3 (a weak Ca\(^{2+}\) mobilizer) which after one minute of thrombin stimulation comprises 90% of the IP3 in the platelet (Daniel et al., 1987). IP4 may also facilitate refilling of internal Ca\(^{2+}\) stores by stimulating Ca\(^{2+}\) influx through the plasma membrane and/or re-sequestration to the dense-tubules (Ashby et al., 1990). Within seconds of agonist stimulation the internal cytosolic Ca\(^{2+}\) concentration in platelets increases from about 100 nM to several \(\mu\)M (Rink et al., 1982). The release of Ca\(^{2+}\) from internal stores is closely
associated with shape changes, aggregation and secretion (Rink and Hallam, 1984). The agonist-induced rise in internal calcium is at least partially dependent on the presence of Ca$^{2+}$ in the surrounding medium.

There are numerous effector systems in platelets which depend on elevated Ca$^{2+}$ to elicit a physiological response. Calmodulin, a 17 kDa protein, is the major Ca$^{2+}$ binding protein in platelets. After binding up to four Ca$^{2+}$ ions, calmodulin binds to and activates myosin light chain kinase (MLCK). This kinase phosphorylates the 20 kDa light chain of myosin, allowing the ATP-dependent interaction of myosin and actin and the generation of contractile forces. Platelet shape change is closely correlated to partial myosin phosphorylation suggesting a role for myosin-actin contraction in the shape change response (Daniel et al., 1984). More extensive myosin phosphorylation has been related to granule centralization, a process required for secretion. Other targets for Ca$^{2+}$ in activated platelets include a group of Ca$^{2+}$-dependent proteases called calpains which may be involved in cytoskeletal re-organization/aggregation (Yoshida et al., 1983). In addition to protein kinase C, there is substantial evidence suggesting that both phospholipase A$_2$ and PI-PLC are regulated by intracellular Ca$^{2+}$ (Kramer et al., 1986).

A.2.1.2 Diacylglycerol/Protein kinase C

DAG, the other second messenger generated by PLC acts in concert with Ca$^{2+}$ to activate conventional protein kinase C isoforms (PKC $\alpha$, $\beta$ and $\gamma$) and serves to amplify the signals generated by PLC leading to platelet aggregation and secretion. This is supported by the experimental observation that low levels of phorbol ester and calcium ionophore synergize to induce aggregation and secretion (Yamanishi et al., 1983). The major substrate for PKC in platelets is a 47 kDa protein known as pleckstrin whose phosphorylation is used as a marker for platelet PKC activation but whose function in platelets is controversial (Sano et al., 1983). DAG/Ca$^{2+}$-activated PKC (i.e. PLC-
dependent activation) has been associated with aggregation (without shape change), secretion and arachidonic acid metabolism. Some have proposed that PKC provides the ‘inside-out’ signal required for activation of the fibrinogen receptor, allowing aggregation (Shattil and Brass, 1987). PKC also has a complicated regulatory role in platelet activation. For instance, there is strong evidence that PKC is involved in feedback inhibition of PI-PLC, the biochemical event that initiates its activation (Watson et al., 1988).

A.2.2 Phospholipase D

Activation of phospholipase D (PLD) resulting in the formation of phosphatidic acid (PA) occurs in platelets in response to agonists such as thrombin and collagen (Halenda et al., 1996). The major PLD substrate in platelets is phosphatidylcholine and dual-labeling experiments have indicated that PLD-derived PA is not converted to DAG by a phosphatidate phosphohydrolase as has been observed in neutrophils. Also, phosphatidylcholine does not appear to be hydrolysed by a PC-specific PLC in platelets. In thrombin-stimulated platelets, the PLD pathway only contributes about 15% of the total PA generated, the rest being formed from phosphoinositides via PLC. The PLD-activating potency of platelet agonists varies appreciably with collagen 2.5 fold stronger than thrombin and PAF being unable to activate platelet PLD (Halenda et al., unpublished observation). Tyrosine kinases, Ca\(^{2+}\), G-proteins and PKC have been shown to contribute to the activation of platelet PLD. While the role of PLD in platelet activation has not been investigated thoroughly, some investigators have proposed a function for this enzyme in platelet secretion and regulation of PLA\(_2\) activity.
A.2.3 Phospholipase A<sub>2</sub>

Activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) is important in stimulus-response coupling in platelets (Kramer et al., 1993). PLA<sub>2</sub> cleaves the sn-2 acyl bond of membrane phospholipids yielding arachidonic acid (AA) and the corresponding lypo-phospholipid. The preferred substrate of PLA<sub>2</sub> is phosphatidylcholine but other lipids are hydrolysed as well. As will be discussed below, PLA<sub>2</sub> is also essential for PAF synthesis. After de-acylation, AA is rapidly metabolized to a diversity of potent cellular mediators (eicosanoids) via the cyclooxygenase and lipoxygenase pathways. The potent platelet agonist TXA<sub>2</sub> is formed from prostaglandin endoperoxides derived from oxygenation of AA by cylooxygenase. TXA<sub>2</sub>, although highly labile, can diffuse out of the platelet and bind to receptors on the same or adjacent platelets leading to direct activation of PLC via G-proteins. There is also evidence that arachidonic acid itself could function as a second messenger in platelets. The arachidonic acid pathway seems to be the primary receptor-mediated effector that allows so-called weak agonists (i.e. ADP) to stimulate secretion and aggregation though autocrine activation of PLC. In contrast, strong agonists (thrombin or collagen) can stimulate PLC directly. Platelet regulation of PLA<sub>2</sub> is complex. Evidence suggests that activation is dependent on elevated cytosolic Ca<sup>2+</sup>. PKC may indirectly potentiate PLA<sub>2</sub> activation by phosphorylating (and inhibiting) an endogenous protein inhibitor of PLA<sub>2</sub> named lipocortin (Kramer et al., 1986; Kramer et al., 1993). PLA<sub>2</sub> activation in platelets may also require alkalinization of the cytosol via Na<sup>+</sup>/H<sup>+</sup> exchange. In summary, arachidonic acid metabolism after PLA<sub>2</sub> activation in platelets, coupled with release of pre-formed mediators such as ADP-serotonin, serves as a crucial mechanism for in vivo feedback amplification of the original stimulatory signal leading to a full hemostatic response.
A.2.4 Platelet Inhibition via Activation of Adenylate Cyclase

The major mechanism by which inhibitory agonists attenuate or prevent platelet response is through activation of adenylate cyclase resulting in elevation of intra-platelet cAMP levels (Mills, 1982). cAMP is metabolized by various phosphodiesterases, thus eliminating the second messenger when the stimulating agonist is no longer present. Agents that increase platelet cAMP and thus are inhibitory to platelet activation are NO (endothelial-derived relaxation factor, EDRF), adenosine, prostacyclin (PGI2), PGE1 and PGD2. Prostaglandins and adenosine bind to platelet receptors that are coupled to adenylate cyclase via the G protein, Gs. Prostacyclin is the most potent inhibitor of platelet aggregation. Adenylate cyclase in platelets is inhibited by several compounds, all of which are excitatory agonists, including thrombin, ADP and adrenaline whose receptors are coupled to the enzyme through Gi. It should be noted that these agents do not stimulate platelet aggregation or secretion through reduction in cAMP concentrations since other adenylyl cyclase inhibitors do not cause platelet activation (Haslam et al., 1978).

In the simplest sense, the effects of cAMP on the platelet are directed at reversing or blunting platelet changes mediated by elevation of Ca2+. Elevated cAMP leads to reversal of agonist-induced myosin light chain and pleckstrin (p47) phosphorylation and reversal of cytoskeletal assembly (Haslam et al., 1978). Several mechanism for cAMP (PKA) directed inhibition of platelet activation have been described; (a) PKA phosphorylates a Ca2+ uptake system, removing Ca2+ from the cytosol (Feinstein et al., 1983), (b) PKA mediates phosphorylation of MLCK, decreasing its affinity for Ca2+-calmodulin and thereby decreasing MLCK activity, resulting in abrogation of the shape change response (Conti and Adelstein, 1981) (c) cAMP mediates inhibition of thrombin-stimulated formation of DAG and PA suggesting possible inhibition of PLC by PKA. Earlier studies from our laboratory have demonstrated that forskolin inhibits PAF-induced activation of rabbit platelets, independent of cAMP induction, possibly through direct
modification or antagonism of the PAF receptor (Wong et al., 1993). These conclusions were reached after it was shown that (a) a cAMP analog could not mimic the effects of forskolin and (b) an inactive analog of forskolin (dideoxyforskolin), which does not activate adenylate cyclase, also reduced PAF binding to its receptor.

B. Platelet-activating Factor (PAF)

B.1 Physiology and Metabolism

The agonist used in these studies, platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine), is a phospholipid with a broad range of biological activities in addition to platelet stimulation (Chao and Olson, 1993; Prescott et al., 1990; Shimizu et al., 1992; Venable et al., 1993; Nojima, 1991; Shukla, 1992). Generally, it exerts effects at very low concentrations (nM or pM range) and is known as an autocoid or “local hormone”.

In 1970, researchers proposed that a ‘soluble factor’ was released from leukocytes in immunized rabbits which induced platelets to release histamine and serotonin, a finding confirmed independently a year later (Henson, 1970; Siraganian and Osler, 1971). Benveniste, Henson and Cochrane (1972) soon reported the leukocyte-dependent aggregation of rabbit platelets (Benveniste et al., 1972). When leukocytes isolated from IgE-sensitized rabbits were treated with specific antigen, this induced the rapid aggregation of platelets. They argued that upon stimulation, IgE-sensitized basophils degranulated and released a ‘soluble factor’ which they subsequently called “platelet-activation factor”. They suggested a role for PAF in acute allergic reactions. The lipid character of PAF was described shortly thereafter (Benveniste, 1974; Benveniste et al., 1977; Pinckard et al., 1979). Several years later Demopoulos, Pinckard and Hanahan demonstrated that a semi-synthetic glycerophospholipid, 1-O-alkyl-2-acetyl-sn-glycero-3-
phosphocholine had identical physicochemical and biological (induced release and aggregation in platelets) characteristics to the native PAF (Demopoulos et al., 1979). Concurrently, this compound was synthesized by another group from bovine heart choline plasmalogens using the same approach (Blank et al., 1979). It possessed powerful anti-hypertensive activity in a rat model of hypertension. Following the total chemical synthesis by Benveniste of 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine in 1979, naturally occurring PAF was purified from stimulated rabbit basophils and extensively characterized by gas-liquid chromatography and mass-spectral analysis (Benveniste et al., 1979; Hanahan et al., 1980). It proved to be identical to 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine.

Modification of the molecule at the sn-1, 2, or 3 position greatly decreases or even abolishes its biological activity. Many cell types including platelets have been shown to synthesize and release PAF upon stimulation, and at the same time show a response to this lipid; these include but are not limited to endothelial cells, neutrophils, macrophages, eosinophils and kidney cells. PAF is synthesized in cells by two routes, the remodeling and the de novo pathways. The remodeling pathway involves PLA$_2$ degradation of a 1-0-alkyl-2-acyl-sn-glycerol-3-phosphocholine (with liberation of arachidonic acid) followed by the action of an acetyltransferase to yield PAF. Stimulation of PAF production therefore is coupled to eicosanoid production with the two pathways acting synergistically. In the de novo pathway alkylacylglycerol serves as a substrate for a cholinephosphotransferase (Nojima, 1991). PAF is degraded by pathways involving acetylhydrolase and mono-oxygenase metabolism (Snyder, 1995; Snyder, 1995).

Numerous studies suggest that PAF is involved as a signalling mediator in both normal and pathophysiological states. Normal physiological responses mediated by PAF in addition to platelet activation, include stimulation of glycogenolysis in liver, increased vascular permeability, hypotension, decreased cardiac output, smooth muscle contraction, activation of neutrophils, eosinophils and macrophages, and ovulation (Venable et al.,
PAF also plays a role in many pathological states such as allergic disorders, acute inflammation, asthma, convulsions, and endotoxin-induced and anaphylactic shock (O'Flaherty and Wykle, 1983).

B.2 The Platelet-activating Factor Receptor and Signal Transduction

Purification and characterization of the PAF receptor was hampered by the low number of receptors per cell and the hydrophobic nature of PAF (resulting in elevated non-specific binding) with a number of candidate proteins observed on sodium-dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. $^3$H-PAF binding experiments were first done using human platelets (Valone et al., 1982). This group demonstrated two classes of binding, high and low, with the former correlating with PAF-induced platelet aggregation. PAF receptors are widely distributed on cells including neutrophils, eosinophils, macrophages, monocytes, smooth muscle cells, endothelial cells, epithelial cells, and in tissues such as lung, liver, brain, and uterus. Interestingly, rat platelets which are insensitive to PAF are completely devoid of high affinity PAF binding sites (receptors) (Sanchez-Crespo et al., 1981).

Molecular characterization of the receptor was enabled by the cloning of a cDNA for the PAF receptor from guinea-pig lung in 1991 (Honda et al., 1991). This analysis indicated that the PAF receptor is a member of the G-protein coupled serpentine receptor superfamily with seven transmembrane segments with a predicted molecular weight of 38982 Da and 342 amino acids. The PAF receptor has limited sequence homology with other G-protein-linked receptors; the highest similarity (29%) is with the N-formyl peptide receptor. The human PAF receptor gene has greater than 80% sequence homology with its guinea-pig counterpart. Several amino acids in the PAF receptor are highly conserved compared with other members of the G-protein coupled receptor superfamily: for example, aspartic acid in the second transmembrane segment, one cysteine each in the first
and the second extracellular loops (which may form a disulfide bond) and three prolines in
the sixth and seventh transmembrane segment. There are also two putative N-
glycosylation sites, one near the N-terminus and the other in the second extracellular loop
(at residues Asn-4 and Asn-169 respectively). A cysteine residue is also conserved in the
C-terminal tail and is a putative palmitoylation, membrane anchoring site. There is also a
cluster of nine Ser/Thr residues found near the C-terminal cytoplasmic tail. These may be
involved in agonist-induced signal desensitization/down-regulation and are putative G-
protein-linked receptor kinase (GRK) targets (Takano et al., 1994). PAF receptor
cDNA's have also been cloned from human leukocytes, heart and eosinophils and from
rat, mouse and monkey (Izumi and Shimizu, 1995).

The PAF receptor is coupled to (and activates) numerous cellular signal
transduction pathways including phospholipases A$_2$, C and D, PI 3-kinase, protein kinase
C, MAPK, protein-tyrosine kinases and Ca$^{2+}$ channels leading to an increase in calcium
influx as well as release from stores. G-proteins are involved in upstream regulation of
many of these enzymes (Chao and Olson, 1993; Shimizu et al., 1992). The class of G-
proteins mediating transduction from the PAF receptor depends on the cell type and the
effector system involved (Izumi and Shimizu, 1995). In platelets for instance, it was
shown that a single pertussis toxin-sensitive $\alpha_{41}$ containing G-protein is involved in
regulation of both adenylate cyclase and phospholipase C (Brass et al., 1988). PAF has
been shown to have an inhibitory effect on adenylate cyclase activation stimulated by
agents such as forskolin and PGI$_2$ in platelets. This indicates that the PAF receptor is
coupled with a pertussis toxin sensitive G-protein, $G_i$ (Izumi and Shimizu, 1995).

Protein-tyrosine kinases may play an important role in signal transduction
pathways originating from G-protein coupled receptors as well as growth factor receptors
and this seems to be the case with the PAF receptor also (Izumi and Shimizu, 1995).
Using anti-phosphotyrosine antibodies several studies show that PAF induces tyrosine
phosphorylation of numerous cellular proteins in rabbit platelets, human neutrophils, rat
liver Kupffer cells, and human B cell lines (Izumi and Shimizu, 1995). This response was usually very rapid and blocked by PAF receptor antagonists. Several of the proteins tyrosine-phosphorylated after PAF stimulation have been identified and they include pp60$^\text{src}$ in rabbit and human platelets and A431 cells, PLC$\gamma_1$ in rabbit platelets and a human B-cell line, the src-related Fyn and Lyn in a human B cell line and MAPK in CHO cells expressing the cloned PAF receptor. The mechanism by which the PAF receptor stimulates protein-tyrosine kinases and pathways dependent on them is not understood but preliminary evidence suggests that the src-related tyrosine kinases could play an important role. Experiments using inhibitors indicate that protein-tyrosine kinases are upstream of several of the signal transduction pathways activated by the PAF receptor such as phospholipase C and D, MAPK, phospholipase A$_2$ and PI 3-kinase (Izumi and Shimizu, 1995).

There is substantial evidence that adenylate cyclase and protein kinase C antagonize PAF effects through mechanisms involving functional dissociation of the receptor from G-proteins, down-regulation of surface receptors and modification of G-proteins (Izumi and Shimizu, 1995). It was shown recently in mutagenesis experiments that the carboxyl-terminal cytoplasmic tail of the PAF receptor is not required for forward activation of multiple pathways but it plays a critical role in signal attenuation; this is probably via GRK’s (G-protein linked receptor kinases) (Izumi and Shimizu, 1995). A characteristic of PAF responses in platelets is that PAF down-regulates its own receptors resulting in a significant decrease in response to PAF after an initial pre-treatment; a process known as homologous desensitization. Divalent cations such as Ca$^{2+}$ enhance specific binding of PAF by 8-10 fold primarily through an increase in the affinity of the receptor for PAF (Izumi and Shimizu, 1995).
B.3 Physiological Responses of Platelets to Platelet-activating Factor

All platelets (except rat) possess high affinity receptors for PAF and are typically activated at very low concentrations of this agonist (Chignard et al., 1985; Vargaftig et al., 1981; Benveniste and Chignard, 1985; Vermyleen et al., 1983; Pinckard, 1983; Benveniste et al., 1981). Rabbit and guinea-pig platelets are among the most sensitive with EC_{50}'s for serotonin secretion or aggregation in the sub-nanomolar range while porcine platelets are about five orders of magnitude less sensitive (Duronio et al., 1990). PAF induces the full complement of physiological responses in platelets except for phosphatidylserine exposure (procoagulant activity). PAF also induces polyphosphoinositide turnover and activation of membranous protein kinase C although at least in rabbit platelets the two events are independent of each other. PAF is able to activate pre-existing membranous PKC without a requirement for translocation from the cytosol (Salari et al., 1990; Pelech et al., 1990; Salari et al., 1990).

PAF stimulation of platelets leads to tyrosine-phosphorylation of numerous platelet proteins and pre-incubation of platelets with the tyrosine-kinase inhibitor erbstatin blocks numerous PAF-induced platelet responses (Salari et al., 1990). The PAF receptor contains several tyrosine residues in its intracellular loops and tail suggesting perhaps a functional link between homologous down-regulation of the receptor and PTK's. Experiments using vanadate suggest that protein-tyrosine phosphorylation does play a role in the regulation of the PAF receptor surface expression (Chao and Olson, 1993). Activation of numerous second-messenger systems occurs as is mentioned above.

C. Phosphatidylinositol 3-kinase

Phosphatidylinositol (phosphoinositide) 3-kinases comprise a family of lipid kinases that phosphorylate the 3’-position of the inositol ring of phosphatidylinositol (PI),
phosphatidylinositol 4-phosphate (PI-4-P) and phosphatidylinositol 4,5-bisphosphate (PI-4,5P_2). This results in the formation of the so-called 3-phosphoinositides (3-PPI’s), phosphatidylinositol 3-phosphate (PI-3-P), phosphatidylinositol 3,4-bisphosphate (PI 3,4P_2) and phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PI 3-kinase and the 3-PPI’s constitute a branch of phosphoinositide metabolism that is separately regulated and functionally distinct from the conventional polyphosphoinositide cycle, as will be discussed here.

C.1 Conventional Polyphosphoinositide Metabolism

Polyphosphoinositides are a quantitatively minor constituent (0.1 %) of total cellular phospholipids. Hokin and Hokin (1955) were the first to show that these lipids turned-over in response to an external stimulus (i.e. were metabolically sensitive). However, it was not until 20 years later that this ‘phosphatidylinositol response’ was linked to mobilization of Ca^{2+} in cells (Michell, 1975). The conventional (canonical) PPI cycle consists of rapid kinase/phosphatase-mediated interconversions between phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI 4-P) and phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2) (Downes and MacPhee, 1990).

Phosphatidylinositol 4-kinase phosphorylates PI to PI 4-P and phosphatidylinositol 4-phosphate 5-kinase completes the phosphorylations giving PI 4,5-P_2. In resting cells PI 4-P and PI 4,5-P_2 are each about 10% as abundant as PI (Verhoeven et al., 1987). The majority of the PPI’s have stearate and arachidonate in the sn-1- and sn-2- positions respectively.

Upon cell stimulation, phosphoinositide-specific phospholipase C (PI-PLC) hydrolyzes PI 4,5-P_2 to inositol 1,4,5-trisphosphate (Ins (1,4,5)P_3) and 1, 2-sn-diacylglycerol (DAG) (Berridge and Irvine, 1989). These second messengers stimulate release of Ca^{2+} from internal stores and activation of protein kinase C (PKC) as described
above. Simultaneous with PLC activation, the equilibria/mass balance among the PPF's shifts to the right (towards PIP2) (Berridge and Irvine, 1989). This shift, coupled with the much higher rate of turnover between PPF's compared to PLC consumption of PIP2, ensures that an adequate supply of this important signal precursor is available during cell stimulation.

The obvious physiological role for the conventional PPF's/kinases is to ensure a sufficient supply of substrate for PI-PLC during cell stimulation (Carpenter and Cantley, 1990). There is evidence to suggest, however, that these lipids have other functions besides being intermediates in phosphoinositide turnover. PI 4,5-P2 has been shown to bind to the actin-binding proteins, gelsolin, profilin, myosin type 1 and the glycophorin-band 4.1 complex from the red blood cell (Anderson and Marchesi, 1985; Adams and Pollard, 1989; Lassing and Lindberg, 1985; Jamney and Stossel, 1987). In particular, this lipid induces dissociation of gelsolin and profilin from actin filaments implying a possible role in cytoskeletal regulation.

C.2 Discovery of a New Phosphoinositide Kinase (type I PI 3-kinase)

The existence of more than one species of PI kinase in mammalian cells had been suspected for many years. Twenty-seven years ago Harwood and Hawthorne described two types of PI kinase in liver that were differentially activated by nonionic detergents (Harwood and Hawthorne, 1969). Interest in a link between cellular transformation and the phosphoinositide cycle was piqued when it was shown that highly purified preparations of pp60v-src had PI and PI-4-P kinase activities that could be inhibited in parallel with its protein tyrosine-kinase activities (Sugimoto et al., 1984). A similar PI kinase activity co-immunoprecipitated with p68v-src (the transforming protein of the avian sarcoma virus UR2), v-abl and the polyoma virus middle T antigen (mT):pp60c-src complex, all of which are protein-tyrosine kinases (Whitman et al., 1985; Fry et al., 1985; Macara et al., 1984).
The PI kinase activities were initially thought to be intrinsic due to their co-regulation and tight association. It was shown subsequently that in vitro expressed src-kinase lacked the lipid kinase activity and that the lipid kinase activity was separable from the mT-pp60c-src complex in very high salt and detergent solutions; thus this activity must originate from the host cell (Piwnica-Worms et al., 1986; Kaplan et al., 1987; Kaplan et al., 1986).

The PI kinases were extensively characterized in fibroblasts where two distinct types were observed, only one of which associated with tyrosine kinases (Whitman et al., 1987). This species, termed type I, eluted earlier on a Mono Q column than the much more abundant type II activity, and was inhibited by nonionic detergent. Type II PI kinase was inhibited by adenosine and activated by nonionic detergents. The type I PI kinase was also shown to co-purify with the activated platelet-derived growth factor (PDGF) receptor in PDGF stimulated cells but not in control cells. Thus, this established a connection between type I PI kinase and tyrosine-phosphorylation in non-transformed cells as well (Whitman et al., 1987). In a major advance it was then shown that the type II PI kinase (PI 4-kinase) phosphorylated the 4-position on the inositol ring (as expected) while the type I enzyme phosphorylated PI to give a previously undescribed lipid, PI-3-P; thus the term, PI 3-kinase (Whitman et al., 1988).

C.3 3-Phosphoinositides are Formed In Vivo in Oncogene-Transformed and Growth Factor Stimulated Cells

After labelling polyoma middle-T antigen transformed fibroblasts with 3H-inositol, Whitman et al. (1988) demonstrated that an inositol lipid with properties identical to the in vitro produced PI-3-P was present in vivo, although at only 3.3 % abundance compared to PI-4-P. Another group also provided unambiguous structural proof for the presence of PI-3-P in 1321N1 astrocytoma cells (Stephens et al., 1989).
PI 3-kinase also phosphorylates PI-4-P and PI-4,5 P$_2$ \textit{in vitro} giving PI-3,4 P$_2$ and PI-3,4,5 P$_3$ (Carpenter et al., 1990). The presence of these 3-phosphoinositides (3-PPI's) was first demonstrated in PDGF-stimulated fibroblasts and fMLP-stimulated neutrophils (Auger et al., 1989; Traynor-Kaplan et al., 1989; Traynor-Kaplan et al., 1988; Stephens et al., 1991). Unlike PI-3-P, the other 3-PPI's were only detected in transformed or growth-stimulated cells, not quiescent cells. The 3-PPI's appearance correlates with recruitment of PI 3-kinase to the PDGF receptor in stimulated fibroblasts (Auger et al., 1989). These observations provided solid evidence that the PI kinase recruited to tyrosine-kinase receptors (i.e. PDGF) and complexed with activated oncogene products is the enzyme responsible for generation of the novel 3-PPI's \textit{in vivo} (i.e. PI 3-kinase). The possibility that the 3-PPI's provided a critical signal for mitogenesis and transformation spurred interest in this new area.

C.4 Structural and Functional Characterization of Phosphoinositide 3-kinases

In the following discussion I will focus on the well characterized tyrosine-kinase regulated PI 3-kinase. Other less characterized isoforms that are regulated differently (i.e. G-proteins) will be covered later in less detail. PI 3-kinase is present in all eukaryotic organisms and in all tissues; in mammals the highest specific activity is in liver and spleen. Correlation of the presence of an 85 kDa polypeptide with PI 3-kinase activity in middle T/pp$^{60c-src}$ complexes and immunoprecipitates of activated PDGF receptors led to the purification of PI 3-kinase to homogeneity from rat liver for the first time (Kaplan et al., 1987; Carpenter et al., 1990). This PI 3-kinase (predominantly cytosolic except present in membrane fraction of transformed and growth-factor stimulated cells) is a tightly associated heterodimer consisting of 85 kDa and a 110 kDa subunits (Fry and Waterfield, 1993). Prior experimental evidence showed that association between PI 3-kinase (p85 subunit) and tyrosine kinases required an activated tyrosine kinase (Kazlauskas and
Identification of consensus phosphotyrosine containing PI 3-kinase binding sequences in these proteins led to a prediction that the p85 subunit contained at least one src homology (SH) 2 (SH2) domain, thus providing a mechanism for the ubiquitously observed association between PI 3-kinase and activated protein-tyrosine kinases (Mayer and Hanafusa, 1990).

C.4.1 The p85 Regulatory Subunit

The p85 subunit was cloned/sequenced first (Escobedo et al., 1991). This subunit comprises a family of at least three different but highly homologous isoforms, α, β and γ which display tissue-specific expression patterns. p85 (724 amino acids) contains a number of important functional motifs including an amino-terminal SH3 domain and two SH2 domains (as predicted). Between the SH3 and amino-terminal SH2 domain is a sequence homologous to the C-terminal part of the Break-cluster region (Bcr) gene product. Flanking the Bcr region are two proline rich sequences which could serve to mediate protein-protein interactions through binding of SH3 domains independent of tyrosine phosphorylation (Kapeller et al., 1994). A region between the SH2 domains is required for binding to the p110 catalytic subunit and has also been shown to contain a lipid binding site for PI 4,5-P2, PI 4-P and PI (Klippel et al., 1993; End et al., 1993).

Expression studies confirmed inferences made from sequence analysis that p85 lacked PI 3-kinase activity and was probably the regulatory subunit. That the p110 subunit was the catalytic subunit was confirmed experimentally when PI 3-kinase activity was associated with a 110 kDa polypeptide from bovine thymus in the absence of detectable p85 (Shibasaki et al., 1991). The specificity and avidity/strength of binding of p85 to tyrosine-phosphorylated sequences is increased when p85 is complexed with the p110 catalytic subunit (Fry et al., 1992). It is clear that both SH2 domains of p85 (evidence suggests cooperatively) enable recruitment of the p110 catalytic subunit by binding to specific
motifs containing phosphotyrosine in receptors, protein-tyrosine kinases or their substrates. The p85 SH2 domains bind to tyrosine phosphorylated sequences that are distinct from those bound by SH2 domains of other proteins such as PLC gamma and ras-GAP (ras-GTPase-activating protein). This sequence is pY-M/V/I/E-X-M (Cantley and Songyang, 1994). This allows access of the kinase subunit to the membrane where the substrates are located. Thus this mechanism of PI 3-kinase recruitment to membranes follows the paradigm established by other SH2 containing signalling enzymes such as PLC gamma, rasGAP, v-crk etc. The p85 subunit is also known to be a substrate for tyrosine kinases and in fact this may be a major mechanism by which it interacts (through SH2 domains) with non-receptor src-like protein-tyrosine kinases (Fukui and Hanafusa, 1991). There is evidence that in addition to the phosphotyrosine-p85 interaction acting as coupling mechanism for membrane localization of p110 that it may function to increase the specific activity of the enzyme through conformational changes transduced to the p110 subunit (Carpenter et al., 1993).

SH3 domains bind to protein sequences rich in proline residues and this module may function in p85 to localize the heterodimeric PI 3-kinase to components of the cytoskeleton. A GST fusion protein containing the p85 SH3 domain bound with high affinity to the microtubule-binding protein dynamin (Gout et al., 1993). It also stimulated the intrinsic GTPase activity of this protein. It has been shown that the SH3 domains of non-receptor tyrosine kinases, c-abl, pp56^{ck}, pp59^{lyn}(t), pp56^{lyn} and pp60^{v-src} associate with the proline-rich sequences in p85 (Prasad et al., 1993; Prasad et al., 1993). The presence of both the SH3 domain and proline-rich sequences in the p85 subunit suggests that self-association may occur in vivo. This could represent another level of regulation. Indeed, it has been shown that the p85 SH3 domain associates in vitro with full-length recombinant p85 (Kapeller et al., 1994). There is evidence, also in vitro, that binding of an SH3 domain to p85 promotes activation of the lipid kinase activity (Pleiman et al., 1994).
No function has yet been attributed to the p85 Bcr homology domain. This sequence is related to those found in rhoGAP, n-chimerin, p190, Bem2, Bem3, 3BP1 and the C-terminal part of the Bcr gene product. All of these proteins have been shown to stimulate the hydrolysis of GTP on rho, rac and/or CDC42 (i.e. GAP activity) (Diekmann et al., 1991). So far the p85 subunit has not been shown to possess GAP activity toward any known protein. It is tempting to speculate that the N-terminal third of p85 encompassing the SH3 domain and Bcr regions may be involved in processing signals to or from members of the family of small G-proteins.

C.4.2 The p110 Catalytic Subunit

The p110 catalytic subunit (1068 amino acids) shows significant sequence homology (55%) to vps34p, a low abundance 100 kDa protein from the yeast, *Saccharomyces cerevisiae*, which had been isolated on the basis of its involvement in the sorting of proteins to the yeast vacuole (Hiles et al., 1992). p110 also contains several conserved motifs constituting the active site of protein kinases. Thus it is likely that protein kinases and this family of lipid kinases share a common ancestry. A second yeast protein, TOR2, shares homology with the catalytic subunit. TOR2 is the probable target protein for the rapamycin-FKBP12 complex and may serve to promote progression through the G1 phase of the cell cycle (Kunz et al., 1993). There are at least two distinct but related 110 kDa catalytic subunits (α and β) which associate with p85 to form heterodimeric PI 3-kinase. This is exemplified by a doublet observed in this region on SDS-PAGE gels for enzyme isolated from rat liver (Carpenter et al., 1990). p110 has also been purified from bovine thymus as both an isolated subunit and a heterodimer with p85 (Shibasaki et al., 1993; Shibasaki et al., 1991). The p110 catalytic subunit also possesses intrinsic serine/threonine protein kinase activity that originally was thought to be due to a tightly associated but separate protein (Carpenter et al., 1993). The possible role of this
activity in auto-regulation of PI 3-kinase and as a kinase targeting other proteins is discussed below.

C.5 Regulation of Phosphoinositide 3-kinase

PI 3-kinase activity, as well as numerous other signal transduction pathways such as PLC and ras/MAPK are increased in the overwhelming majority of transformed and growth-factor stimulated cells and in activated terminally differentiated cells such as neutrophils and platelets. Our knowledge of upstream regulatory (activating and inhibitory) pathways impinging on PI 3-kinase has increased greatly in the last several years. This has been compounded by the discovery of several additional isoforms of phosphoinositide 3-kinase which are differentially regulated (Zvelebil et al., 1996). In addition, regulation of phosphoinositide 3-kinase can vary between cell type and between different stimuli for the same cell type.

Although the 3-PPi’s, PI 3,4-P₂ and PIP₃ are usually dramatically elevated after stimulation (agonist sensitive) the levels of PI-3-P remain essentially unchanged. It now appears that PI-3-P may be synthesized by a phosphatidylinositol-specific PI 3-kinase (high degree of homology to vps34) in mammalian cells that is distinct from the broad-specificity ‘phosphoinositide 3-kinase’ discussed so far (Stephens et al., 1994). This could imply a different role for PI-3-P in mammalian cells perhaps analogous to that of PI-3-P in yeast which is formed by the VPS34 gene product.

Many of the mechanisms for regulation of the heterodimeric form of phosphoinositide 3-kinase have already been discussed in the context of describing structural characteristics of this enzyme (see above). Here I will briefly summarize the major routes for regulation of heterodimeric PI 3-kinase with some examples where each mechanism has been implicated.
C.5.1 Translocation

For phosphoinositide 3-kinase to be active it must have access to its substrates. This necessarily involves translocation of what (in quiescent cells) is a normally cytosolic protein to a juxtamembrane position at the plasma membrane. Activation of tyrosine kinases is the best understood mechanism that enables recruitment of heterodimeric PI 3-kinase to the plasma membrane (Carpenter and Cantley, 1996). Nature has employed tyrosine phosphorylation in a number of variations to allow recruitment and activation of PI 3-kinase. PI 3-kinase may be recruited and directly bind via the p85 SH2 domains, to receptors that possess intrinsic tyrosine kinase activity and autophosphorylate themselves (e.g. the PDGF receptor), or receptors without intrinsic kinase activity but whose cytoplasmic domains are tyrosine phosphorylated to create a binding site (i.e. hemopoietic receptors). Recruitment of PI 3-kinase to a receptor may also be indirect via a tyrosine-phosphorylated docking protein (i.e. IRS-1 for the insulin receptor). Cytosolic protein-tyrosine kinases associated with the intracellular domain of certain B-cell or T-cell receptors such as the CD4/CD8/\textit{lk} system may be activated by ligand engagement of the receptor (Vogel and Fujita, 1993). This often involves dephosphorylation of a C-terminal auto-inhibitory phosphotyrosine allowing binding of PI 3-kinase via both the SH2 and SH3 domains of the receptor-associated tyrosine kinase. It has been hypothesized that it is mainly the SH3 domain of Src-family kinases rather than the SH2 which mediates interaction with PI 3-kinase.

C.5.2 Allosteric Regulation of PI 3-kinase

In addition to recruitment to membranes the specific activity of the catalytic subunit could be increased by (a) p85 SH2 domain mediated binding to tyrosine-phosphorylated sequences or (b) binding of proline-rich sequences in p85 to SH3 domains
in other proteins (or the reciprocal) as has been demonstrated \textit{in vitro} for p56\textsuperscript{lk} (Pleiman et al., 1994). Backer et al. (1992) were the first to show that monophosphopeptides containing a YXXM motif found in IRS-1 were able to activate PI 3-kinase in vitro albeit with much less potency than native phosphotyrosyl IRS-1. More recently this group showed that full activation of PI 3-kinase by tyrosylphosphorylated proteins requires occupancy of both SH2 domains of p85 (Rordorf-Nikolic et al., 1995).

C.5.3 Phosphorylation

The p85 subunit is tyrosine-phosphorylated in PDGF-stimulated, polyoma middle T antigen-transformed and NGF-stimulated cells (Kaplan et al., 1987). There is preliminary evidence that this results in an increase in kinase activity (Ruiz-Larrea et al., 1993). However, tyrosine phosphorylation of p85 is probably not a major regulatory mechanism as it is often not observed.

There seems to be a role for serine phosphorylation of PI 3-kinase in regulation of the lipid kinase activity of this enzyme. Carpenter's group originally described the co-purification of an unusual Mn\textsuperscript{2+} dependent protein serine/threonine kinase activity with PI 3-kinase from rat liver (Carpenter et al., 1993). This protein kinase activity phosphorylated a serine residue in the p85 subunit causing an 80 % decrease in the intrinsic phosphoinositide activity. Treatment of the complex with protein phosphatase 2A restored \textit{in vitro} activity. After all attempts to separate the protein kinase activity from PI 3-kinase failed it was shown that in fact the serine/threonine protein kinase activity is intrinsic to the p110 subunit and the enzyme possess a dual kinase specificity (Dhand et al., 1994). Perhaps this intrinsic negative regulation by the p110 subunit serves to minimize generation of the 3-phosphoinositides in quiescent cells. The possibility that this novel activity may have an effector function was raised when it was discovered that p110
phosphorylates IRS-1 on serine residues in insulin-stimulated cells (Freund et al., 1995; Tanti et al., 1994; Lam et al., 1994).

C.5.4 Regulation of PI 3-kinase by G-proteins

C.5.4.1 Small G-proteins

There is indirect evidence suggesting that the heterodimeric form of PI 3-kinase may also be regulated by (or downstream of) small GTP-binding proteins. In systems where PI 3-kinase activation is known to be largely regulated through tyrosine kinases, PI 3-kinase activity has been found associated with RasGAP, p21^{rho} and p21^{ras} (Sjolander and Lapetina, 1992; Sjolander et al., 1991). Rho has also been implicated as a regulator of PI 3-kinase in platelets and fibroblasts (Kumagai et al., 1993; Zhang et al., 1993). This association may be mediated through the Bcr domain in the p85 subunit. In support of this contention it has been shown recently \textit{in vitro} that GTP-bound Rac and CDC42 associate with the p85 subunit leading to activation of lipid kinase activity (Zheng et al., 1994).

An exciting development has been the recent finding that Ras associates with the catalytic subunit (p110) of heterodimeric PI 3-kinase in a strictly GTP-dependent manner \textit{in vitro} (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996). Additional work showed that Ras, when overexpressed, stimulates 3-PPI production in intact cells and that dominant negative Ras mutants severely reduce PI 3-kinase activation in response to growth factor ligation of tyrosine kinase receptors. These developments firmly entrench Ras as an upstream regulator of PI 3-kinase in addition to protein tyrosine kinases; with the two mechanisms possibly being required for optimal activation of PI 3-kinase in cell stimulation (Rodriguez-Viciana et al., 1996).
C.5.4.2 Heterotrimeric G-proteins

In myeloid cells such as neutrophils, ligand engagement of G-protein-linked receptors leads to accumulation of PIP$_3$ through a mechanism that appears to involve heterotrimeric G-proteins and is independent of protein-tyrosine kinases (Stephens et al., 1993). This led to the purification and characterization recently by two groups of a new and immunologically distinct isoform of phosphoinositide 3-kinase termed PI3K$_\gamma$ which is activated by G-protein $\alpha$ and $\beta\gamma$-subunits (Stoyanov et al., 1995; Stephens et al., 1994). This isoform consists of 101 kDa and 117/120 kDa subunits, the latter possessing the lipid kinase activity. The catalytic subunit of PI 3-kinase gamma is similar to the $\alpha$ and $\beta$ subunit of the classical tyrosine kinase regulated PI 3-kinase except it does not contain a p85 binding domain and therefore does not bind p85. The 101 kDa subunit shows no regions of homology to any known protein and of course is insensitive to tyrosine-phosphorylated peptides which activate the classic p85/p110. This species then represents a form of PI 3-kinase that is not regulated by the p85 adapter subunit.

The phosphoinositide 3-kinases, as a family of related lipid kinases, are regulated by numerous input or activating signals that vary between isoforms, stimulus and cell-type much like the paradigm established by the phospholipase C family. There is accumulating evidence that full activation of certain PI 3-kinase isoforms (e.g. the heterodimeric form) in some systems may require simultaneous inputs from both tyrosine kinases and G-proteins (Rodriguez-Viciana et al., 1996; Rodriguez-Viciana et al., 1996).

C.6 Function of Phosphoinositide 3-kinase

The question of a role for PI 3-kinase (or more particularly the 3-phosphoinositides) in cellular responses to growth factors/agonists that is independent of the classical phosphoinositides is a very active area in biological research. Publications in
this area at present usually average from 10-20 per week. It is certainly a challenge to uncover functions uniquely dependent on PI 3-kinase in the complex array of signalling events and pathways that are stimulated on agonist binding to a membrane receptor.

In general, cellular functions that appear to be regulated by PI 3-kinase include mitogenesis, inhibition of apoptosis, vesicular trafficking/secretion and cytoskeletal rearrangements (regulation of actin and integrin function) among others (Carpenter and Cantley, 1996). Early studies were primarily correlative in that inferences were made by associating a given cellular response to the presence or absence of PI 3-kinase or its lipid products. In the last few years many more tools have become available to use for probing the function of this enzyme.

### C.6.1. Correlation of Cellular Responses with PI 3-kinase Activity

Most of the initial work on the function of PI 3-kinase addressed its involvement in mitogenesis and transformation. Indeed the association of PI 3-kinase with transforming oncogene products led to the discovery of this new phosphoinositide kinase. These studies showed that association of PI 3-kinase with activated tyrosine kinases (e.g. mT-pp60<sup>-src</sup> complex or pp60<sup>v-src</sup>) was absolutely necessary for oncogenic transformation (Cantley et al., 1991). These conclusions were reached by mutating the oncogene product to specifically remove the binding site for PI 3-kinase. Later, it was discovered that association was not sufficient for transformation because some non-transforming oncogene mutants were defective in membrane localization and in generating PIP<sub>3</sub> but still associated with PI 3-kinase (Fukui and Hanafusa, 1989). Therefore, the model was revised to suggest that PI 3-kinase activation (generation of 3-PPI's) was necessary for transformation.

Much the same approach was employed regarding the involvement of PI 3-kinase in growth-factor mediated stimulation of mitogenesis. The general observation was that
phosphatidylinositol (3,4) bisphosphate (PI (3,4)P₂) and PI (3,4,5)P₃ formation correlated with growth factor stimulation as they were not detected in quiescent cells and PI 3-kinase was recovered in anti-PDGF receptor immunoprecipitates only from activated cells. More convincing data came from mutational studies of the PDGF receptor. Removal of the specific PI 3-kinase binding sites on this receptor abolished its ability to stimulate mitogenesis and 3-PPI formation. Restoration of the PI 3-kinase binding site but not that of PLCγ₁ or rasGAP, for example, on the receptor rescued its mitogenic capability and 3-PPI generation. The main problem with the above approach is that the noted effects could be due to disrupted binding of a signalling molecule that shares the same binding site with PI 3-kinase on the receptor (i.e. Nck).

A close correlation between the kinetics of PI 3-kinase activation and cellular responses (respiratory burst) was also observed in neutrophils stimulated with fMLP (Traynor-Kaplan et al., 1989; Traynor-Kaplan et al., 1988). The appearance of PIP₃ in these cells also temporally correlated with actin polymerization thus representing the first correlation between PI 3-kinase activation and a biochemical endpoint (Eberle et al., 1990). Of course, the activation of PI 3-kinase in terminally differentiated cells such as neutrophils raised speculation about a role for this enzyme in functions independent of growth.

C.6.2. Cloning of PI 3-kinase Provides New Functional Insights

Cloning and sequencing of the p110 catalytic unit of phosphoinositide 3-kinase shows that it bears significant sequence homology (55%) to vps34p, a low abundance 100 kDa protein from the yeast, Saccharomyces cerevisiae. This led to early suggestions that PI 3-kinase was involved in vesicular/membrane/protein trafficking analogous to this proven role of vps34p in yeast. Recent evidence suggests however, that a protein distinct from the heterodimeric/broad specificity enzyme termed 'phosphatidylinositol-specific
phosphoinositide 3-kinase' which only phosphorylates phosphatidylinositol may in fact be the true mammalian homologue of vps34p (Stephens et al., 1994).

Protein database sequence analysis has revealed several other proteins that have homology to the kinase domain of PI 3-kinase but do not possess lipid kinase activity. These include the Ataxia telangiectasia (ATM) gene product, the DNA-dependent protein kinase (DNA PK), the yeast TOR proteins and their mammalian homologues, FRAP and RAFT1 (Savitsky et al., 1995; Hartley et al., 1995; Kunz et al., 1993). Thus the family of proteins with homology to PI 3-kinase is growing rapidly.

Several independent molecular approaches have been used to elucidate cellular functions dependent on PI 3-kinase. Antibodies have been produced which block the activity of the p110α subunit. When these antibodies are micro-injected into quiescent fibroblasts they block DNA synthesis and growth in response to PDGF and EGF but not colony-stimulating factor (CSF)-1, bombesin or lysophosphatidic acid (Roche et al., 1994). Plasmids containing the mammalian p110 subunit which is either constitutively active (p110*) or under the control of an inducible promoter have been transfected into cells and the resulting biochemical or physiological effects determined. Hu et al. (1995) found that expression of p110* results in c-fos induction and elevation in cellular levels of GTP-bound Ras. However, expression of mammalian p110 in yeast profoundly inhibits growth, an effect which is countered by co-expression of the p85 subunit (Kodaki et al., 1994). Cellular overexpression of mutant p85 subunits which lack the p110 binding site sequence (Δp85) but are still able to bind phosphotyrosine, blocks recruitment to receptors of endogenous PI 3-kinase and results in a dominant negative effect. Δp85 has been used to show the dependence of PDGF-stimulated membrane ruffling and insulin-induced GLUT4 translocation to the plasma membrane on heterodimeric PI 3-kinase (Wennstrom et al., 1994; Cheatham et al., 1994). Other complementary approaches to the Δp85 method involve micro-injection or expression of fusion proteins containing the p85 SH2
domains or phosphotyrosine-containing peptides with the PI 3-kinase SH2 domain binding sequence (Kotani et al., 1994; Jhun et al., 1994).

C.6.3. Inhibitors of Phosphoinositide 3-kinase

The discovery of potent inhibitors of PI 3-kinase has greatly facilitated the delineation of cellular functions dependent on PI 3-kinase activation as well as potential pathways that are downstream of this enzyme (Powis and Phil, 1994).

Wortmannin was originally isolated as an antifungal antibiotic from a culture of the mold, *Penicillium* (Brian et al., 1957). This highly toxic compound, which possesses anti-inflammatory properties, was later shown to block induction of the respiratory burst in neutrophils, monocytes and macrophages at extremely low concentrations (1-10 nM) (Baggiolini et al., 1987). Nakanishi et al. (1992) discovered that this compound inhibited myosin light chain kinase at micromolar concentrations. However it became apparent that wortmannin was targeting another enzyme when it was shown that the compound also inhibited antigen-stimulated histamine release from RBL-2H3 cells and human basophils in the nanomolar range (Kitani et al., 1992).

That wortmannin inhibited PI 3-kinase purified from several different systems and in whole cells with IC$_{50}$'s (inhibitory concentration) in the range of 1-10 nM was reported soon thereafter (Yano et al., 1993; Arcaro and Wymann, 1993). Wortmannin is an irreversible inhibitor of PI 3-kinase and forms a covalent adduct/bond through amino or thiol groups near the ATP-binding site of the catalytic subunit (Norman et al., 1996; Wymann et al., 1996). PI (3,4)P$_2$ and ATP compete with wortmannin. Wortmannin inhibits both the lipid and protein kinase activity of the p110$\alpha/\beta$ subunits. The wortmannin sensitivity of the various isoforms of PI 3-kinase varies considerably. The yeast vsp34, plant PI 3-kinase and PI-specific enzyme (vsp34 homologue) in humans are
considerably less sensitive with IC_{50}'s of about 100 nM with the classical heterodimeric form being most sensitive.

As we and others have shown, wortmannin is unstable (subject to hydrolysis) at 37 °C in media at physiological pH 7.4 with a half-life of about one hour with respect to its ability to inhibit immunopurified PI 3-kinase (Woscholski et al., 1994; Scheid et al., 1995). Thus if long incubations with wortmannin are desired fresh drug must be added at regular intervals to maintain a given steady-state concentration.

A derivative of the naturally occurring flavonoid, quercetin, 2-(4- morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) was recently found to be a potent and specific inhibitor of PI 3-kinase (Vlahos et al., 1994). LY294002 is a competitive inhibitor of the ATP-binding site of PI 3-kinase with an IC_{50} of 1.4 μM and is selective in that it does not inhibit PI 4-kinase or several other ATP-requiring protein or lipid kinases tested in vitro (Vlahos et al., 1994).

Since LY294002 and wortmannin inhibit PI 3-kinase by completely different mechanisms their combined use in experiments significantly lessens the chance that an effect is due to inhibition of a target other than PI 3-kinase.

C.6.4. Cellular Functions of Phosphoinositide 3-kinase

C.6.4.1 Mitogenesis

The early work on PI 3-kinase strongly suggested a role for this enzyme in cell growth and transformation. Although there are numerous examples of the necessity/sufficiency for PI 3-kinase activation in cell growth it seems that its importance depends on cell-type and stimulus used. An example of the dispensability of PI 3-kinase activation for growth in response to some stimuli was recently demonstrated (Roche et al., 1994). They showed that inhibitory antibodies to the p110α subunit block the DNA
synthesis that occurs in response to PDGF and EGF but not CSF-1, bombesin or lysophosphatidic acid. Also, our laboratory demonstrated that even though IL-4 activates PI 3-kinase in cells it is not capable of stimulating proliferation thus implicating other pathways in addition to PI 3-kinase for a full mitogenic response (Gold et al., 1994).

C.6.4.2 Apoptosis

Many cytokines share the property of inhibiting programmed cell death (apoptosis) in addition to stimulating mitogenesis in hemopoietic cells. We have shown recently, using wortmannin and LY294002, that PI 3-kinase activation is necessary for the prevention of apoptosis in cells treated with IL-4, Steel factor and IL-3 but not GM-CSF or IL-5 (Scheid et al., 1995). That PI 3-kinase lies on pathways leading to prevention of apoptosis was also demonstrated in PC12 cells maintained in NGF, EGF, insulin or serum (Yao and Cooper, 1995).

C.6.4.3 Intracellular Vesicular/Protein Trafficking

There is growing evidence that PI 3-kinase activity (3-phosphoinositides) may be required at discrete steps in the trafficking and processing of proteins within the cell. The best evidence that PI 3-kinase may be important for trafficking of proteins to the lysosome comes from work involving the PDGF receptor (Joly et al., 1995; Joly et al., 1994). This group demonstrated that wortmannin causes a dramatic decrease in the rates of down-regulation and degradation of the PDGF receptor. This effect was not through a decrease in internalization but rather reflects the necessity of PI 3-kinase to signal diversion of the receptor to the lysosomal degradative pathway.

PI 3-kinase dependent protein trafficking is also important in a key insulin response in target cells. Insulin-stimulated GLUT4 (glucose transporter) exocytosis requires
activated PI 3-kinase perhaps to regulate components involved in membrane budding, fusion or movement (Corvera and Czech, 1996).

C.6.4.4 Regulation of the Cytoskeleton

As mentioned in the discussion on conventional polyphosphoinositides, these lipids seem to participate in regulation of actin polymerization. No direct role for PI 3-kinase or the 3-PPI's in cytoskeletal modifications has been demonstrated although this enzyme is necessary for some forms of cell motility and adherence. For example, cells containing PDGF receptor mutants that do not bind or activate PI 3-kinase do not exhibit membrane ruffling or chemotaxis in response to PDGF (Wymann and Arcaro, 1994; Wennstrom et al., 1994). This blockage can be rescued by microinjection of fibroblasts with constitutively activated V12 Rac placing PI 3-kinase upstream of Rac in fibroblasts. Interestingly, α-actinin has recently been shown to bind to PI 3-kinase through the p85 subunit (Shibasaki et al., 1994). Finally, PI 3-kinase may be involved in stimulus-dependent activation of integrin receptors leading to cell adhesion (Metzner et al., 1996; Kinashi et al., 1995).

C.7. Molecular Targets of Phosphoinositide 3-kinase

New tools to selectively manipulate PI 3-kinase activity in vivo along with the availability of chemically synthesized 3-PPI's has greatly accelerated discovery of downstream biochemical pathways as well as putative direct targets for these novel lipids (Watanabe et al., 1995; Reddy et al., 1995).

Since the 3-phosphoinositides are resistant to degradation by all phospholipases C so far tested it is the lipids themselves rather than a metabolite which are the likely second messengers (Serunian et al., 1989). Several groups have documented that PI (3,4,5)P₃
and PI (3,4)P₂ directly activate calcium-independent PKC isoforms in vitro (Singh et al., 1993; Nakanishi et al., 1993; Toker et al., 1994). The specificity and potency of this activation for the novel phosphoinositides versus the classical phosphoinositides is presently unclear. PI 3-kinase has been functionally located upstream of protein kinase C isoforms λ and ε in PDGF signalling (Moriya et al., 1996; Akimoto et al., 1996). We recently found that PKC δ associates with PI 3-kinase in an activation dependent manner in human erythroleukemia cells and rabbit platelets (Ettinger et al., 1996). This provides a possible mechanism for PI 3-kinase dependent activation of PKC δ (or possibly the reverse pathway) in vivo. Evidence has accumulated rapidly suggesting that PI 3-kinase functions upstream of two important signalling kinases, pp70⁶⁰ kinase and akt/PKB in pathways leading to mitogenesis and cell division (Petritsch et al., 1995; Weng et al., 1995; Cheatham et al., 1994; Dahl et al., 1996; Burgering and Coffer, 1995; Franke et al., 1995).

C.8. Metabolism of 3-Phosphoinositides

The transient spike of PI (3,4)P₂ and PIP₃ produced in stimulated cells suggests pathways are in place to mediate the metabolism of these lipids; in particular, phosphatases since these lipids are not substrates of PLC (Serunian et al., 1989). Several proteins have been isolated and characterized from various tissues that de-phosphorylate PIP₃, PI (3,4)P₂ and PI(3)P (Jackson et al., 1995; Kabuyama et al., 1996). Some of these enzymes also hydrolyze various inositol polyphosphate isomers. It would seem that, at least when assayed in vitro, different phosphatases metabolize PIP₃ as opposed to the other isomers. For instance, PIP₃ is a substrate for a 5-phosphatase while the other 3-PPI’s are degraded by distinct 3-phosphatases (Woscholski et al., 1995). It is not clear whether phosphatases exist that have absolute specificity for the 3-PPI’s. Two groups have recently cloned a novel SH₂ containing inositol polyphosphate 5-phosphatase (referred to as SHIP) that forms complexes with Shc and Grb-2 (Damen et al., 1996; Kavanaugh et al., 1996). This
protein hydrolyzes PI (3,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ but not PI(4,5)P$_2$. Regulation of SHIP by association with tyrosine-phosphorylated proteins may be involved in regulation of the lipids. For example, the generation of PI (3,4)P$_2$ could be primarily from 5-phosphatase mediated breakdown of PI (3,4,5)P$_3$ rather than a PI 3-kinase-mediated phosphorylation of PI (4)P.

D. Platelets and Phosphoinositide 3-kinase

Detection of the novel polyphosphoinositides (3-PPI's) in activated platelets closely followed their discovery in neutrophils and spurred interest in the possibility that PI 3-kinase might have a role in control of cellular processes other than mitogenesis (Kucera and Rittenhouse, 1990). Much more is known about regulation of PI 3-kinase in platelets than the downstream biochemical targets or the role of this enzyme in platelet responses (Rittenhouse, 1995). The obvious inability to genetically manipulate PI 3-kinase in platelets is the underlying reason for this discrepancy. In subsequent sections I will summarize what is currently known about activation, regulation and role of PI 3-kinase in blood platelets activated by physiological agonists.

D.1. Activation of PI 3-kinase in Agonist Stimulated Platelets

The stimulated formation of 3-PPI's in platelets was first reported independently by two groups in 1990 (Nolan and Lapetina, 1990; Kucera and Rittenhouse, 1990). Agonists that induce 3-PPI formation in platelets include thrombin, thrombin receptor activating peptide (TRAP), U46619 (a stable TXA$_2$ analogue), anti-CD9 monoclonal antibody, vWF, concanavalin A and lyso-phosphatidic acid among others (Nolan and Lapetina, 1990; Kucera and Rittenhouse, 1990; Nolan and Lapetina, 1991; Yatomi et al.,
1993; Jackson et al., 1994; Sultan et al., 1990; Cunningham et al., 1990; Torti et al., 1995).

As is the case in most other mammalian cells the level of PI-3-P is unchanged when platelets are stimulated thus reducing the likelihood that this species is involved in platelet activation (Kucera and Rittenhouse, 1990; Sultan et al., 1990). The levels of the other two 3-PPI's are acutely increased on platelet activation. In general, PIP₃ exhibits distinctly different kinetics of formation and magnitude of elevation in response to agonists compared to PI 3,4P₂ and the species do not show an obvious product-precursor relationship. This suggests an independent regulation of the two lipids that could arise by any number of different mechanisms. In thrombin-stimulated platelets for example, PIP₃ is rapidly induced to a maximum of 2-3 fold basal between 30 and 60 seconds followed by a slow decay (Kucera and Rittenhouse, 1990). PI 3,4P₂ formation on the other hand usually lags behind PIP₃ with maximums of 5-20 fold over basal reached much later (i.e. 2-5 minutes). This could imply that a major source of PI (3,4)P₂ in stimulated platelets is via 5-phosphatase mediated degradation of PIP₃. If this conversion occurred rapidly levels of PIP₃ would not accumulate to the same extent as PI (3,4)P₂.

D.2. Regulation of Phosphoinositide 3-kinase in Platelets

The regulation of PI 3-kinase in platelets is complex. Reports in the literature suggest that most if not all of the mechanisms for upstream activation of PI 3-kinase discussed above are operative in platelets (Rittenhouse, 1995). In addition some pathways unique to this cell type are likely to exist; fibrinogen receptor and/or cytoskeletal-dependent regulation, for instance. In the context of regulation it is important to ask what is the relative contribution of various pathways upstream of PI 3-kinase to the level of 3-PPI's at a given time point. Thus, what proportion of the 3-PPI is due to initial agonist
interaction with the platelet receptor, autocrine stimulation by secreted agonist or fibrinogen-receptor engagement/aggregation?

There is much evidence to suggest that production of PI 3,4P$_2$ and PIP$_3$ in platelets is controlled by different regulatory mechanisms, different isoforms of phosphoinositide 3-kinase or even other phosphoinositide kinases. This could reflect distinct roles for these two 3-PPi's in platelet function.

PI 3,4P$_2$ may be produced in platelets by 3-phosphorylation of PI-4-P (PI 3-kinase) or 4-phosphorylation of PI-3-P (PI 4-kinase). There are data supporting both routes of synthesis. Lapetina’s group showed that a 150 kDa phosphatidylinositol 3-phosphate 4-kinase is present in platelets and is inhibited, like PI 3-kinase, by nonionic detergents, (Yamamoto et al., 1990). Labelling studies performed by one group in particular have suggested that in stimulated platelets, PI-3-P is the precursor of PI 3,4P$_2$ which is then phosphorylated by a 5-kinase to give PLP$_3$ (Cunningham et al., 1990). This conclusion regarding the origin of PLP$_3$ in platelets is contradicted by metabolic studies in most other cells and most recently by direct labelling evidence from Rittenhouse’s laboratory indicating that PI 4,5P$_2$ is the precursor of PIP$_3$ (Carter et al., 1994).

Phorbol esters by themselves induce a moderate elevation in 3-PPi’s (especially PI 3,4P$_2$) in platelets thus implicating PKC in upstream regulation (Yamamoto and Lapetina, 1990; Kucera and Rittenhouse, 1990). That staurosporine inhibits agonist-induced 3-PPi formation is probably due to blockade of protein-tyrosine kinases rather than inhibition of PKC as suggested in early studies (Yamamoto and Lapetina, 1990). Treatment of permeabilized platelets with the PKC pseudo-substrate peptide (RFARK) inhibits 3-PPi generation in response to thrombin whereas okadaic acid, a serine/threonine phosphatase inhibitor potentiates the response (King et al., 1991). Thus protein kinase C is required for full agonist-induced activation of PI 3-kinase in platelets but in itself is insufficient for full stimulation. Recent work shows that phorbol-ester activated PKC seems to be
upstream of the heterodimeric (p85/p110) PI 3-kinase as opposed to PI 3-kinase γ (Zhang et al., 1996).

A major mechanism for regulation of PI 3-kinase in platelets is by G-proteins. Virtually all of the evidence for this originates from the work of a single group (Rittenhouse, 1995). The first clue came when they showed that GTP-γS (non-hydrolyzable GTP analogue) potently stimulates formation of PI 3,4P₂ and PIP₃ in saponin-permeabilized platelets incubated with [γ-³²P]-ATP (Kucera and Rittenhouse, 1990). In later work they provide convincing data that the small G-protein Rho is primarily responsible for the GTP-dependent activation of PI 3-kinase in platelets (Zhang et al., 1993). Indeed Rho is present in p85 immunoprecipitates from thrombin-stimulated platelets (Zhang et al., 1995). The effect of Rho on PI 3-kinase in platelets may be analogous to the recently discovered relationship between Ras and PI 3-kinase in other cells (Rodriguez-Viciana et al., 1994). In addition to low molecular weight G-proteins, members of the heterotrimeric family may also be involved. Initially, this was shown when G-protein βγ-subunits were able to stimulate PI 3-kinase activity in platelet cytosol (Thomason et al., 1994). A more detailed examination revealed that an additional PI 3-kinase isoform is present in platelets, γ, which is regulated by heterotrimeric G-protein βγ subunits (Zhang et al., 1995). Thus at least two isoforms of PI 3-kinase have been identified in platelets, heterodimeric p85/p110 and PI 3-kinase γ which are differentially regulated by Rho and G-protein βγ, respectively. The site(s) on platelet PI 3-kinase which interacts with Rho or βγ has not been determined.

Protein-tyrosine phosphorylation is a basic mechanism for activation of the heterodimeric (p85/p110) isoform of PI 3-kinase in cells, and platelets are no exception. Although platelet agonist receptors do not possess intrinsic tyrosine-kinase activity, platelet activation by most agonists results in activation of protein tyrosine activity and tyrosine phosphorylation of numerous substrates (Dhar and Shukla, 1993). Platelets are rich in non-receptor protein-tyrosine kinases of the src and related families, constituting
about 0.2% of total platelet protein (Clark et al., 1994). Thrombin stimulation has been shown to result in the rapid (5 sec) association of PI 3-kinase with p60^src^ and p59^fyn^ in platelets (Gutkind et al., 1990). In thrombin-treated porcine platelets the non-receptor protein-tyrosine kinase p72^iyi^ has been shown to associate with PI 3-kinase in a phosphotyrosine-dependent manner (Yanagi et al., 1994). PI 3-kinase protein and activity are increased in anti-phosphotyrosine immunoprecipitates from stimulated platelets although for most agonists neither the p85 or p110 subunit of the heterodimer are tyrosine-phosphorylated. This implies that PI 3-kinase (heterodimeric) must associate with other tyrosine-phosphorylated proteins as a mechanism of recruitment to membranes in platelets.

The use of specific tyrosine-kinase inhibitors also suggests a close correlation between tyrosine-phosphorylation and synthesis of PI 3,4P^2^ in stimulated platelets. Tyrphostin AG-213 was shown to potently inhibit thrombin-stimulated generation of PI 3,4P^2^ (Guinebault et al., 1993; Yatomi et al., 1994). This correlated with inhibition of aggregation, secretion and the amount of p85 subunit detected in anti-phosphotyrosine immunoprecipitates (Guinebault et al., 1993; Yatomi et al., 1994).

The cytoskeleton may play an important role in regulation of PI 3-kinase in platelets (Fox, 1993; Fox, 1994). When platelets are activated with thrombin, the membrane cytoskeleton (TX-100 insoluble fraction) becomes enriched in a number of signalling protein and lipid kinases including diacylglycerol kinase, PI 4-kinase, PI 3-P 5-kinase, phospholipase C, PKC, FAK, pp60^src^ and PI 3-kinase (Zhang et al., 1992; Grondin et al., 1991; Guinebault et al., 1995). The PI 3-kinase associated with the cytoskeleton in activated platelets has a higher specific activity (up to 4-fold) than the Triton-soluble form (Grondin et al., 1991). One study using western blotting and kinase assays of the cytoskeleton-associated PI 3-kinase shows that up to 30 % of the p85 subunit is translocated to the cytoskeleton and that this represents about 70 % of total platelet PI 3-kinase activity (Grondin et al., 1991). Inhibition of ligand binding to the
fibrinogen receptor, leading to blockade of aggregation, decreases this association. The relocation of p85/p110 to the cytoskeleton correlates temporally with aggregation, tyrosine phosphorylation of numerous platelet proteins and PI 3,4P₂, but not PIP₃ accumulation, which precedes these responses (Guinebault et al., 1995). Tyrosine kinase inhibitors also potently inhibit cytoskeletal association of PI 3-kinase.

The differences in regulation of PI 3,4P₂ and PIP₃ are further exemplified by studies involving the platelet fibrinogen receptor (integrin αIIβ₃) (Calvete, 1995). In thrombin-stimulated platelets pre-incubation with RGDS reduced (to a maximum of 60%) formation of PI 3,4P₂ in a dose-dependent manner. This correlated well with inhibition of aggregation and total tyrosine phosphorylation (Sultan et al., 1991). Platelets from patients with Glanzmann's thrombasthenia fail to aggregate or form PI 3,4P₂ in response to thrombin (Sultan et al., 1991). The late phase of PI 3,4P₂ accumulation is stimulated by addition of Ca²⁺ which is required by the fibrinogen receptor to bind ligand, but in contrast the PIP₃ response is unaffected by RGDS or Ca²⁺(Sorisky et al., 1992). A recent study showed that adhesion of resting platelets (no previous activation of agonist receptors) by fibrinogen receptor binding to immobilized fibrinogen specifically stimulated PI 3,4P₂ formation (Gironcel et al., 1996).

From these data one can conclude that synthesis of the majority of late phase PI 3,4P₂ in platelets requires ligand engagement of the fibrinogen receptor and aggregation leading to association of PI 3-kinase isoforms with the cytoskeleton through a mechanism involving tyrosine phosphorylation.

D.3. Function of Phosphoinositide 3-kinase in Platelets

Relatively little is known about the function(s) of PI 3-kinase in platelets. It is probable that this enzyme, if it does have a function, may augment or contribute to a function(s) controlled by one or more of the many other pathways activated by agonists in
platelets. A central question then is what is the relative role of PI 3-kinase in platelet function(s) compared to the other more established activation pathways as discussed above? Is activation of PI 3-kinase indispensable to any functions or can its absence be compensated for by another pathway?

Some data on possible functions for PI 3-kinase in platelet activation have emerged recently with the availability of the PI 3-kinase inhibitors wortmannin and LY294002. In TRAP-stimulated human platelets, inhibition of PI 3-kinase with either inhibitor did not affect actin assembly or platelet granule (dense or alpha) release (Kovacsovics et al., 1995). However, it was found that wortmannin inhibited platelet aggregation induced by TRAP or by binding of the monoclonal antibodies P256 or LIBS-6 to the fibrinogen receptor. PI 3-kinase, it was suggested, was required for irreversible aggregation and to maintain the fibrinogen receptor in its active state. They argue that PI 3-kinase stimulation downstream of fibrinogen receptor engagement provides a positive feedback signal in an ‘inside-out’ direction to preserve the activated state.

In the only other study to date, wortmannin was found to inhibit platelet aggregation induced by lyso-phosphatidic acid (Zhang and Rittenhouse, 1995). These authors conclude, in contrast to Kovacsovics’ group, that aggregation is inhibited because PI 3-kinase is necessary for the initial agonist induced activation of the fibrinogen receptor. PI 3-kinase activation downstream of fibrinogen receptor engagement they claim is not functionally important. Wortmannin was also shown to inhibit agonist induced activation of the fibrinogen receptor in platelets using a novel ELISA method (Gao and Shattil, 1995).

To summarize the current state of knowledge, PI 3-kinase activation does not appear to be necessary for platelet shape change (actin assembly) or secretion but does promote the aggregation response. The limited data (cited above) supporting a role for PI 3-kinase in platelet aggregation have led to completely opposing viewpoints as to the underlying mechanism.
Phosphorylation of the major PKC substrate in platelets, the p47 phosphoprotein pleckstrin, is partially dependent on PI 3-kinase as it is inhibited by wortmannin (Toker et al., 1995; Zhang et al., 1995). It was shown that synthetic PI 3,4P$_2$ and PIP$_3$ when added to permeabilized platelets causes wortmannin insensitive phosphorylation of pleckstrin. These lipids are also able to overcome inhibition of pleckstrin phosphorylation by wortmannin in stimulated platelets. Wortmannin is unable to inhibit phorbol ester mediated pleckstrin phosphorylation. The conclusions reached from these findings were that p47 phosphorylation in activated platelets is mediated both by PLC/DAG mediated activation of PKC and by (a slower) PI 3-kinase mediated activation of calcium-independent PKC isoforms.

The role of pleckstrin in platelet function is not well understood. Recent data suggests that this protein may inhibit agonist-induced phosphoinositide hydrolysis mediated by various isoforms of phospholipase C (Abrams et al., 1995). Somewhat paradoxically, pleckstrin phosphorylation may also inhibit PI 3-kinase activity as it was shown that recombinant phosphopleckstrin inhibits the G-protein $\beta y$ activatable isoform but not the heterodimeric isoform of PI 3-kinase in vitro (Abrams et al., 1996). Other evidence supports a role for PKC-mediated pleckstrin phosphorylation in platelet secretion and activation of the fibrinogen receptor (Dallavia et al., 1996; Gabbeta et al., 1996). There is a report that pleckstrin undergoes self-association and that this process is reversed by PKC-mediated phosphorylation (Mcdermott and Haslam, 1996). Thus it is probable that pleckstrin (phosphorylation) plays a complex regulatory role in platelet function perhaps as a major effector for protein kinase C.
E. Current Problem

As outlined in the above discussion, platelets are hemopoietic cells that play a critical role in initiation of hemostasis and wound healing but which also contribute to a wide range of significant cardiovascular and inflammatory disorders. There is evidence that PAF, a major inflammatory mediator, may regulate platelet function in vivo. Recent preliminary findings suggest that activation of the novel lipid kinase, phosphatidylinositol 3-kinase, is involved in certain aspects of platelet activation in addition to its more familiar role in cell growth. Currently there is no information available on the importance of PI 3-kinase in PAF-induced platelet activation. Research aimed at elucidating the role of PI 3-kinase in PAF-induced platelet responses will add to our understanding of the mechanisms of platelet activation by this important biological mediator.
OBJECTIVES

The goals of this project were:

1. To determine whether PAF activates phosphoinositide 3-kinase in platelets

2. To correlate changes in the generation of 3-phosphoinositides with the kinetics of various platelet responses to PAF

3. To determine the potency of the PI 3-kinase inhibitors, wortmannin and LY294002 on PAF-induced generation of 3-phosphoinositides

4. To correlate inhibition of phosphoinositide 3-kinase in PAF-stimulated platelets with inhibition of various responses to ascertain their PI 3-kinase dependency.

5. To characterize proteins that associate with, and may regulate, PI 3-kinase during platelet activation.
METHODS

A. Platelet Isolation

A.1 Rabbit Platelets

Platelets were isolated by the methods of Pinckard et al. (1979). Whole blood from New Zealand white rabbits was expressed directly into the anticoagulant of Aster and Jandl (25 g trisodium citrate dihydrate, 14 g citric acid and 20 g glucose per litre) at a ratio of 9:1 and used immediately. All subsequent manipulations were at room temperature. Anticoagulated blood (30 ml) was diluted with 10 ml Tyrode's buffer pH 6.5 containing 0.1 mM EGTA and centrifuged for 12 minutes at 190 g. The platelet-rich plasma (8 ml) was withdrawn, underlayered with 2 ml Histopaque solution (57 mg/ml polysucrose, 90 mg/ml sodium diatrizoate, density 1.077 g/ml, Sigma), and then spun at 600 g for 12 minutes. Platelets, which formed a band at the Histopaque/plasma interface, were removed, diluted with Tyrode's buffer + EGTA and the Histopaque separation repeated. The platelet suspension was diluted again in Tyrode's + EGTA, pelleted at 600 g then washed three times in Tyrode's buffer + EGTA. After resuspension, platelets were counted in an automated Coulter counter. Erythrocyte or leukocyte contamination was never greater than 0.1% (data not shown). For experiments, platelets were gently resuspended to the desired concentration in Tyrode's buffer pH 7.2 containing 1 mM Ca^{2+}. Porcine platelets were isolated in the same manner.

A.2. Human Platelets

Washed human platelets were isolated according to the protocol of Packham, Kinlough-Rathbone and Mustard (Kinlough-Rathbone et al., 1970). It differed from the
above protocol by the inclusion of apyrase, heparin and prostaglandin I_2 in all steps after the initial low-speed spin to inhibit premature activation.

**B. Platelet Aggregation**

Platelets were resuspended to 2.5-5.0 X 10^8/ml in Tyrode's solution + 1 mM Ca^{2+}. Aggregation was determined by the turbidimetric method in an aggregometer (Biodata Corp.). Platelet suspensions (0.5 ml) were transferred to a siliconized glass cuvette containing a small magnetic stir bar. After two minutes pre-incubation at 37 °C, the cuvette was placed in the measurement chamber at a stirring speed of 1100 r.p.m. The aggregating agent (i.e. PAF, thrombin) was added in a small volume (50 µl) and light transmission at 609 nm was recorded continuously with a potentiometric pen recorder. Inhibitors, if used, were pre-incubated for 10 minutes at 37 °C with the platelet suspension.

**C. Platelet Secretion (dense-granule release):**

The dense-granule release reaction was measured in rabbit platelets pre-loaded with ^3H-hydroxytryptamine (serotonin) essentially as described previously (Salari et al., 1990). Rabbit platelets (isolated as above) were resuspended to 2 X 10^9/ml in Tyrode's-Hepes buffer pH 6.5 containing 1 µg/ml prostaglandin I_2 (PGI_2). ^3H-hydroxytryptamine (0.3 µCi/ml) was added and the platelets incubated at 37 °C for 2 hr. with occasional mixing. Platelets were washed 3 times to remove label then re-suspended to 2 X 10^8/ml in Tyrode's-Hepes, pH 7.2 containing 1 mM Ca^{2+}. After pre-incubation at 37 °C for 2 minutes, PAF (10 µl) was added, the tube was vortexed briefly and incubated for another 2 minutes. The reaction was stopped by adding 0.5 ml ice-cold 20 mM EDTA pH 8.0, pelleting the platelet suspension and counting 500 µl of the supernatant medium in a
scintillation counter. The data was expressed as percentage of $^3$H counts released into the supernatant relative to total counts in unstimulated platelets. Test compounds were incubated with platelets for 10 minutes at 37 °C before PAF addition.

D. Measurement of PI 3-kinase Activation in Platelets

D.1. Detection and Quantitation of Intracellular Phosphatidylinositol (3,4,5) trisphosphate

D.1.1 Labelling of platelets with $^{32}$P-orthophosphate

Platelets, isolated as above, were re-suspended in Tyrode's-Hepes buffer pH 7.0 to $5 \times 10^9$ per ml and labeled with 0.75 mCi/ml H$_3$[32P]O$_4$ (ICN, HCl and carrier free) for 90 minutes at 37 °C with occasional mixing. Excess label was removed by centrifugation and the platelets were washed twice at room temperature with fresh medium before being suspended at $3 \times 10^9$/ml in Tyrode's-Hepes buffer + 1 mM Ca$^{2+}$. Platelet suspensions (0.5 ml) were stimulated with agonist at 37 °C in an aggregometer to facilitate simultaneous measurement of the aggregation response and to better control reaction conditions (see above). Stimulations were terminated by adding 50 µl concentrated HCl (1 N final) followed by chilling in an ice-bath.

D.1.2. Extraction of Total Cellular Lipids

Platelet phospholipids were extracted by a modification of the method of Gold et al. (1994). Chilled platelet suspensions were transferred to 15 ml polypropylene tubes then vortexed for 20 sec. with 1.88 ml 2:1 MeOH:CHCl$_3$ (v:v) and allowed to stand at room temperature for 20 minutes. Phases were separated by vortexing the mixture with 0.63 ml H$_2$O then 0.63 ml CHCl$_3$ followed by centrifugation. The lower phase was
recovered then the residual upper phase re-extracted with 0.5 ml 2:1 CHCl₃:MeOH (v:v) and the organic layers combined. Two washes of the organic layer with 0.5 ml 1:0.9 MeOH:0.1 M EDTA (v:v) to remove divalent metal cations were performed. The extract was transferred to glass tubes and dried under nitrogen.

D.1.3. Thin-layer Chromatography (TLC) Analysis of Phosphatidylinositol (3,4,5) trisphosphate

The lipid film was dissolved in 100 μl 95:5 CHCl₃:MeOH then 25 μl (in 5 x 5 μl aliquots with drying in between) applied to the origin of an 20 cm x 20 cm oxalate-treated silica-gel TLC plate (Si 60, E. Merck, aluminum backed). After drying briefly, the plate was developed to the top in one of two solvent systems. The first, system A, consisted of CHCl₃:acetone:MeOH:conc. acetic acid:H₂O 80:30:26:24:14 (v:v). System B consisted of n-propanol:2 M acetic acid 13.7:7 (v:v). The plates were dried thoroughly in a flow hood, then exposed to autoradiography film (Kodak XAR), typically for 2-4 hours at -80 °C. The spot corresponding to ³²P-labelled PIP₃ in stimulated platelets was located by co-migration with an authentic standard produced by in vitro phosphorylation of PI (4,5) bisphosphate using immunopurified rabbit platelet PI 3-kinase (see below). The PIP₃ spots were excised from the TLC plate and quantified by scintillation counting. Values were routinely normalized to total ³²P-dpm in the extract.

D.1.4. Validation of TLC Method for Measurement of Phosphatidylinositol (3,4,5) trisphosphate: De-acylation and High-pressure Liquid Chromatography (HPLC) Analysis of Glycerophosphoinositides

The identity of the PIP₃ spot was periodically confirmed by HPLC analysis of the corresponding glycerophosphoinositide using procedures and conditions similar to those
described previously (Gold et al., 1994). The putative PIP₃ spot (from platelet lipids or *in vitro* phosphorylation) was scraped from the TLC plate and the lipid deacylated by adding 1.8 ml of methylamine reagent (methanol:25% methylamine:n-butyl alcohol 45.7:42.8:11.4 v:v:v) and incubating for 50 min at 53 °C. After removal of silica, the mixture was dried *in vacuo*, resuspended in water, and dried a second time. The dried lipids were re-dissolved in 2 ml of water, extracted three times with 2 ml of n-butyl alcohol:light petroleum ether:ethyl formate (20:4:1, v:v:v), dried again then resuspended in 160 μl of water and stored at -80 °C until HPLC analysis. Ammonium phosphate, pH 3.8 was added to each sample to a final concentration of 10 mM as well as ADP and ATP (60 nmole each) as internal standards. The deacylated samples were then separated on a Partisil 10 SAX ion exchange column. Following injection, the column was washed with water for 10 min, then eluted with a 20 min linear gradient of 0-0.25 M ammonium phosphate pH 3.8 followed by a 50 min linear gradient of 0.25-1.0 M ammonium phosphate 3.8 at a flow rate of 1 ml/min. ³²P radioactivity was quantitated by scintillation counting of 0.5 ml column fractions using Ecolite+ (ICN). Under these conditions ADP and ATP consistently eluted at about 32 and 47 minutes respectively. The identity of the ³²P-glycerophosphatidylinositol (3,4,5) trisphosphate was confirmed by its elution time (56 min) relative to those for [³²H]inositol P₃ (53 min) and [³²H]inositol P₄ (68 min).

D.2. Detection and Quantitation of Intracellular Phosphatidylinositol (3,4) bisphosphate

PI (3,4)P₂ is not resolved from the much more abundant PI (4,5)P₂ using one-dimensional TLC. However PI (3,4)P₂ can be measured by de-acylation of the PIP₂ zone on the TLC plate followed by HPLC separation of the glycero-phosphoinositides (g-phosphoinositides).

The PIP₂ zone was located, scraped from the plate, de-acylated and samples prepared for HPLC exactly as indicated above. For resolution of gPI (3,4)P₂ from gPI
(4,5)P$_2$ a shallower gradient was employed. After injection and a 10 min. H$_2$O wash, a 60 min. linear gradient to 0.25 M ammonium phosphate 3.8 was run followed by a 50 min. linear gradient to 1.0 M ammonium phosphate 3.8. 0.25 ml fractions were collected. Under these conditions, gPI (3,4)P$_2$ and gPI (4,5)P$_2$ eluted at 45.5 min. and 48 min. respectively.

D.3. **In vitro** Determination of Phosphatidylinositol 3-kinase activity

D.3.1 Preparation of Platelet Lysates and Immunoprecipitation

Platelets in Tyrode's-Hepes buffer pH 7.2 containing 1 mM Ca$^{2+}$ were solubilized with an equal volume of ice-cold TX-100 lysis buffer (2% Triton X-100, 20 mM Tris-HCl pH 8, 137 mM NaCl, 20 % glycerol, 4 mM EDTA, 2 mM Na$_3$VO$_4$, 2 mM Na$_3$MoO$_4$, 2 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 2 µg/ml aprotinin, 20 mM NaF, 2 µM pepstatin, 20 µg/ml soybean trypsin inhibitor). After 10 minutes on ice, detergent insoluble material was removed by centrifugation for 15 min at 14,000 rpm in the cold. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce). In some cases the Tyrode's-Hepes medium was removed followed by two washes prior to addition of lysis buffer.

In experiments to determine the *in vivo* inhibition of platelet PI 3-kinase by wortmannin, Tyrode's medium containing the inhibitor was removed and platelets washed twice in drug-free buffer before detergent lysis.

D.3.2 Kinase Reaction

Platelet lysates (210 µg total protein) were immunoprecipitated with a monoclonal anti-phosphotyrosine antibody (4G10, UBI) or antisera to the p85 (UBI) or p110 (Santa
Cruz) subunits of PI 3-kinase for 3 hr at 4 °C and the immune complexes collected on protein A-Sepharose (25 µl packed beads). The precipitates were washed twice with lysis buffer containing 50 µM sodium orthovanadate (for 4G10 immunoprecipitation only) and three times with 10 mM Tris-HCl pH 7.4. Sonicated phosphatidylinositol (10 µg) (Avanti Polar Lipids, Alabaster, AL) was mixed with the beads and kept for 10 minutes on ice. The kinase reaction was initiated by adding 40 µl of kinase buffer (50 µM ATP, 30 mM Hepes pH 7.4, 30 mM MgCl₂, 200 µM adenosine) containing 10 µCi of [γ-³²P]ATP. After 15 minutes at room temperature, 0.1 ml of 1 N HCl and 0.2 ml of chloroform:methanol (1:1 v:v) were added to stop the reaction. The samples were vortexed, then the lower organic layer (70 µl) was transferred to new tubes and kept at -20 °C until analysis. Compounds tested for their effects on PI 3-kinase in vitro were incubated with anti-p85 immunoprecipitates for 10 minutes at room temperature prior to addition of PI.

D.3.3 Thin-layer Chromatography

³²P-labelled phosphatidylinositol 3-phosphate was separated from residual [γ-³²P]ATP by chromatography on oxalate-treated TLC plates (Si 60, 20 x 20 cm, aluminum backed, E. Merck) using a solvent system of chloroform:methanol:water:28% ammonia (90:70:15:5, v:v:v:v). TLC plates were exposed to X-ray film at -80 °C then radioactivity incorporated into PI-3-P (PI 3-kinase activity) was measured by excising the spot from the plate followed by liquid scintillation counting.

E. Immunoblotting of Platelet Lysates and anti-PI 3-kinase Immunoprecipitates

Immunoprecipitates (prepared as above) were washed 5 times in 1% TX-100 lysis buffer, then mixed with 30 µl SDS sample buffer containing 1 mM 2-mercaptoethanol.
Proteins were eluted from the beads by boiling for 3 minutes. Whole platelet lysates (20 µg protein) were mixed with 1/5th volume of 5X sample buffer. Proteins were then separated by SDS-PAGE (7.5%) and transferred to nitrocellulose by semi-dry blotting. The membranes were blocked overnight with 10 mM Tris-HCl pH 8, 150 mM NaCl (TBS) containing 5% bovine serum albumin, 1% ovalbumin and 0.05% azide. The primary antibody (4G10 monoclonal anti-PY at a final concentration of 0.1 µg/ml or anti-p85 at 1:1000 for example) was diluted in TBS containing 1% bovine serum albumin, 0.2% ovalbumin and 0.05 % azide and incubated with the filter for 2 hours at room temperature. After extensive washing with TBS containing 0.05% Tween 20, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase-coupled secondary antibody. Bound antibodies were detected using enhanced chemiluminescence (Amersham Corp.) as suggested by the manufacturer. In some instances, blots were re-probed with other antibodies after stripping with 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol at 50 °C for 30 min.
RESULTS

A. Activation of PI 3-kinase in PAF-stimulated Platelets

A.1 Analysis of 3-phosphoinositide Formation in PAF-stimulated Platelets

Activation of PI 3-kinase in PAF-stimulated platelets was determined using several different methods. The most direct is to measure, in vivo, the generation of the putative lipid second messengers, PI(3,4,5)P3 and PI (3,4)P2. 32P-PIP3 was detected in PAF-stimulated, 32P-labelled platelets by lipid extraction followed by one-dimensional TLC. In time course studies, PIP3 levels rose rapidly to a maximum of 225 % of basal level within 1 minute then slowly fell to slightly above unstimulated levels at 10 minutes when stimulated with 100 nM PAF (Figure 1A and 1B). The concentration-dependence of PIP3 formation is shown in Figure 2 for platelets stimulated with various levels of PAF for 1 minute. PAF concentrations were deliberately chosen based on their ability to induce no visible aggregation (0.01 nM), reversible aggregation (.075 nM and .10 nM) and irreversible aggregation (1 nM and 10 nM). These data show no measurable stimulation of PIP3 at sub-aggregatory (0.01 nM) PAF concentrations. At PAF concentrations leading to reversible aggregation PIP3 appears to be increased modestly up to about 120 % of basal. PIP3 is sharply increased when platelets are stimulated with PAF (1 or 10 nM) leading to irreversible aggregation. Similar elevations in PIP3 were also observed in thrombin-stimulated rabbit platelets (data not shown).

The identity of PIP3 was confirmed in two ways. The first method was by co-chromatography using TLC with authentic PIP3 synthesized in vitro using commercial PI (4,5)P2 as a substrate for immunopurified platelet PI 3-kinase (Figure 1A). Secondly, the de-acylated putative PIP3 spot from the TLC plate co-eluted on a strong-anion exchange HPLC column with authentic glycerophosphatidylinositol (3,4,5) trisphosphate (gPIP3)
Figure 1A. **PIP₃ is rapidly elevated in PAF-stimulated platelets.** ³²P-labeled rabbit platelets were stimulated for various times with 100 nM PAF at 37 °C in a stirred cuvette then total phospholipid extracts separated by chromatography on oxalate-treated silica gel TLC plates. Autoradiogram of TLC plate after 2 hours exposure. PIP₃ in platelet extracts is located by co-elution with authentic PIP₃(S) generated enzymatically. This experiment is representative of at least three giving similar results.
Figure 1B. The PIP₃ spot was excised and ³²P quantitated by scintillation counting. PIP₃ levels in PAF-stimulated samples are expressed as the percentage of PIP₃ counts in unstimulated platelets (basal PIP₃=6,000 cpm). Data are means ± S.E. of triplicate samples from a single experiment representative of several. The maximum in PIP₃ varied between 30 sec. and 1 min. for different experiments consistent with the kinetics of aggregation.
Figure 2A. PAF induces a concentration-dependent increase in PIP₃ in platelets. 
³²P-labeled rabbit platelets were stimulated at 37 °C under stirring conditions for 1 minute with various concentrations of PAF then total phospholipid extracts separated by chromatography on oxalate-treated silica gel TLC plates. PAF concentrations were chosen that resulted in no visible aggregation (0.01 nM), reversible aggregation (0.075 and 0.1 nM) and irreversible aggregation (1 and 10 nM) when added to platelets. Autoradiogram of TLC plate after 2 hours exposure. PIP₃ in platelet extracts is located by co-elution with authentic PIP₃(S) generated enzymatically.
Figure 2B. \( ^{32}P \)-was quantitated by phosphorimager analysis using local background correction. PIP\(_3\) levels in PAF-stimulated samples are expressed as the percentage of PIP\(_3\) counts in unstimulated platelets (basal PIP\(_3\)=6,000 cpm). Data are in triplicate from a single experiment representative of several.
produced by de-acylation of standard PIP₃ (Figure 3). Also, gPIP₃ eluted, as predicted, between the standard inositol phosphates Ins (1,4,5)P₃ and Ins (1,3,4,5)P₄ as previously shown (Gold et al., 1994). As discussed later, the PI 3-kinase inhibitors, wortmannin and LY294002 could prevent the PAF-induced increase in PIP₃ seen in platelets which supports the contention that the PIP₃ formed originates from activation of a PI 3-kinase.

One-dimensional TLC alone cannot be used to measure PI (3,4)P₂ in labelled cells as this species is not separated from the vast excess of labelled PI (4,5)P₂ (Carpenter and Cantley, 1990). Therefore to detect this lipid in platelets, the PI bisphosphate zone was excised from the TLC plates, de-acylated, then the glycerophosphoinositides run on a strong-anion exchange HPLC column. Under these conditions, gPI (3,4)P₂ (identified by co-elution with standard de-acylated PI (3,4)P₂) eluted at 45 min while gPI (4,5)P₂ eluted at 49 min as shown in Figure 4. PI (3,4)P₂ was not detected in unstimulated platelets or after 5 seconds PAF stimulation. However, at one minute stimulation high levels of PI (3,4)P₂ were present which decayed to less than half this level by 10 minutes (Figure 4). The fold stimulation of PI (3,4)P₂ over basal levels estimated from the peak areas in Figure 4 was at least twenty (20) times.

A.2. PI 3-kinase activity Associated with Tyrosine-phosphorylated Proteins

A major mechanism for cellular activation of PI 3-kinase is ligation of the SH2 domains of the p85 regulatory subunit with specific sequences in proteins containing phosphotyrosine residues, following activation of tyrosine kinases (Carpenter et al., 1993). This results in recruitment of heterodimeric PI 3-kinase to membranes (access to lipid substrate) as well as an increase in specific activity through conformational changes in the p85 subunit which influence the activity of the p110 catalytic subunit. Thus, determination of phosphotyrosine-associated PI 3-kinase activity can be predictive of its activation in cells. PAF-stimulated platelets were lysed in TX-100 detergent, insolubles removed by
Figure 3. De-acylated putative PIP₃ in platelets co-elutes with authentic de-acylated PIP₃ on a strong-anion exchange (SAX) HPLC column. Identification of PIP₃ in PAF-stimulated platelets. A, The putative ³²P-PIP₃ spot from TLC of PAF-stimulated platelets (Figure 1) was excised, de-acylated with methylamine then co-injected with tritiated IP₃ and IP₄ standards on a strong-anion exchange HPLC column and eluted with a linear gradient of ammonium phosphate pH 3.8 buffer as detailed in Experimental Procedures. Column fractions were analysed by scintillation counting. B, ³²P-Glycero-PIP₃ (gPIP₃) produced by deacylation of authentic ³²P-PIP₃ was co-injected with IP₃ and IP₄ and eluted under the same conditions as in A.
Figure 4. PI 3,4P₂ formation is induced in PAF-stimulated platelets. The PI-bisphosphate zone (unresolved PI 3,4 and PI 4,5P₂ isomers) from TLC of PAF-stimulated platelets was de-acylated then the glycerophosphoinositides resolved on a strong-anion exchange HPLC column using a shallow salt gradient as detailed in Experimental Procedures. Shown are representative tracings from four separate sample injections (unstimulated (A), 5 sec (B), 1 min (C) and 10 min (D) PAF stimulation) after column fractions were counted in a scintillation counter. The arrow indicates the elution position of authentic glycerol-PI 3,4P₂ produced from PI 3,4P₂ synthesized in vitro. gPI 4,5P₂ is the large peak eluting at 49 min.
centrifugation then the lysate immunoprecipitated with monoclonal antibody to
phosphotyrosine (4G10). Stimulation with 10 nM PAF resulted in a time-dependent
increase in anti-phosphotyrosine associated PI 3-kinase activity that reached a maximum
(3 fold over basal) by 45 seconds stimulation then fell to slightly above basal by 10
minutes (Figure 5).

B. Role of PI 3-kinase in PAF-stimulated Platelets

B.1. Correlation of PI 3-kinase Activation with Platelet Functional Responses:

Valid comparisons can be made between activation of PI 3-kinase and the
aggregation response in platelets because these two parameters can be simultaneously
determined in the same sample. At early time points the kinetics of PI 3-kinase activation
(anti-Tyr(P) or PIP₃) closely paralleled the aggregation response when these were plotted
on the same graph (Figure 6). However at later times both measures of PI 3-kinase
activity were reduced to near basal while aggregation (irreversible) is still maintained.
PIP₃ generation appears to parallel, if not precede, aggregation in PAF-stimulated
platelets.

In rabbit platelets stimulated with increasing concentrations of PAF there are two
distinct phases of aggregation. At low agonist concentrations platelets exhibited reversible
(or primary phase) aggregation in which the platelets show an initial aggregation followed
by a disaggregation. At higher PAF concentrations the response is irreversible
(secondary phase) and platelet aggregates are maintained (Figure 7A). The transition
from reversible to irreversible aggregation occurs over a very narrow PAF concentration
range (i.e. exhibits a threshold effect). When the anti-phosphotyrosine
immunoprecipitable PI 3-kinase activity was simultaneously measured in platelets
Figure 5A. PI 3-kinase activity associated with tyrosine-phosphorylated proteins is increased in PAF-stimulated platelets. Rabbit platelets (3 x 10^8/ml) were stimulated for various times with 10 nM PAF, detergent-lysed then the supernatant remaining after removal of TX-100 insoluble material immunoprecipitated with monoclonal antibody to phosphotyrosine (4G10). PI 3-kinase activity was assayed in the immunoprecipitates using PI as a substrate. ^32P-labeled PI-3-P was separated from residual ^32P-ATP by thin layer chromatography. Figure (A) is autoradiogram after 8 hrs. exposure to film at -80 °C. Note: 'p' is negative control without platelets. Radioactivity at origin is residual ^32P-ATP, the amount of which is unrelated to signaling.
Figure 5B. PI-3-P was quantitated in a scintillation counter and expressed graphically as percentage relative to control.
Figure 6. Aggregation and activation of PI 3-kinase are tightly linked events in PAF-stimulated platelets. Platelet aggregation determined by the turbidimetric method can be simultaneously determined in samples that are then assayed for PI 3-kinase activation. The time course for PAF-induced aggregation is co-plotted with PIP₃ formation and phosphotyrosine-associated PI 3-kinase activity.
stimulated with increasing levels of PAF, the enzyme activity increased sharply over the same concentration range (Figure 7B and 7C). Thus, irreversible aggregation, or platelet activation states necessary for this response, were highly correlated with increases in PI 3-kinase activity associated with tyrosine-phosphorylated proteins.

B.2. Effect of Selective Inhibition of PI 3-kinase on Platelet Functional Responses

B.2.1. Validation of Wortmannin and LY294002 as Inhibitors of PI 3-kinase in Platelets

Wortmannin and LY294002, two potent but mechanistically distinct inhibitors of mammalian PI 3-kinases were tested for their ability to inhibit platelet PI 3-kinase, both in vitro and in vivo. The in vitro $IC_{50}$ for LY294002 was 1.5 μM for immunopurified rabbit platelet PI 3-kinase (using PI as substrate) over an inhibitor range of 0.1-100 μM (Figure 8A). Under identical conditions, the $IC_{50}$ for wortmannin was 5 nM (Figure 8B).

Since wortmannin is an irreversible inhibitor of PI 3-kinase and forms a covalent adduct with the enzyme, inhibition is retained after lysis and immunopurification of PI 3-kinase. Therefore the residual activity in immunoprecipitates may reflect the actual state of the enzyme at the time of lysis. Platelets were pre-incubated with wortmannin for 10 minutes at 37 °C then pelleted and washed twice with Tyrode’s buffer in the absence of drug. Platelets were then lysed with TX-100 lysis buffer and PI 3-kinase activity in anti-p85 immunoprecipitates determined. The $IC_{50}$ for PI 3-kinase inhibition by wortmannin determined in this way was 15 nM (Figure 9). This was independent of the state of platelet activation and unaffected by the presence of BSA in the medium (data not shown). PI 3-kinase inhibition after one minute incubation was 82% of inhibition observed after 10 minutes indicating that wortmannin rapidly permeated the platelet membrane (Figure 10). However, under these conditions it is evident that PI 3-kinase activity was not completely abrogated in immunoprecipitates from platelets incubated with
Figure 7. Attainment of irreversible aggregation is correlated with a large increase in PI 3-kinase activity associated with tyrosine-phosphorylated proteins. A, Aggregation tracings showing transition from reversible (primary phase) to irreversible (secondary phase) aggregation of rabbit platelets stimulated with increasing concentrations (nM) of PAF. As shown for the 1.0 nM PAF tracing, the vertical axis represents increasing light transmittance through the platelet suspension (increasing aggregation). The horizontal axis is time with 1 minute represented by the double-ended arrow. The point of PAF addition is also indicated by an arrow. B (autoradiogram) and C (plot) showing anti-phosphotyrosine (4G10) immunoprecipitable PI 3-kinase activity in platelet samples corresponding to panel A. These data are from a single experiment representative of many giving similar results. The median value of the concentration range of PAF resulting in reversible aggregation can vary by a factor of 2 between individual experiments due to variations in the platelet preparation.
Figure 7A
Figure 7B
Figure 7C

PI 3-kinase activity (% of basal)

[PAF] (nM)
Figure 8. Wortmannin and LY294002 are potent inhibitors of rabbit platelet PI 3-kinase in vitro. Various concentrations of wortmannin (A) or LY294002 (B) were incubated at room temperature for 10 minutes with anti-p85/protein A-sepharose immunoprecipitates from detergent-lysed rabbit platelets. The PI 3-kinase activity was then determined using PI as a substrate as described in Experimental Procedures.
Figure 9. Wortmannin inhibition of PI 3-kinase is retained after detergent-lysis of platelets, immunoprecipitation and numerous washings. Rabbit platelets were incubated with varying concentrations of wortmannin for 10 minutes at 37 °C then pelleted and washed twice with Tyrode's medium without wortmannin before lysis in solubilization buffer. The PI 3-kinase activity in anti-p85 immunoprecipitates of the Triton soluble fraction was determined as in Experimental Procedures. A, Autoradiogram and B, PI 3-kinase activity retained in immunoprecipitates expressed as percentage relative to level in the absence of wortmannin.
Figure 9.
Figure 10. Wortmannin rapidly permeates rabbit platelets. Platelets were incubated with 1000 nM wortmannin for various times at 37 °C, wortmannin was removed by washing, platelets were lysed and the detergent soluble fraction immunoprecipitated with anti-p85 antibody. The PI 3-kinase activity in the immunoprecipitates was then determined.
up to 1.0 μM wortmannin. Incomplete inhibition was not due to loss of wortmannin from PI 3-kinase during immunoprecipitation or subsequent washing. The inhibition of PI 3-kinase by wortmannin \textit{in vitro} is retained after several washings. When platelets were lysed in detergent prior to removal of wortmannin from surrounding medium, the inhibition curve was shifted to the left and the IC\textsubscript{50} lowered to 8 nM. This shows that wortmannin in its active form (able to inhibit PI 3-kinase) is still present in the Tyrodes's medium surrounding the platelets after 10 minutes incubation at 37 °C. Almost complete inhibition was observed at 100 nM wortmannin (Figure 11). In this case the curve resembles that for the purely \textit{in vitro} inhibition (Figure 8B).

The ability of wortmannin and LY294002 to inhibit formation of PIP\textsubscript{3} in vivo in PAF-stimulated whole platelets was also determined. Wortmannin and LY294002 inhibited PIP\textsubscript{3} formation in PAF-stimulated platelets in a dose-dependent manner with IC\textsubscript{50}'s of less than 10 nM (Figure 12A) and 5 μM (Figure 12B) respectively. Similar results were obtained with thrombin-stimulated platelets (data not shown).

We found wortmannin to be labile with respect to its ability to inhibit platelet PI 3-kinase when used in physiological media at near-neutral pH. The half-life for inhibition of PI 3-kinase using wortmannin incubated in RPMI 1640 + 20 mM Hepes pH 7.4 at 37 °C was about 60 minutes (Figure 13). The presence of 10 % fetal calf serum had no effect (data not shown). In contrast, wortmannin was much more stable in 20 mM Tris-HCl pH 7.4 or distilled water.
Figure 11. Concentration-dependence for wortmannin inhibition of PI 3-kinase activity in anti-p85 immunoprecipitates without removal of wortmannin from the medium before platelet lysis. Rabbit platelets were incubated for 10 minutes at 37 °C with various concentrations of wortmannin then detergent lysed without removal of the drug. Rest of protocol is exactly as in legend to Figure 9.
Figure 12A. Wortmannin potently inhibits PAF-induced formation of PIP$_3$ in platelets. $^{32}$P-labelled rabbit platelets were stimulated for 1 minute in a stirred cuvette with 0.2 nM PAF in the absence or presence of increasing concentrations of wortmannin then PIP$_3$ levels determined as in Experimental Procedures. Data are averages ± 1 std. deviation of triplicate determinations from a single experiment representative of three.
Figure 12B. LY294002 potently inhibit PAF-induced formation of PIP₃ in platelets. 
³²P-labelled rabbit platelets were stimulated for 1 minute in a stirred cuvette with 0.2 nM PAF in the absence or presence of increasing concentrations of LY294002 then PIP₃ levels determined as in Experimental Procedures. Data are averages ± 1 std. deviation of triplicate determinations from a single experiment representative of three.
Figure 13. Wortmannin is unstable in nutrient cell-culture medium at physiological pH. Wortmannin (600 nM) was prepared in either RPMI medium containing 20 mM Hepes pH 7.4 without serum or 20 mM Tris.HCl pH 7.4 and incubated in closed tubes at 37 °C for various times. 10 μl aliquots were introduced into the standard PI 3-kinase assay and the resulting activity determined. The initial (t=0) assay concentration of wortmannin is 100 nM. Data are expressed as percentage inhibition of PI 3-kinase by incubated samples relative to maximal (100%) inhibition observed using freshly made wortmannin prepared in cold dH₂O.
B.2.2. Correlation of PI 3-kinase Inhibition with Inhibition of Platelet Functional Responses

B.2.2.1 Aggregation

Both PI 3-kinase inhibitors, LY294002 and wortmannin inhibited PAF-induced aggregation of washed rabbit platelets. The IC₅₀ for inhibition of aggregation by wortmannin was highly dependent on the concentration of PAF used or more precisely, the activation state of the platelets (Figure 14). Platelets stimulated with high levels of PAF (i.e. ≥ 1 nM) which resulted in irreversible aggregation were more resistant to the anti-aggregatory effects of wortmannin than weakly stimulated platelets. The IC₅₀ was found to be approximately 5 μM, with no inhibition observed up to 1 μM wortmannin. These concentrations were much higher than the concentrations needed to block PI 3-kinase activity (Figures 9 and 12A). In contrast, weakly stimulated platelets, in which the aggregation response could be reversed by continual stirring, were potently inhibited, typically with IC₅₀'s lower than 100 nM. There was significant inhibition at 10 nM wortmannin which correlated more closely with the concentration of wortmannin required to inhibit PI 3-kinase activity. As for wortmannin, the effect of LY294002 on platelet aggregation was dependent on the degree of activation of the platelets. The IC₅₀ for LY294002 inhibition of reversibly aggregating platelets was typically 5-10 μM with complete inhibition at 50 μM (Figure 15). Inhibition of aggregation by LY-294002 correlated with inhibition of PI 3-kinase at low levels of stimulation. Strongly stimulated platelets require high levels of drug for appreciable inhibition.
Figure 14. Inhibition of aggregation by wortmannin depends on the activation state of the platelets. Rabbit platelets were pre-incubated for 10 minutes with wortmannin then stimulated for 1 minute with the noted concentrations of PAF and aggregation recorded in an aggregometer. PAF levels were chosen that induced reversible (0.05 or 0.1 nM PAF) or irreversible (1 nM PAF) aggregation in the absence of the inhibitor. Values are means ±SEM of triplicate determinations from a single experiment representative of at least three.
Figure 15. Inhibition of aggregation by LY294002 depends on the activation state of the platelets. See legend to Figure 14.
B.2.2.2 Secretion

The effects of inhibition of PI 3-kinase on platelet dense-granule (δ granule) release were also measured. Dense granule secretion was quantitated as the release into the medium of \(^{3}\)H from platelets pre-loaded with \(^{3}\)H-hydroxytryptamine (serotonin) after stimulation with PAF for 2 minutes. The release reaction was significantly less sensitive to PI 3-kinase inhibitors than was reversible aggregation. The IC\(_{50}\) for wortmannin inhibition of secretion was greater than 1000 nM at a PAF concentration giving 22% release of incorporated label (Figure 16). The inhibition curve displayed a distinct plateau phase over which the release reaction was relatively unaffected ([wt]=100-1000 nM), followed by a sharp inhibition of release at wortmannin concentrations over 1 μM. These high concentrations of wortmannin were much higher than those needed to inhibit PI 3-kinase activity. However there was modest inhibition (up to one-third) at relatively low concentrations of wortmannin (10-100 nM) that more closely paralleled the inhibition of PI 3-kinase activity by the drug. Secretion from dense granules at high concentrations of PAF (i.e. 10 nM) was minimally affected by wortmannin.

Secretion was inhibited by LY294002 in a similar fashion compared to wortmannin (Figure 17). The IC\(_{50}\) was greater than 50 μM (the highest concentration tested) for platelets releasing 12% of dense granule contents and the plateau phase was also seen. A similar percentage of secretion, however, was inhibited at relatively low levels of LY294002 (i.e. 5-10 μM). We tested the possibility that the inhibitors were simply slowing the release response rather than decreasing the overall magnitude. If this was the case then one would expect that with increasing time of PAF stimulation the percentage inhibition of secretion by wortmannin would decrease. As shown in Figure 18 the same relative inhibition by wortmannin is observed after 1, 2 or 5 minutes PAF stimulation.
Figure 16. Wortmannin partially inhibits platelet dense granule release in PAF-stimulated platelets. $^3$H-hydroxytryptamine (serotonin) pre-loaded rabbit platelets were incubated for 10 minutes at 37 °C with wortmannin then activated for 2 minutes with PAF (0.6 or 10 nM). $^3$H-hydroxytryptamine released into the medium was measured by scintillation counting after pelleting the platelets. Maximally stimulated platelets released approximately 80 ± 5% of total incorporated $^3$H. Values are means of triplicate measurements from a single experiment representative of at least two. Error bars are contained within plot symbols.
Figure 17. LY294002 partially inhibits platelet dense granule release in PAF-stimulated platelets. See legend to Figure 16.
Figure 18. Wortmannin inhibition of platelet dense granule release is independent of time of PAF stimulation. $^3$H-hydroxytryptamine pre-loaded rabbit platelets were incubated with wortmannin (100 or 200 nM) or vehicle for 10 minutes at 37 °C then stimulated with 0.5 nM PAF for 1, 2 or 5 minutes. $^3$H-hydroxytryptamine release was determined as outlined in the legend to Figure 16.
C. Co-immunoprecipitation of PI 3-kinase with Tyrosine-phosphorylated Proteins in PAF-stimulated Platelets

As association of PI 3-kinase (p85 subunit) with tyrosine-phosphorylated proteins is a major mode of activation of this enzyme and since platelets are rich in tyrosine kinases, we set out to look for tyrosine-phosphorylated proteins that associate with PI 3-kinase in PAF activated platelets. This investigation could lead to insight into mechanisms of regulation of PI 3-kinase in platelets.

TX-100 soluble fractions from control and PAF-stimulated platelets were immunoprecipitated with anti-phosphotyrosine (4G10) or anti-PI 3-kinase antibodies (p85 and p110 subunit) to recover phosphotyrosine-associated and total PI 3-kinase respectively. In addition, these respective samples would yield the total tyrosine-phosphorylated proteins and the tyrosine phosphorylated proteins associated with PI 3-kinase. Proteins were eluted by boiling beads in SDS-sample buffer, separated by SDS-PAGE, transferred to nitrocellulose, then blots were probed with various antibodies. Immunoblots of the anti-phosphotyrosine immunoprecipitates showed that the level of PI 3-kinase protein (p85 subunit) increased following PAF stimulation for times ranging from 5 seconds to 10 minutes (Figure 19-left panel). The induction of p85 association with tyrosine-phosphorylated proteins followed similar kinetics as the increase in PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates (Figure 5A). When this blot was stripped and re-probed using an anti-phosphotyrosine antibody, it was apparent that many platelet proteins were inducibly tyrosine-phosphorylated following PAF stimulation. Interestingly, there was very little signal in the molecular weight ranges expected for the PI 3-kinase p85 subunit. (Figure 19-right panel). This indicated that the presence of PI 3-kinase in the anti-phosphotyrosine immunoprecipitates was probably due to association with other tyrosine-phosphorylated proteins. This was not unexpected, since it has been shown previously.
Figure 19. The p85 subunit of PI 3-kinase is increased in anti-phosphotyrosine immunoprecipitates from the detergent soluble fraction of PAF-stimulated platelets. Supernatants from detergent lysed rabbit platelets were immunoprecipitated with monoclonal antibody (4G10) to phosphotyrosine. Eluted proteins were separated by SDS-PAGE, transferred to nitrocellulose then blotted with polyclonal antisera to the p85 subunit (left panel) then subsequently stripped and re-probed with anti-phosphotyrosine antibody (right panel). Positions of molecular weight standards are shown on the left. Data are from a single experiment representative of three.
that most platelet agonists do not induce tyrosine-phosphorylation of the PI 3-kinase regulatory subunit (Guinebault et al., 1995; Torti et al., 1995; Yatomi et al., 1994).

The tyrosine-phosphorylated proteins associating with PI 3-kinase in activated platelets were analysed by blotting proteins eluted from anti-PI 3-kinase immunoprecipitates with an anti-phosphotyrosine antibody. As shown in Figure 20 (left panel), a major tyrosine phosphorylated substrate of apparent molecular weight 115 kDa (p115) co-immunoprecipitates with PI 3-kinase (p85 subunit) in platelets as early as 20 seconds after PAF stimulation, reaching a maximum at 1 minute and then decaying at later times. As a control, the anti-phosphotyrosine blot was stripped and re-probed with anti-p85 antibody to indicate constancy of PI 3-kinase protein (lane loading) in all samples (Figure 20-right panel). Immunoprecipitates of activated platelet lysate using rabbit pre-immune serum do not contain this protein (Figure 21). This substrate was also recovered in anti-p110 immunoprecipitates from PAF-stimulated platelets with the same kinetics as described for the anti-p85 immunoprecipitates (Figure 22). A tyrosine-phosphorylated protein co-eluting with p115 was also present in the anti-p85 (Figure 23) and anti-p110 (not shown) immunoprecipitates of rabbit platelets stimulated with 1 U/ml human thrombin. While this protein was the major tyrosine-phosphorylated protein associated with PI 3-kinase in PAF-stimulated platelets, there were also several other proteins observed in many experiments. The p115 protein was always the most prominent band and the most consistently observed. A band corresponding to p115 could be partially depleted from detergent lysates of PAF-stimulated platelets by pre-clearing with anti-p85 antibodies (Figure 24).

Anti-p85 immunoprecipitates from thrombin-stimulated human platelets also exhibit a tyrosine-phosphorylated substrate of similar molecular weight compared to rabbit platelets (Figure 25). Based on the similar association with PI 3-kinase, it is possible that the p110 protein is the human homolog of the rabbit p115. We attempted to identify the p115 protein using antibodies against a number of potential tyrosine phosphorylated
Figure 20. PI 3-kinase co-immunoprecipitates with a major tyrosine-phosphorylated substrate (p115) in PAF-activated platelets but is itself not tyrosine-phosphorylated. Supernatants from detergent-lysed rabbit platelets were immunoprecipitated with polyclonal antisera to the p85 subunit. Eluted proteins were separated by SDS-PAGE, transferred to nitrocellulose then blotted with monoclonal antibody (4G10) to phosphotyrosine (left panel) then subsequently stripped and re-probed with anti-p85 antisera (right panel). Positions of molecular weight standards are shown on the left.
Figure 21. Rabbit pre-immune serum does not co-immunoprecipitate tyrosine-phosphorylated p115 from PAF-stimulated rabbit platelets. Supernatants from unstimulated platelets, or platelets stimulated with 10 nM PAF for 1 minute then lysed in detergent were immunoprecipitated with rabbit pre-immune serum or polyclonal antisera to the p85 subunit. Bound proteins were eluted, separated and blotted with anti-phosphotyrosine antibody as described in the legend to Figure 20. Data are from a single experiment representative of three.
Figure 22. Anti-sera to the p110 catalytic subunit of PI 3-kinase also co-immunoprecipitate tyrosine-phosphorylated p115 from PAF-stimulated platelets. Rabbit platelets were stimulated with 10 nM PAF for various times at 37 °C then lysed in TX-100 detergent and kept on ice for one hour. After centrifugation, the supernatants were immunoprecipitated with polyclonal antiserum to the p110β subunit of PI 3-kinase (Santa Cruz) and bound proteins separated and blotted with anti-phosphotyrosine antibody as detailed in the legend to Figure 20.
Figure 23. A major tyrosine-phosphorylated protein (p115) co-immunoprecipitates with PI 3-kinase in rabbit platelets stimulated with human thrombin. Platelets were stimulated with human thrombin (1 U/ml) for various times then the detergent-soluble fractions immunoprecipitated with polyclonal antibody to PI 3-kinase (p85 subunit). Bound proteins separated by SDS-PAGE, transferred to nitrocellulose then blotted with monoclonal antibody to phosphotyrosine (4G10).
Figure 24. A tyrosine-phosphorylated protein co-migrating with p115 is depleted from the detergent soluble fraction of lysates from PAF-stimulated platelets by incubation with anti-p85 antibody. Rabbit platelets were stimulated with 10 nM PAF for various times then lysed in ice-cold TX-100 detergent and the insoluble fraction removed. 10 μg of the TX-100 soluble fraction was then separated under reducing conditions by SDS-PAGE, transferred to nitrocellulose then blotted with anti-phosphotyrosine antibody (4G10). 10 μg of lysate from 1 min. PAF stimulated samples before (-) and after (+) immunoprecipitation with polyclonal anti-p85 antiserum were also run on the same gel to determine depletion. Experiment is representative of at least three giving similar results.
Figure 25. Human platelets stimulated with thrombin also exhibit inducible association of a tyrosine-phosphorylated protein (p110) with PI 3-kinase. Human platelets were stimulated for various times with thrombin (1 U/ml), lysed then the TX-100 soluble fraction immunoprecipitated with polyclonal antisera to the p85 subunit. Bound proteins were eluted, separated by SDS-PAGE then transferred to nitrocellulose and blotted with anti-phosphotyrosine antibody.
proteins having a similar molecular weight. These experiments ruled out the 110 kDa catalytic subunit of PI 3-kinase, p120 ras-GAP, JAK-2, cbl, focal adhesion kinase (FAK) or integrin subunit β1 as possible candidates. Recent data from our laboratory indicates that tyrosine-phosphorylated p115 is co-immunoprecipitated with PI 3-kinase only in the cytosol or detergent-soluble fraction of PAF-activated platelets and not in the membrane or detergent-insoluble (cytoskeletal) fractions.
A. Activation of PI 3-kinase in PAF-stimulated Platelets

The central question investigated in these studies was to determine the role of the signaling enzyme, phosphoinositide 3-kinase in functional responses of platelets stimulated with the potent lipid mediator, platelet-activating factor. A requirement for a signaling enzyme or pathway to transduce signals and therefore mediate a response to a particular extracellular agonist is that this enzyme be activated by the agonist in question. Therefore the initial portion of this work was focused on determining whether or not PI 3-kinase was activated in PAF-stimulated platelets and if so, to characterize the response. Two independent methods to quantitate activation of PI 3-kinase in platelets were used.

A.1. Direct measurement of 3-phosphoinositides

Quantitation of the 3-phosphoinositide products, phosphatidylinositol 3,4-bisphosphate (PI (3,4)P$_2$) and phosphatidylinositol 3,4,5-trisphosphate (PI (3,4,5)P$_3$) in lipid extracts from $^{32}$P-labeled cells stimulated by agonist or growth-factors is the most direct and accurate means to indicate PI 3-kinase activation (Auger et al., 1989; Carpenter and Cantley, 1990).

In platelets stimulated with a high concentration of PAF (100 nM) PIP$_3$ rapidly increases to 275 % of basal within one minute then decays to 120 % of basal after 10 minutes of stimulation (Figure 1). This is convincing evidence that PI 3-kinase is activated in PAF-stimulated platelets. The kinetics for PIP$_3$ induction closely parallel the physiological response of aggregation determined simultaneously in the same samples indicating a tight linkage between these biochemical and physiological events (Figure 6). The concentration-dependence for PIP$_3$ formation (Figure 2) also shows that PI 3-kinase
is activated at all concentrations of PAF that lead to visible aggregation. However, it is also evident from these data that PIP₃ levels rise rapidly in a high dose-correlation with the attainment of platelet activation states leading to irreversible aggregation.

PI (3,4)P₂ could be measured in the same samples as PIP₃. The kinetics of PI (3,4)P₂ formation were different than that for PIP₃ with a maximum seen at 1 minute stimulation and a slower decay through 10 min. stimulation (Figure 4). This could arise through differential regulation of PI 3-kinase isoenzymes, compartmentalization with respect to both enzyme and substrate or because PI (3,4)P₂ production is secondary (dependent on) PIP₃ synthesis. It is presently not known what proportion of PI (3,4)P₂ formed in stimulated cells originates from direct 3-phosphorylation of PI-4-P versus 5'-dephosphorylation of PIP₃, but pulse-labelling studies suggest that PI (3,4)P₂ is not formed by 4'-phosphorylation of PI-3-P in platelets (Carter et al., 1994). However, either mechanism could result in the observed kinetics.

The kinetics we obtained for PAF-mediated induction of PIP₃ are similar to those produced when platelets are stimulated with another strong agonist, thrombin (Kucera and Rittenhouse, 1990; Nolan and Lapetina, 1991; Rittenhouse, 1995; Zhang et al., 1996). However, thrombin stimulation usually results in a greater induction of PIP₃ (e.g. greater than 5-fold) than that which we observed for PAF (Kucera and Rittenhouse, 1990). The differences between PAF and thrombin with respect to PIP₃ induction are not unprecedented in that the TXA₂ mimetic, U46619 stimulates less PIP₃ than does thrombin and with different kinetics (Kucera and Rittenhouse, 1990). Also, another platelet agonist, lyso-phosphatidic acid induces quite a different pattern of 3-PPI formation in platelets compared to thrombin or PAF (Zhang and Rittenhouse, 1995).

PI (3,4)P₂ is elevated in platelets by strong agonists such as thrombin but with different kinetics than PIP₃, as we have shown for PAF (Rittenhouse, 1995). Generally, there is a lag followed by a slower rise than for PIP₃ leading to a maximum at 3-5 minutes followed by a slow decay. However, the amplitude of the stimulated increase in PI (3,4)P₂
is much greater with 6-50 fold elevations observed (Cunningham et al., 1990; Kovacsovics et al., 1995; Kucera and Rittenhouse, 1990; Nolan and Lapetina, 1991; Nolan and Lapetina, 1990; Thomas and Holub, 1992). Some have proposed that the lag may be due to autocrine production of a factor such as PDGF which subsequently binds to receptors on the same platelet leading to PI (3,4)P$_2$ synthesis (Sultan et al., 1990). More recent evidence, however, suggests that accumulations of PIP$_3$ and PI (3,4)P$_2$ are regulated differently in thrombin activated platelets with the latter at least partially dependent on extracellular calcium and engagement or clustering of the fibrinogen receptor (Sorisky et al., 1992).

In the only other cell-type where this response was examined, neutrophils, PAF was shown to stimulate formation of PIP$_3$ very rapidly with a maximum at 10 seconds (Stephens et al., 1993).

A.2. PI 3-kinase Activity Associated with Tyrosine-phosphorylated Proteins

As we and others have shown, platelet stimulation by strong agonists including PAF results in increased tyrosine phosphorylation of numerous proteins (Salari et al., 1990; Clark et al., 1994). A major mechanism for cellular activation of PI 3-kinase is binding of the SH2 domains of the p85 subunit to specific sequences in proteins containing phosphotyrosine residues, following activation of tyrosine kinases. This results in recruitment of the heterodimeric PI 3-kinase to membranes (access to lipid substrate) as well as an increase in specific activity through conformational changes conferred to the p110 catalytic subunit. Thus, an increase in PI 3-kinase activity associated with tyrosine-phosphorylated proteins often reflects actual in vivo activation of PI 3-kinase.

Immunoprecipitation of detergent lysates from PAF stimulated platelets with monoclonal antibody to anti-phosphotyrosine (4G10) followed by an in vitro kinase assay
showed large time- and concentration-dependent increases in PI 3-kinase activity associated with tyrosine-phosphorylated proteins (Figures 5 and 7).

Comparison of the time courses for PIP3 generation with those for phosphotyrosine-associated PI 3-kinase activity show many similarities with the aggregation response (Figure 6). The time courses show both measures of PI 3-kinase activation follow approximately the same kinetics with a maximum at 30-45 seconds followed by a slow decline. This could indicate that a major mechanism of PI 3-kinase activation in PAF-stimulated platelets is due to association of the enzyme with tyrosine-phosphorylated proteins in addition to G-protein dependent or other mechanisms. Due to the very rapid series of activation events in platelets it is not possible to draw conclusions from a kinetic analysis as to whether or not PI 3-kinase activation is simply a result of aggregation or is in fact an early signal that is required for this response.

In comparing the concentration-response curves for aggregation and anti-phosphotyrosine immunoprecipitable PI 3-kinase activity it is apparent that there is no substantial increase in activity over control when platelets exhibit only a primary phase or reversible aggregation (Figure 7). However the PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates rises sharply over a narrow concentration range when platelets are sufficiently stimulated to irreversibly aggregate. Thus there is a strong correlation between irreversible aggregation and increases in anti-phosphotyrosine immunoprecipitable PI 3-kinase activity. One possible explanation is that clustering or engagement of the fibrinogen receptor (integrin) is required to provide the necessary ‘outside-in’ signal. This signal could ‘turn-on’ the particular tyrosine kinases required for upstream activation of PI 3-kinase in platelets. There are a number of other possible interpretations of this concentration-dependence in addition to those mentioned above. It is possible that the anti-phosphotyrosine method for determining PI 3-kinase activation cannot detect activated PI 3-kinase at low levels of stimulation because the associating substrate proteins are not as heavily tyrosine-phosphorylated. With this possibility, the
correlation between irreversible aggregation and a jump in anti-phosphotyrosine immunoprecipitable PI 3-kinase activity is only coincidental. Another explanatory mechanism may be that association of heterodimeric PI 3-kinase with tyrosine-phosphorylated substrates is not as important as G-protein mediated activation of other isoforms (p110γ, for example) at low levels of agonist. The concentration-dependence curves for PIP3 and phosphotyrosine-associated PI 3-kinase activity (Figures 2 and 8) share a common property in that both parameters are elevated dramatically in irreversibly aggregating platelets. One possible interpretation, as suggested above, is that G-proteins largely mediate activation of PI 3-kinase at low concentrations of agonist via a direct receptor-associated mechanism. At high activation states resulting in engagement, clustering and cross-linking of the fibrinogen receptor (leading to irreversible aggregation) an indirect protein-tyrosine kinase dependent mechanism may predominate for generation of PIP3.

Thrombin stimulation of platelets also results in an increase in PI 3-kinase activity associated with tyrosine-phosphorylated proteins (Gutkind et al., 1990; Mitchell et al., 1990). Consistent with increases in activity, levels of the p85 subunit of PI 3-kinase are elevated in anti-phosphotyrosine immunoprecipitates from thrombin and Con A stimulated platelets in both Triton X-100 soluble and insoluble (cytoskeletal) fractions (Guinebault et al., 1995; Guinebault et al., 1993; Torti et al., 1995). The time courses observed are similar to our data with PAF. Similar to our results for dependence of phosphotyrosine-associated PI 3-kinase activity on attainment of irreversible aggregation is a study by Guinebault et al. (1995) showing that relocation of p85 to the cytoskeleton was measurable only when aggregation was 25% of maximum. They did not, however equate relocation of PI 3-kinase with reversible/irreversible aggregation.
B. Effect of PI 3-kinase Inhibitors on Platelet Responses to PAF

The tools available to study the role of PI 3-kinase (or other signaling pathways) in platelets are limited compared to nucleated cells. The methods of cDNA transfection or protein microinjection which are currently used to complement inhibitors in the study of cellular PI 3-kinase function are not feasible in platelets. Despite these limitations, the use of two potent PI 3-kinase inhibitors, wortmannin and LY294002 provides a powerful approach to analyze the role of PI 3-kinase in platelet function. These two inhibitors act via completely different mechanisms (Nakanishi et al., 1995; Vlahos et al., 1994). Wortmannin is an irreversible inhibitor forming a covalent adduct with the catalytic (p110) subunit. LY294002 (a derivative of the flavonoid, quercetin) on the other hand is a reversible, competitive inhibitor of the kinase ATP-binding site. The likelihood that these inhibitors would elicit the same physiological effect on platelet function via a target other than PI 3-kinase is extremely remote. The need for simultaneous use of these inhibitors is highlighted by a recent report showing that wortmannin inhibits PLA$_2$ with an IC$_{50}$ similar to that for PI 3-kinase (Cross et al., 1995). Use of these inhibitors also allows one to address the role of PI 3-kinase in platelets stimulated with low concentrations of PAF where the biochemical detection of enzyme activity is not as reliable (see previous sections). Our laboratory has recently provided convincing evidence that wortmannin is inhibiting a target other than PI 3-kinase upstream of MAP kinase in cytokine-stimulated cells (Scheid and Duronio, 1996).

Prior to evaluating the effect of these inhibitors on platelet function it was necessary to show that wortmannin and LY-294002 were indeed inhibitors of PI 3-kinase in rabbit platelets; these data not being available in the literature. We expected the compounds to inhibit rabbit platelet PI 3-kinase with potencies similar to those noted for other mammalian PI 3-kinases due to the high degree of isoform homology between mammalian species (Zvelebil et al., 1996). The inhibitory potencies of wortmannin and
LY-294002 against rabbit platelet PI 3-kinase were determined both in vitro and in vivo. Recently it was shown that the different isoforms of PI 3-kinase vary in their sensitivity to wortmannin (Carpenter and Cantley, 1996). Therefore, it is especially important to quantitate the potency of these inhibitors in vivo (i.e. against PAF-induced PIP₃ formation) since this measures the overall (net) inhibition of 3-PPi's generated by all isoforms of PI 3-kinase activated by PAF in platelets.

B.1. Validation of Wortmannin and LY294002 as Inhibitors of Platelet PI 3-kinase

The in vitro IC₅₀'s we found for wortmannin and LY294002 inhibition of immunopurified platelet PI 3-kinase (5 nM and 1.5 μM respectively) agree with those found previously for the heterodimeric (p85/pl10) form of PI 3-kinase in platelets and mammalian cells in general (Norman et al., 1996; Vlahos et al., 1994; Thomason et al., 1994; Nakanishi et al., 1995). Near complete inhibition was observed at 100 nM and 30-50 μM respectively.

As wortmannin is an irreversible covalent inhibitor of PI 3-kinase the inhibitory effect is still preserved in anti-p85 immunoprecipitates from wortmannin-incubated platelets that have been thoroughly washed prior to lysis (Figure 9). Theoretically, persistent inhibition in immunoprecipitates is proportional to the actual inhibition of 3-PPi formation in platelets at the time of stimulation. The IC₅₀ we observed (15 nM) and the fact that complete inhibition of PI 3-kinase in the immunoprecipitates is never seen is similar to observations by others (Gao et al., 1996; Jackson et al., 1996; Yano et al., 1993; Barker et al., 1995). Perhaps PI 3-kinase(s) are compartmentalized in the cell such that a certain fraction is not accessible to a cell permeable inhibitor such as wortmannin. This explanation is supported by our observation that complete inhibition (left-shift of concentration-response curve) is achieved when platelets are lysed without prior removal of wortmannin from the medium (Figure 11). In addition, this effect indicates that there is
still active wortmannin remaining in the Tyrode’s medium after 10 minutes incubation with platelets at 37 °C.

Both wortmannin and LY294002 potently inhibited PAF-stimulated PI 3-kinase activity in whole platelets (Figure 12). The IC_{50}’s we obtained for wortmannin and LY294002 (< 10 nM and 5 μM respectively) inhibition of PAF-stimulated PIP₃ in platelets are in good agreement with data obtained using thrombin receptor directed agonist peptide (TRAP), lyso-phosphatidic acid or β-PMA (Toker et al., 1995; Zhang et al., 1995; Kovacsovics et al., 1995; Zhang and Rittenhouse, 1995). In addition, the IC_{50}’s we found for inhibition of PAF-generated PIP₃ are similar to those found for the heterodimeric PI 3-kinase in platelets as opposed to the γ-isoform which is less sensitive to wortmannin (Zhang et al., 1996). This could imply that in PAF-stimulated platelets, activation of the heterodimeric isoform of PI 3-kinase is the major route for PIP₃ generation. These data confirm that both wortmannin and LY-294002 inhibit PAF-stimulated PI 3-kinase activity in rabbit platelets at concentrations reported to have little effect on other signalling pathways. Therefore, these compounds can be used in combination to effectively probe the dependence of various responses on PI 3-kinase in PAF-stimulated platelets.

We characterized the stability of wortmannin in physiological media (RPMI 1640 + 20 mM Hepes pH 7.4) in comparison to a tris buffer of the same pH or distilled water (Figure 13). These studies were prompted by previous reports that indicated that wortmannin is hydrolyzed in aqueous media to a metabolite which is a much less potent inhibitor of PI 3-kinase (Woscholski et al., 1994). An analogue of wortmannin, demethoxyviridin, is also unstable in aqueous solution (Woscholski et al., 1994). The derived half-life of wortmannin in the test medium would then dictate the sequence and duration that this drug is added to platelets or cells. Unexpectedly the degradation of wortmannin was not due to the presence of 10% fetal calf serum thereby implicating some component of the RPMI nutrient media. This finding shows that for long incubations of
this inhibitor with cells it is necessary to perform repeated additions at regular intervals to maintain a steady-state concentration. Due to the rapid permeability of platelets to wortmannin (Figure 10) and the short incubation times used in these studies repetitive additions of the drug were not necessary.

B.2. Aggregation

Agonist-stimulated aggregation in washed platelets is a complex phenomenon requiring millimolar concentrations of Ca$^{2+}$ in the external medium, stirring conditions and sustained activation of the fibrinogen receptor (GP IIbIIIa) to allow interplatelet bridging of the adhesive ligand, fibrinogen (Calvete, 1995). Aggregation is a fundamental platelet response and is necessary for haemostasis and the initiation of blood clotting (Siess, 1989). We used the PI 3-kinase inhibitors LY294002 and wortmannin to probe the dependence of the aggregation response on the PI 3-kinase pathway.

Both agents inhibited PAF-induced aggregation in a dose-dependent manner but the potency depended on the concentration of PAF used for stimulation which correlated with the activation state of the platelets (Figures 14 & 15). The IC$_{50}$'s for inhibition of reversible aggregation (only a primary phase observed) by wortmannin and LY-294002 were < 100 nM and 5-10 µM, respectively. Examination of these data (Figures 14 and 15) shows that substantial (greater than 20 %) inhibition of reversible aggregation occurs at 10-20 nM wortmannin and 2-5 µM LY294002. On the other hand, when the PAF concentration was increased resulting in irreversible aggregation (primary + secondary phase) the IC$_{50}$'s for inhibition were dramatically higher; 5 µM and 50-100 µM respectively and the inhibition was less complete. The inhibitory effects noted at high concentrations of these compounds is almost certainly due to non-specific inhibition of other targets which contribute to the aggregation response in addition to PI 3-kinase. Other enzymes that are important in platelet function and known to be inhibited by higher
concentrations of wortmannin include myosin light chain kinase (MLCK), phospholipase D, phospholipase A₂, phospholipase C and a PI 4-kinase (Nakanishi et al., 1992; Bonser et al., 1991; Cross et al., 1995; Nakanishi et al., 1995).

Under these conditions the IC₅₀'s for inhibition of PAF-stimulated PIP₃ formation were approximately 10 nM and 5 μM for wortmannin and LY-294002, independent of the concentration of PAF used (see above). Thus, for platelet activation states resulting in reversible aggregation, inhibition of aggregation and PI 3-kinase activity are highly correlated while they are not at high states of activation (irreversible aggregation). Since inhibition of PIP₃ formation by these inhibitors is independent of PAF concentration (data not shown) the trivial explanation that the results (PAF dependency of inhibition) are due to incomplete blockade of PI 3-kinase at high agonist concentrations, is unlikely.

These data suggest that PI 3-kinase plays an important role in processes leading to reversible (primary) aggregation but not irreversible (secondary) aggregation. Perhaps other signalling pathways resistant to these PI 3-kinase inhibitors play a greater role at high PAF concentrations. An alternative explanation is that high (above threshold) concentrations of PAF leads to dense-granule secretion (i.e. release of ADP) and/or generation of arachidonic acid metabolites such as TXA₂, both of which are platelet agonists. These compounds could then act on the same (autocrine) or neighbouring platelets to activate signaling pathways necessary for irreversible (secondary phase) aggregation but not involving PI 3-kinase. Our observation that both inhibitors invoke the same effect on reversible aggregation despite having different mechanisms for blocking PI 3-kinase greatly increases the probability that the effects seen are due to blockade of PI 3-kinase as opposed to non-specific inhibition of other pathways. These results also implicate the phosphoinositide 3-kinase pathway in events leading to initial but not sustained activation of the fibrinogen receptor.

The majority of work using inhibitors to uncover platelet responses dependent on PI 3-kinase have utilized wortmannin. Interpretation of early studies (pre-dating its
identification as a PI 3-kinase inhibitor) is complicated by the use of very high concentrations of this inhibitor (i.e. 3-6 μM) that are known to inhibit other important enzymes such as myosin light chain kinase, phospholipase A₂, phospholipase D or even a phosphatidylinositol 4-kinase (Nakanishi et al., 1992; Bonser et al., 1991; Cross et al., 1995; Nakanishi et al., 1995). In human platelets it was found that ADP-induced aggregation of platelet-rich plasma or washed platelets was potently inhibited by 3-6 μM wortmannin even in the presence of U46619, a TXA₂ analogue (Hashimoto et al., 1994). This suggests that the point of blockade (PI 3-kinase or alternative pathway) is downstream of TXA₂ receptor interaction (which is necessary for ADP-induced irreversible aggregation). Interestingly, no effect of high-dose wortmannin on ADP-induced shape change (including spheration, pseudopod formation or granule centralization) was observed, effectively ruling out PI 3-kinase in these responses. Several studies, however, have indicated a role for PI 3-kinase in platelet aggregation using wortmannin at more relevant concentrations. Wortmannin inhibits 100 nM PMA and 5 μM l-lysophosphatidic acid induced platelet aggregation with IC₅₀'s of 10 nM and approximately 5 nM respectively (Yatomi et al., 1992; Zhang and Rittenhouse, 1995). Also, wortmannin inhibits (IC₅₀ between 9 & 90 nM) aggregation induced by the combination of serotonin and epinephrine (Shah and Saeed, 1995). These results parallel the ability of wortmannin to inhibit PAF-induced platelet aggregation. In the most extensive work to date, wortmannin and LY294002 were shown to inhibit platelet aggregation induced by 25 μM TRAP with IC₅₀'s of 50 nM and 25 μM respectively (Kovacsovics et al., 1995). These investigators concluded from other experiments that activation of PI 3-kinase is necessary for prolonged maintenance of the platelet integrin GPIIb/IIIa in an activated state and for irreversible aggregation possibly through PI 3-kinase stimulation downstream of fibrinogen receptor engagement. This contrasts with our data that suggests that PI 3-kinase may have a role in initial activation of the fibrinogen receptor. The dependence of the inhibitory effect of these compounds on the
concentration of agonist used to stimulate the platelets has not been previously reported in the literature.

B.3. Secretion

Platelet agonists such as PAF and thrombin induce secretion from several types of granules which serve partly to induce and amplify secondary (irreversible) aggregation by autocrine action of the secreted substances (e.g. ADP and TXA$_2$). Our data on the effects of PI 3-kinase inhibition on PAF-induced platelet dense granule release support a minor role for PI 3-kinase in this important platelet response at low activation states. The IC$_{50}$'s for inhibition of PAF-induced secretion from platelet dense-granules even at low levels of PAF (i.e. 0.5 nM) are much higher (see above) than the corresponding IC$_{50}$'s for inhibition of PI 3-kinase under the same conditions. The IC$_{50}$ for wortmannin inhibition of secretion was approximately 3 μM, and for LY-294002 it was greater than 50 μM, at PAF concentrations giving 22 % and 12 % of maximal release respectively (Figures 16 & 17). Inhibition of dense-granule secretion observed at these high concentrations of inhibitors is likely due to non-specific inhibition of multiple targets, other than PI 3-kinase, involved in the secretion response. However an interesting aspect of the inhibition curves is that up to 30 % of the total release of serotonin could be blocked at low concentrations of the inhibitors (less than 100 nM wortmannin or less than 10 μM LY294002) (Figures 16 & 17). This suggests that a fraction of the PAF-induced secretion from dense-granules (about 30%) depends on PI 3-kinase activation in platelets. Similar to the results of aggregation studies shown above, secretion from platelets treated with higher concentrations of PAF was not inhibited by blockade of PI 3-kinase, except at concentrations much higher than those required for PI 3-kinase inhibition. Also, the PI 3-kinase inhibitors do not simply slow the release reaction (Figure 18).
Our results agree with those of Yatomi et al. (1992) who showed a dose-dependent inhibition of secretion by wortmannin (IC$_{50}$ = 50-100 nM) when platelets were stimulated with low levels of thrombin, collagen, STA$_2$ (thromboxane A$_2$ analogue), PMA or PMA + ionomycin. The only other study examining the effects of wortmannin on platelet dense-granule secretion found no inhibition whatsoever, probably attributable to use of high (above threshold) concentrations of TRAP (Kovacsovics et al., 1995).

C. Co-immunoprecipitation of PI 3-kinase with Tyrosine-phosphorylated Proteins in Activated Platelets

Anti-phosphotyrosine immunoprecipitates from PAF-stimulated platelets showed increased levels of the p85 subunit of PI 3-kinase with the increase in protein paralleling the increase in associated PI 3-kinase activity (Figure 19). However, at later times the level of p85 subunit was maintained whereas the activity was decreased to near basal levels by 10 minutes (Figure 5). A possible explanation for this difference is down-regulation of the lipid kinase activity by the endogenous serine/threonine kinase activity of the catalytic subunit. Also, it is possible that at later times an associated 3-phosphatase activity is increased. The increase in PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates is probably due to association of p85 or p110 subunits with tyrosine-phosphorylated substrates since the p85 subunit is not tyrosine-phosphorylated in PAF-stimulated platelets (Figure 20). In agreement with our findings, strong platelet agonists generally do not induce tyrosine phosphorylation of the p85 subunit (Guinebault et al., 1995; Torti et al., 1995; Yatomi et al., 1994). The only exception noted in the literature is a recent one involving thrombopoietin stimulation of human platelets (Chen et al., 1995). This strongly implies that for many other platelet agonists in addition to PAF, PI 3-kinase inducibly associates with tyrosine-phosphorylated proteins serving perhaps as a major mechanism for activation of heterodimeric PI 3-kinase in platelets.
The major tyrosine-phosphorylated substrate increased in anti-p85 or anti-p110 immunoprecipitates from PAF-stimulated rabbit platelets has an apparent molecular weight of 115 kDa which is referred to here as p115 (Figures 20 and Figure 22). A substrate with the same mobility on SDS-PAGE gels was also present in anti-PI 3-kinase immunoprecipitates from thrombin-stimulated rabbit platelets (Figure 23). This association is specific for PI 3-kinase as rabbit pre-immune sera did not precipitate a tyrosine-phosphorylated protein of similar molecular weight from PAF-stimulated platelets (Figure 21). p115 co-immunoprecipitates with PI 3-kinase with the same kinetics as the increase in PI 3-kinase protein or PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates. Therefore it is at least possible that association of tyrosine-phosphorylated p115 with PI 3-kinase is involved in activation of this isoform of PI 3-kinase in PAF-stimulated platelets. A band corresponding to p115 could be specifically depleted in blots of platelet whole lysates following pre-clearing of the lysates with polyclonal antibody to the p85 subunit (Figure 24). In thrombin-stimulated human platelets anti-p85 immunoprecipitates contained a tyrosine-phosphorylated substrate of slighter faster mobility corresponding to a molecular weight of 110 kDa (p110) (Figure 25).

At least two possible mechanisms could account for the PAF-induced co-immunoprecipitation of tyrosine-phosphorylated p115 with PI 3-kinase in platelets. In one model, p115 is not associated with PI 3-kinase in unstimulated platelets. On PAF stimulation protein-tyrosine kinases are activated and phosphorylate p115 on tyrosine residues in sequences that allow binding to the SH2 domains of PI 3-kinase. Tyrosine-phosphorylated p115 then associates (and perhaps activates) with PI 3-kinase. In another model p115 is always (constitutively) associated with PI 3-kinase but is inducibly phosphorylated on tyrosine residues when platelets are stimulated with PAF. We have conducted preliminary experiments involving anti-p85 immunoprecipitation of platelet lysates followed by 2-dimensional SDS-PAGE and silver staining which suggest that p115
is not constitutively associated with PI 3-kinase (data not shown). These data do not preclude other mechanisms for association of tyrosine-phosphorylated p115 with PI 3-kinase including the involvement of adapter or intervening proteins (indirect association) and SH3-polyproline interactions for example.

We attempted to identify p115 using antibodies against a number of potential tyrosine-phosphorylated proteins having a similar molecular weight. In addition to being tyrosine-phosphorylated and in the correct molecular weight range these candidate proteins have been shown to associate with PI 3-kinase in other systems or platelets. For example, tyrosine-phosphorylated ras-GAP has been found to co-immunoprecipitate with PI 3-kinase in IGF-1 stimulated rat HTC cells and PDGF stimulated CHO cells (Sanchez-Margalet et al., 1995; Kaplan et al., 1990) JAK-2 forms a complex with PI 3-kinase and other signalling proteins in IL-11-stimulated hematopoietic cells (Fuhrer and Yang, 1996). Focal adhesion kinase (FAK) has been found to associate with PI 3-kinase in thrombin stimulated human platelets, polyoma middle-T transformed fibroblasts and PDGF stimulated fibroblasts (Chen and Guan, 1994; Chen and Guan, 1994; Bachelot et al., 1996; Chen et al., 1996). In activated lymphoid cells (A20 B-cells or Jurkat T-cells), the most prominent tyrosine-phosphorylated substrate associated with PI 3-kinase has been identified as the product of the \( c-cbl \) oncogene, p120\(^{c-cbl} \) (Hartley et al., 1995; Meisner et al., 1995). Our immunoblotting experiments ruled out the possibility that p115 is the 110 kDa subunit of PI 3-kinase, p120 ras-GAP, JAK-2, cbl, FAK or integrin \( \beta_1 \).

Results of preliminary experiments show that p115 co-immunoprecipitates with PI 3-kinase only in the cytosol and detergent soluble fractions of activated platelets. This suggests that PI 3-kinase which is resident or translocated to the membrane or the cytoskeleton in PAF-stimulated platelets does not associate with tyrosine-phosphorylated p115.

An association of PI 3-kinase with prominent tyrosine-phosphorylated substrates in this molecular weight range has not previously been reported in platelets. Yatomi et al.
(1994) reported no bands in this region from anti-p85 immunoprecipitates of thrombin-stimulated human platelets. However, they also found no increase in PI 3-kinase protein levels (p85 subunit) in anti-phosphotyrosine immunoprecipitates from thrombin-stimulated platelets that is often reported and accompanies the increase in PI 3-kinase activity. Another group also reported no detectable association of tyrosine-phosphorylated proteins with PI 3-kinase in Con A-stimulated human platelets, yet showed that p85 subunit was increased in anti-phosphotyrosine immunoprecipitates (Torti et al., 1995). Examination of anti-p85 immunoprecipitates in the cytoskeletal fraction of thrombin-stimulated human platelets found no such associated proteins (Guinebault et al., 1995). It is possible that the absence of similar bands seen by others is due to lower sensitivity of detection. As shown in Figure 20 there are other tyrosine-phosphorylated proteins that co-immunoprecipitate with PI 3-kinase in PAF-stimulated platelets although they are minor (either in protein level or degree of phosphorylation) in comparison to p115. \( pp72^{syk} \) is tyrosine-phosphorylated and associates with PI 3-kinase in thrombin-stimulated porcine platelets (Yanagi et al., 1994). Also, the non-receptor tyrosine-kinases, \( src, lyn, fyn \) are tyrosine-phosphorylated and associate with PI 3-kinase in thrombin-stimulated human platelets (Gutkind et al., 1990). Perhaps the p115:PI 3-kinase association is specific to or much more prominent in rabbit platelets than for other species or alternatively, specific for PAF signaling. The latter possibility however, seems unlikely, as thrombin also induces co-immunoprecipitation of a p115 tyrosine-phosphorylated protein with PI 3-kinase in rabbit platelets (see above). This suggests that the thrombin receptor and PAF receptor share a common tyrosine-kinase mediated pathway leading to phosphorylation and association of p115 with PI 3-kinase.

Another candidate for this protein is the recently identified Gab1 (Grb2-associated binder-1) protein which is tyrosine-phosphorylated and associates with the PI 3-kinase p85 subunit in EGF-stimulated A431 cells (Weidner et al., 1996; Holgadomadruga et al., 1996). This protein bears significant sequence and domain homology to IRS-1 and may
serve as an adapter between certain receptor tyrosine-kinases such as the EGF or the c-Met receptor and downstream pathways. This possibility will be investigated as soon as antibodies to Gab1 can be obtained. Efforts are also currently underway to purify and sequence platelet p115.

D. Mechanisms leading to Activation of Platelet PI 3-kinase following Ligation of the PAF Receptor

PI 3-kinase is only one of the signaling pathways activated in platelets following binding of PAF to its membrane receptor. As is the case for other pathways activated downstream of the PAF receptor it is likely that several mechanisms exist for activation of the various isoforms of PI 3-kinase in platelets. These include but are not limited to G-proteins (heterotrimeric or low-molecular weight), tyrosine-kinase and cytoskeletal mediated processes. It is also likely that the contribution of these various pathways to the level of 3-PPI's in the platelet at various times after agonist addition changes. The above-mentioned processes could be classified as direct (membrane proximal) mechanisms for PAF receptor activation of platelet PI 3-kinase that may be common to the majority of nucleated cells containing these receptors. In addition, the unique structural and physiologic characteristics of the platelet and its specialized functions may provide unique pathways for activation of PI 3-kinase. In particular, activation of PI 3-kinase following engagement and/or clustering of the integrin $\alpha_{IIb}\beta_3$ by fibrinogen, which is required for aggregation, occurs for other platelet agonists (usually detected as PI (3,4)P$_2$ formation) such as thrombin and is likely to occur with PAF as well. The latter mechanism may be classified as indirect and could also involve protein-tyrosine kinases or G-proteins.

Analyzing the kinetics of PI 3-kinase activation could be used to differentiate between the direct and indirect mechanisms but this is difficult in platelets due to the extremely rapid rate of biochemical and functional changes. Despite these limitations our
data for activation of PI 3-kinase in PAF-stimulated platelets support a tyrosine-kinase based mechanism that may occur through direct or indirect pathways; at the same time these data do not rule out the involvement of other possibilities such as G-proteins. The kinetics for PIP\(_3\) formation and increases in PI 3-kinase activity associated with tyrosine-phosphorylated proteins in PAF-stimulated platelets are very similar and both correlate with the aggregation response as measured by turbidometry (Figure 6). The rapid association of a major tyrosine-phosphorylated protein (p115) with PI 3-kinase in rabbit platelets also supports a tyrosine-kinase based model. A large fraction of this tyrosine-kinase regulated PI 3-kinase activity may be triggered by or be dependent upon sustained engagement/crosslinking of the fibrinogen receptor. This is suggested from the concentration-dependence for both PIP\(_3\) generation and phosphotyrosine-associated PI 3-kinase activity where there is a high correlation between attainment of irreversible aggregation and PI 3-kinase activation (see Figure 2 and Figure 7).

The dependence of PI 3-kinase activation in PAF-stimulated platelets on engagement of the fibrinogen receptor by its adhesive ligand fibrinogen could be tested experimentally. Use of the peptide RGDS and/or exclusion of Ca\(^{2+}\) from the medium has been shown to specifically block fibrinogen binding to its receptor and inhibit aggregation in thrombin-stimulated platelets (Sultan et al., 1991). In these studies it was also shown that total tyrosine phosphorylation and activation of PI 3-kinase (as production of PI (3,4)P\(_2\)) were simultaneously blocked. There is additional evidence in the literature supporting a crucial role for fibrinogen binding in 'late-phase' activation of PI 3-kinase. Platelets from patients with Glanzmann's thrombasthenia (absent fibrinogen receptor) fail to aggregate and form PI (3,4)P\(_2\) in response to thrombin (Sultan et al., 1991). In addition antibodies are available which specifically block fibrinogen binding to its receptor. These studies would complement the data reported here which are correlative in nature.

At low concentrations of PAF giving reversible aggregation, the data suggest a more prominent involvement of G-protein mediated pathways in direct stimulation of
PIP₃ production. The potency for inhibition of PIP₃ generation in PAF-stimulated platelets described in this work (IC₅₀=10 nM) implies that this lipid is generated predominantly by the tyrosine-kinase regulated, heterodimeric (p85/π10) PI 3-kinase rather than the G-protein regulated isoforms (Zhang et al. 1996).

Since the integrin α₃β₃ does not possess intrinsic tyrosine-kinase activity, engagement by fibrinogen must lead to activation of protein-tyrosine kinases indirectly. This could occur by direct or indirect association of the cytosolic domain of the fibrinogen receptor with proteins such as FAK or src-family kinases in focal contacts, for example. These tyrosine-kinases would then phosphorylate p115 or another tyrosine kinase ultimately leading to p115 phosphorylation followed by its association with and activation of PI 3-kinase.

E. A Model for the Involvement of PI 3-kinase in Platelet Activation Leading to Reversible Aggregation

Results of the experiments reported here suggest that activation of PI 3-kinase in PAF-stimulated platelets contributes to reversible but not irreversible aggregation. At low concentrations of PAF resulting in only a primary phase (or reversible) aggregation, PI 3-kinase (detected as formation of PLP₃) is modestly activated. This may be through a direct mechanism involving coupling of the PAF receptor to PI 3-kinase through G-proteins. Blocking activation of the 'directly-activated' PI 3-kinase using low concentrations of wortmannin or LY294002 impairs reversible aggregation of platelets. This suggests, at least for PAF, that activation of PI 3-kinase is important for (or contributes to) exposure of the fibrinogen receptor via an ‘inside-out’ mechanism to allow binding of the adhesive ligand, fibrinogen. This possibility could be tested directly by assaying for activation of the fibrinogen receptor; using antibodies directed at ‘activation-dependent’ epitopes in the extracellular domain, for example (Gao and Shattil, 1995). PIP₃ may directly alter the
membrane microenvironment surrounding the cytoplasmic domains of the integrin subunits resulting in a conformational change in the extracellular domains to allow binding of fibrinogen. PIP₃ could also act on the integrin indirectly perhaps via activation of PKC (Ca²⁺ and DAG independent isoforms) and phosphorylation of pleckstrin (p47) for example. This would allow for a PLC-independent pathway for activation of the fibrinogen receptor at low agonist levels through PI 3-kinase.

At high concentrations of PAF, especially those resulting in irreversible aggregation of platelets (secondary phase), PI 3-kinase is extensively activated resulting in much higher levels of PIP₃ production. This could arise through an 'indirect' mechanism involving distinct isoforms of PI 3-kinase (heterodimeric, for example) activated by protein-tyrosine kinases downstream of clustering or engagement of the fibrinogen receptor and irreversible aggregation. In PAF-stimulated rabbit platelets, association of tyrosine-phosphorylated p115 with PI 3-kinase may be the major mechanism for the indirect, 'outside-in' activation of PI 3-kinase. Paradoxically, this 'second-wave' of PI 3-kinase activation may not be functionally important in the aggregation response as blockade by wortmannin or LY294002 has little effect. Other pathways, such as PLC for example, which are not sensitive to these inhibitors, may supplant or replace PI 3-kinase at high agonist levels as the functionally relevant pathway in the aggregation response. The alternative pathways may be either direct or indirect based on engagement of the fibrinogen receptor or through release and autocrine action of mediators such as ADP or TXA₂. A cartoon illustrating the involvement of PI 3-kinase in reversible aggregation induced by PAF is shown in Figure 26.

F. Recommendations for Future Work

There are many possibilities for future research in this area. With regard to the role of PI 3-kinase in cellular adhesive interactions, is PI 3-kinase important for
aggregation in response to all agonists and how universal is it amongst mammalian species? Does PI 3-kinase contribute to activation of other integrin receptors besides $\alpha_{\text{mb}}\beta_3$? Is the role of PI 3-kinase in integrin function confined to platelets or can it be extended to other cell types? An important task would be to elucidate the mechanism for PI 3-kinase activation of integrin $\alpha_{\text{mb}}\beta_3$. Do the lipid products or the protein kinase activity of PI 3-kinase regulate the cytoplasmic domains of integrins? The recent availability of synthetic 3-PPF's could allow one to address this problem. Are these interactions direct or via intermediate pathways such as protein kinase C or other possible targets of PI 3-kinase? Does PI 3-kinase regulate aggregation by mechanisms other than those involving integrins? Does PI 3-kinase play a functional role in platelets other than aggregation? Of great interest would be the functional impairment, if any, in platelets from PI 3-kinase 'knock-out' animals.

The anti-aggregatory effects of PI 3-kinase inhibitors reported in this work suggest that they may have some clinical utility as anti-platelet drugs. Indeed, work is in progress at several pharmaceutical companies employing wortmannin or LY294002 as lead compounds for drug development. Specific issues of course would be specificity and toxicity. There is also current interest in small and large molecule inhibitors of integrin $\alpha_{\text{mb}}\beta_3$ that exert their effects on the extracellular domains by blocking ligand binding and show promise as anti-thrombotic agents.

Identification and characterization of p115 as a possible novel regulator of heterodimeric PI 3-kinase in platelets is currently an area of major interest in our laboratory.
Figure 26. A Proposed Model for the Involvement of PI 3-kinase in Reversible Platelet Aggregation Induced by PAF
CONCLUSIONS

These studies have shown, for the first time, that platelet-activating factor (PAF) stimulates the formation of 3-phosphoinositides (and therefore activates phosphoinositide 3-kinase) in rabbit platelets. The pattern of formation of PIP3 and PI 3,4P2 in platelets exposed to PAF was similar to that noted for the classical platelet agonist, thrombin, in human platelets. The formation of PIP3 but not PI 3,4P2 was closely correlated with the kinetics of platelet aggregation indicating a tight coupling between these events. PI 3-kinase activity associated with tyrosine-phosphorylated proteins was increased in PAF-stimulated platelets with similar kinetics to PIP3 generation and aggregation. This supports the possibility that a significant proportion of PI 3-kinase activation in platelets stimulated with high levels of PAF is regulated by protein-tyrosine kinases. The results of concentration-response studies also support a complex mechanism for activation of PI 3-kinase with G-proteins possibly being the major regulator of PIP3 production at low PAF concentrations. Aggregation or engagement of adhesion receptors may be upstream of the association of PI 3-kinase activity with tyrosine-phosphorylated proteins perhaps reflecting the necessity for these events for tyrosine-phosphorylation of the major PI 3-kinase associating protein (p115) in PAF-stimulated platelets.

In PAF-stimulated platelets PI 3-kinase is not a target of protein-tyrosine kinases as we could not detect tyrosine phosphorylation of either subunit.

We confirm that PI 3-kinase immunoprecipitated from rabbit platelets with polyclonal antiserum to the p85α subunit is inhibited by wortmannin and LY294002 with potencies similar to that reported for other mammalian PI 3-kinases. Both LY294002 and wortmannin inhibited PAF-induced PIP3 formation in whole platelets with potencies suggesting that activation of the heterodimeric p85/p110 prevails over the PI 3-kinase γ isoform in rabbit platelets at high levels of agonist.
Inhibition of PAF-induced aggregation by these PI 3-kinase inhibitors depends on the activation state of the platelets. Platelets stimulated with high levels of PAF are largely insensitive to the effects of wortmannin and LY294002. On the other hand, platelets exhibiting reversible or primary aggregation at lower PAF concentrations are inhibited at concentrations that correlate with inhibition of PIP\textsubscript{3} generation. Thus, we conclude that PI 3-kinase plays an important role in the primary phase of or reversible aggregation induced by PAF. At higher activation states resulting in irreversible (secondary) aggregation, other wortmannin and LY294002 insensitive pathways may play an increasingly important role. Alternatively, irreversible aggregation induced by dense-granule release or arachidonic acid metabolites acting in an autocrine fashion involves other pathways not sensitive to PI 3-kinase inhibitors (independent of PI 3-kinase).

We found dense-granule (serotonin) release at high PAF concentrations to be insensitive to PI 3-kinase inhibitors. At low activation states resulting in 12-20% release, this response is partially inhibited by PI 3-kinase inhibitors. About one-third of the total release under these conditions can be blocked by wortmannin or LY294002 at concentrations consistent with specific inhibition of PI 3-kinase. A possible explanation is that a subset of the platelet dense granules depend on PI 3-kinase activation for release.

We also report the co-immunoprecipitation of a previously unreported 115 kDa tyrosine-phosphorylated protein with PI 3-kinase in PAF-stimulated platelets. This protein co-immunoprecipitates with PI 3-kinase in close correlation with the increase in PI 3-kinase activity/protein levels in anti-phosphotyrosine immunoprecipitates. We could not identify this protein by immunoreactivity to antibodies directed against a number of tyrosine-phosphorylated signalling proteins in this molecular weight range. We propose that tyrosine-phosphorylated p115 activates heterodimeric PI 3-kinase in PAF stimulated platelets by possibly serving as an adapter or docking protein to enable recruitment of the enzyme to the plasma membrane.
To conclude, this work has revealed that PI 3-kinase promotes the first or primary phase of aggregation in PAF-stimulated rabbit platelets and plays a minor role in dense-granule secretion. Therefore the PI 3-kinase pathway plays an active role in platelet responses to agonists such as PAF in addition to other established pathways.
REFERENCES


Feinstein, M.B., Egan, J.J., Shaafi, R.I., and et.al (1983). The cytoplasmic concentration of free calcium in platelets is controlled by stimulators of cyclic AMP production (PGD2 ,PGE1, forskolin). Biochemical Biophysical Research Communications 113, 598


Torti, M., Ramaschi, G., Montsarrat, N., Sinigaglia, F., Balduini, C., Plantavid, M.,
Breton, M., Chap, H., and Mauco, G. (1995). Evidence for a glycoprotein IIb-IIIa- and
aggregation-independent mechanism of phosphatidylinositol 3',4'-bisphosphate synthesis in

An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. Nature
334, 353-356.

Traynor-Kaplan, A.E., Thompson, B.L., Harris, A.L., Taylor, P., Omann, G.M., and
Sklar, L.A. (1989). Transient increase in phosphatidylinositol 3,4-bisphosphate and
phosphatidylinositol trisphosphate during activation of human neutrophils. Journal of
Biological Chemistry 264, 15668-15673.

phospholipid platelet-activating factor by human platelets. Journal of Immunology 129,
1637-1641.

and present status of research on platelet-activating factor (PAF-acether). [Review].

activating factor: a phospholipid autacoid with diverse actions. Journal of Lipid Research
34, 691-702.

of the phosphomonoester groups of polyphosphoinositol lipids in unstimulated human


Vigon, I., Florindo, C., Fichelson, S., Guenet, J.L., Mattei, M.G., Souyri, M., Cosman,
member of the hematopoietic cytokine receptor family: molecular cloning, chromosomal
location and evidence for a function in cell growth. Oncogene 8, 2607-2615.

Vigon, I., Mormon, J.P., Cocault, L., Mitjavila, M.T., Tambourin, P., Gisselbrecht, S., and
Souyri, M. (1992). Molecular cloning and characterization of MPL, the human homolog
of the v-mpl oncogene: identification of a member of the hematopoietic growth factor
receptor superfamily. Proceedings of the National Academy of Sciences of the United
States of America 89, 5640-5644.


