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I am submitting herewith a thesis written by Xiao-Fen Wang entitled "Effect of Aggregation on Regulation of Cytosolic Phospholipase A_2 in Human Platelets." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Roger C. Carroll, Major Professor

We have read this thesis
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Associate Vice Chancellor and
Dean of The Graduate School

EFFECT OF AGGREGATION ON THE REGULATION OF CYTOSOLIC PHOSPHOLIPASE A_2 ACTIVITY IN HUMAN PLATELETS

A Thesis

Presented for the

Master of Science Degree

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Xiao-Fen Wang

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DEDICATION

This work is dedicated to my parents, Ding-Yi Wang and Fong-Bao Wang, to my husband, life friend, Qi Chao, and to my lovely son, Zhi-Xia Chao.

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ABSTRACT

Ro 44-9883, a potent and selective GPIIb/IIIa antagonist, is an antithrombotic peptidomimetic developed using the arginine-glycine-aspartate-serine (RGDS) sequence as a chemical lead compound. Inhibition of aggregation, as detected by an aggregometer, by Ro 44-9883 resulted in complete inhibition of ^{14}C-serotonin secretion. This correlated with an inhibition of thromboxane A_2 (TxA_2) formation, detected by EIA, which was induced by weak agonists such as ADP or low dose thrombin receptor agonist peptide (TRAP). The inhibition of serotonin secretion as well as TxA_2 formation was overcome by higher doses of TRAP. Ro 44-9883 had no effect on serotonin secretion in Glanzmann's thrombasthenic platelets. Inhibition of serotonin secretion was not completely reversed by the TxA_2 mimetic, U46619, or exogenously added arachidonic acid, but additionally required lysophosphatidic acid. Interestingly, Ro 44-9883 inhibition of TxA_2 formation was not due to a lack of cytosolic phospholipase A_2 ($cPLA_2$) activation as assayed in vitro. Moreover, blocking aggregation with Ro 44-9883 had no effect on phospholipase C generated IP_3, as measured by ^{3}H-IP_3 radioreceptor assay, spectrophotometrically measured cytosolic calcium flux and pH changes, diacylglycerol formation, as detected by ^{32}P-labeling of the phosphatidic acid pool and thin-layer chromatography (TLC), nor on protein kinase C and myosin kinase activities, as detected by ^{32}P-labeling, SDS-PAGE and autoradiography. However, tyrosine phosphorylation of $p125^{FAK}$ (focal adhesion kinase) in both low and high dose TRAP-stimulated platelets was blocked by Ro 44-9883 pretreatment, as observed by SDS-PAGE and western blotting. These results suggest that aggregation is specifically required for weak agonists induced

intracellular activity of $cPLA_2$, possibly by regulating phospholipid substrate availability or interaction of $cPLA_2$ with the membrane.

Our study also suggested unactivated platelets have a major portion of $cPLA_2$ associated with the membrane, which is not mediated by actin, and possibly mediated by interaction by an unknown mechanism with several membrane proteins, as evaluated by immunoprecipitation, SDS-PAGE and western blotting. Platelet activation results in $cPLA_2$ phosphorylation and a shift of $cPLA_2$ from the membrane to the cytosol with a large portion associated with the cytoskeleton since this $cPLA_2$ could be released by DNase I treatment to depolymerize actin filaments. This cytosolic/cytoskeletal fraction also contained most of the phosphorylated $cPLA_2$ with no ^{32}P-labeled $cPLA_2$ found in membrane fractions, as detected by ^{32}P-labeling, IPPT, SDS-PAGE, and autoradiography. Blocking aggregation neither affected phosphorylation of $cPLA_2$, nor the redistribution of $cPLA_2$ between the cytosol and the membrane. These results suggest that membrane protein clustering in connection with aggregation, perhaps related to tyrosine phosphorylation of $p125^{FAK}$, is required for the phospholipid substrates of $cPLA_2$ to be exposed. Additionally, these results suggest a more complex regulation of $cPLA_2$ than previously proposed.

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PART I

INTRODUCTION

Blood platelets are anucleate cell fragments derived from megakaryocytes and play an important role in hemostasis and thrombosis. Platelets can adhere to exposed extracellular matrix proteins of subendothelium in an injured blood vessel wall. This adhesion causes platelets to release activating signals, which further stimulates circulating platelets to undergo adhesion, shape change, aggregation, and secretion. The formation of a hemostatic platelet plug will be reinforced by the effects of thrombin, which converts fibrinogen to fibrin. Defective function of platelets due to congenital factors can cause bleeding problems such as Bernard-Soulier Syndrome (deficient in Glycoprotein Ib, GPIb) and Glanzmann's thrombasthenia (deficient in GPIIb/IIIa) (Gerrard et al., 1988). Activated platelets not only play a role in hemostasis, but also in pathologic conditions. One of the well-known examples is that the formation of excessive thrombi on ruptured atherosclerotic plaques can block blood flow, resulting in ischemic necrosis, stroke, and myocardial infarction. (Scharf et al., 1987).

Platelet Structure and Function

Unstimulated platelets have discoid shape with a mean diameter around 5 um. When platelets are activated by agonists, they undergo shape change to spiny spheres following the formation of pseudopodia (Ware et al., 1995).

The plasma membrane of platelets is composed of a bilayer of phospholipids, which also contains cholesterol, glycolipids and glycoproteins. The prominent

phenomenon in the plasma membrane of platelets is that the phospholipids are asymmetrically distributed. The negatively charged phospholipids, including phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE), are mostly located in the inner leaflet of plasma membrane, but the others are distributed more evenly (Schick, 1994). This patter of distribution is considered to regulate coagulation since PS, translocated from the inner to the outer leaflet of the plasma membrane during platelet activation, accelerates the steps of coagulation (Walsh et al., 1994). The aggregated platelets generate thrombin from prothrombin by the prothrombinase complex composed of activated factors V and X plus negatively charged phospholipids, especially PS. The generated thrombin converts fibrinogen to fibrin which reinforces the platelet plug.

Platelets contain two internal membrane systems. The open canalicular membrane system is an important access by which external elements enter the inside of platelets and secreted contents go to the outside of activated platelets. It is also a source of membrane for the filopodia formation and spreading in platelet activation reaction (White, 1974; White, 1994). The other major membrane reticular system, the dense tubules, contains several of the enzymes for arachidonic acid metabolism. These are prostaglandin endoperoxide synthetase, thromboxane synthetase, and diglyceride lipase. It is also a storage site of calcium, and releases calcium to regulate cytoplasmic calcium concentration and the function of several calcium-dependent proteins (Gerrard, 1988).

The most outer layer of the platelets is glycocalyx, which extends from the trilaminar plasma membrane. The glycocalyx is comprised of the complex carbohydrate

residues of several glycoproteins (GP) which are important components of platelet membrane. Glycoprotein receptors, such as GPIIb/IIIa, GPIb/IX, and GPIa/IIa, are the binding sites for adhesion molecules, which are involved in platelet activation, adhesion and aggregation (White, 1994).

CD9 is a transmembrane glycoprotein and its function in platelets is not clearly understood. β2-microglobulin (β2-m), a small invariant protein associated with MHC class I antigens on platelets, is involved in immune responses. CD32 and CD62 are also transmembrane platelet surface proteins. CD32 or Fcγ-RII is involved in immune complex binding and activation. CD62 or P-selectin exists in alpha granule membrane in resting platelets, translocates to the surface of platelets upon activation, and is involved in platelet-leukocyte adhesion (Ware et al., 1995). Some monoclonal antibodies to the CD9 and β2-m can trigger platelet activation through interaction with the Fcγ-RII (Carroll et al.,1990; Rubinstein et al., 1991).

Platelet organelles include dense bodies, alpha granules, lysosomes, peroxisomes and mitochondria. The first two organelles have prominent effects in platelet activation. The dense bodies contain adenosine diphosphate (ADP), serotonin, and calcium. Released ADP from dense granules of the activated platelets serves as a positive feedback element to recruit more platelets for the formation of aggregates at the injured vessels. Alpha granules contain platelet specific proteins, such as platelet factor 4 and β-thromboglobulin, and other adhesive glycoproteins such as fibrinogen, vWF, fibronectin, thrombospondin, and vitronectin, which are involved in platelet adhesion reaction (Ware et al., 1995). Coagulation factor V in alpha granules is a potent coagulation promoter (Bode et al.,

1985). Alpha granules also contain a group of mitogenic factors including platelet-derived growth factor, endothelial cell growth factor, epidermal growth factor, and transforming growth factor-β, which are related to various cell growth responses (Deuel et al., 1986; King et al., 1984; Rifkin et al., 1993; Ware et al., 1995)

The platelet cytoskeleton is divided into two components, membrane skeleton and cytoplasmic actin filaments. The membrane skeleton contains short actin filaments, located on the inner surface of the membrane. Actin filaments are also considered to attach to the cytoplasmic domains of transmembrane glycoproteins such as GPIb/IX, GPIa/IIa, and probably GPIIb/IIIa, through linking proteins such as actin-binding protein, talin, vinculin and spectrin. The function of the membrane skeleton is to regulate platelet shape and maintain membrane stability. The actin filaments in the cytoplasm are also involved in platelet contractile events (Fox, 1993).

The platelet cytoskeleton undergoes dynamic changes following stimulation, in which polymerization of actin monomers and cross-linking of actin filaments are both increased (Fox et al., 1984; Jennings et al., 1981), and interaction of myosin, actin, and other proteins are required for centralization of granules, extension of filopodia, and clot contraction (Gerrard, 1988). Platelet activation directly and indirectly involves reorganization of the platelet cytoskeleton, as demonstrated by several previous studies including work from this laboratory (Carroll et al., 1982). Platelet aggregation mediated by interaction of fibrinogen and GPIIb/IIIa is required to induce cytoskeleton reorganization, and linkage of the GPIIb/IIIa to the cytoskeletal core. Some of the important signal transducing enzyme were determined to partially associate with platelet

cytoskeleton such as tyrosine kinases, $pp60^{c\text{-}src}$ and $p72^{syk}$, (Pumiglia et al., 1993; Tohyama et al., 1994), low molecular weight G proteins, Rap2B and Rap 1b (Torti et al., 1994; Fischer et al., 1994), PLC, diacylglycerol kinase and several proteins with tyrosine phosphorylation dependent on platelet aggregation (Grondin et at., 1991; Zhang et al., 1992).

Platelet Adhesion and Aggregation

In achieving hemostasis and thrombosis, platelet function can be divided into two distinct processes, adhesion and aggregation.

Platelets circulating in the blood vessel usually do not adhere to the normal endothelium, but they can adhere to the exposed subendothelium of injured vessels within seconds. This adhesion consists of two phases, platelet pseudopodia formation followed by platelet spreading which closely adheres to subendothelium (Turitto et al.,1983). Collagen and von Willebrand factor (vWF) are major extracellular proteins for initiating platelet adhesion. Subendothelial vWF can come from three different sources, endothelium, plasma and platelet alpha granules (Sixma, 1994; Coller, 1985). Platelet receptors binding to these molecules are GPIa/IIa for collagen (Kunicki et al., 1988), and GPIb for vWF (Coller, 1985). Deficiency in GPIb, called Bernard-Soulier Syndrome, and in vWF, called von Willebrand's disease, shows reduced adhesion of the platelets, and thus impaired hemostasis clinically (Gerrard, 1988). Other adhesion proteins, fibronectin, vitronectin, and fibrinogen are also involved in the platelet adhesion reaction.

Platelets adherence to subendothelium leads to platelet activation. Activated platelets release ADP, serotonin, and generate TxA_2, which further amplifies the platelet activation response and recruits surrounding platelets into a growing platelet plug. Platelet aggregation, which is essential for thrombus formation, is mediated by fibrinogen binding to the functional platelet GP IIb/IIIa complex (Phillips et al., 1988). When platelets are activated, the shielded fibrinogen binding sites (GP IIb/IIIa) on platelets become exposed by an unknown mechanism. The binding of fibrinogen in the plasma to GP IIb/IIIa crosslinks and thus aggregates adjacent platelets. Patients with Glanzmann's thrombasthenia (defect of GP IIb/IIIa) show deficient aggregation of the platelets and thus impaired hemostasis (Gerrard, 1988).

GP IIb/IIIa (fibrinogen receptor; $\alpha_{IIb}\beta_3$), an integrin receptor, is essential for the aggregation reaction. It has the basic structure of integrin receptors consisting of an α subunit (IIb), and a β subunit (IIIa) which are both transmembranous proteins. GPIIb has four calmodulin-like domains which bind calcium, and both α and β subunits have ligand binding regions (Phillips et al., 1988). Calcium in the extracellular environment is required to maintain the functional structure of GP IIb/IIIa (Kunicki et al. 1981). Calcium chelators can disrupt the functional conformation of GP IIb/IIIa, and thus block aggregation in the stimulated platelets (Pidard et al., 1986). The tetrapeptide sequence, arginine-glycine-aspartate-serine (RGDS), is one of the recognition sequences for the fibrinogen receptor GP IIb/IIIa. Other adhesion proteins containing this sequence also can bind to GP IIb/IIIa on the activated platelets and mediate an adhesion reaction. They include vWF, vitronectin, fibronectin, and thrombospondin (Plow et al., 1985).

Ro 44-9883, a non-peptide fibrinogen receptor antagonist with a high efficiency and specificity in blocking aggregation, has been produced by using RGDS sequence as a chemical structure model (Alig et al., 1992). In comparison with other platelet aggregation inhibitors, Ro 44-9883 shows higher selectivity for GPIIb/IIIa than other synthetic peptides containing a RGDS sequence which could also bind to other integrin receptors. It also has a shorter plasma half-life than anti-IIb/IIIa antibody, which may decrease the side effects when Ro 44-9883 is clinically used (Alig et al., 1992). In addition, it has a very low Ki for inhibition of platelet aggregation induced by all platelet agonists in comparison to other synthetic peptides, (Gartner et al., 1985; Shebuski et al., 1989). It has clinically been tested to determine its efficiency as an antithrombotic agent in treating unstable angina due to the formation of the uncontrolled platelet aggregates (Theroux et al., 1994).

Signal Transduction in Stimulated Platelets

Blood platelets are widely used as a model cell for studying signal transduction. The platelet can be stimulated by variety of agonists, such as collagen, thrombin, ADP, TxA_2, serotonin, epinephrine, platelet-activating factor, and vasopressin. These agonists are usually divided into strong and weak groups according to whether they can induce full platelet activation without the self amplifying effects of platelet autocrine factors such as ADP and TxA_2. Strong agonists include collagen and thrombin. Weak antagonists, such as ADP, serotonin, epinephrine, platelet-activating factor, and vasopressin, and low doses of strong agonists induce platelet activation dependent on the generation and release of

autocrine factors. Antagonists of platelets includes another arachidonic acid metabolite produced in endothelial cells, prostacyclin (PGI_2), which inhibits and even reverses platelet activation (Cox and Carroll et al. 1984; Ware, 1995).

The role of the GPIIb/IIIa complex in platelet stimulus response coupling has been the topic of intense study. Activation by ADP and other weak agonists invokes platelet shape change and aggregation, but dense granule secretion relies on TxA_2 formation following aggregation (Detwiler et al., 1980; Zucker, 1980). In these experiments secretion and TxA_2 formation were blocked by inhibiting aggregation, which was either prevented by not stirring the platelets or by using thrombasthenic platelets which lack GPIIb/IIIa. The above studies generally support a role for GPIIb/IIIa mediated aggregation in dense granule secretion by weak agonists and low doses of thrombin and collagen.

Thrombin, a potent platelet agonist, not only plays a central role in hemostasis and thrombosis, but also possesses diverse biological functions of hormone and growth factor (Fenton, 1988). Platelet stimulation by thrombin can cause maximum granule secretion and platelet aggregation through two activating signal transduction pathways, which are composed of the phospholipid metabolites of activated phospholipase C (PLC) and phospholipase A_2 (PLA_2) (Brass et al., 1993).

Activated PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 mobilizes Ca^{2+} from dense tubular system to cytosol, which is required to activate Ca^{2+}-dependent enzyme activities such as myosin kinase which phosphorylates myosin 20-kDa light chains. The

other PLC metabolite, diacylglycerol, activates protein kinase C (PKC) which phosphorylates the 47-kDa protein (Kawahara et al. 1980).

Liberation of arachidonic acid with concurrent generation of lysophospholipids from thrombin-stimulated platelets can be mainly attributed to activation of PLA_2, which hydrolyses arachidonic acid from the inner leaflet membrane phospholipids (Broekman, 1986). Arachidonic acid and lysophospholipids may serve directly as intracellular second messengers (Kim et al., 1989) or they are further converted to lipid mediators such as PAF, and eicosanoids (most importantly in platelets, TxA_2), which are both vasoactive and important inflammatory mediators (Chang et al., 1987).

Arachidonic acid in platelets can undergo several metabolic routes, but conversion to the precursor prostaglandin (PG) H_2 by cyclooxygenase, and finally conversion to TxA_2 by thromboxane synthetase is the most important one. Both PGH_2 and TxA_2 are potent platelet activators. TxA_2 stimulates platelets by G_q-coupled receptor, which leads to activation of PLC, triggering IP_3 mediated release of calcium from dense tubular system as well as diacylglycerol formation and activation of PKC (Brass et al 1993). TxA_2 also serves as an intracellular signal transduction molecule which, like IP_3, directly releases calcium from the dense tubular system (Brace et al. 1985). Aspirin inhibition of platelet activation is due to irreversible inhibition of cyclooxygenase activity by acetylation, which leads to inhibition of TxA_2 production (Picot et al. 1994). Strong agonists such as a high level of thrombin still can fully activate platelets through other pathways (Oates et al., 1988), thus aspirin is not very effective in treating acute thrombotic events.

Generally, PLA_2 of platelets is found in two forms, the secretory form ($sPLA_2$, 14-kDa) and the cytosolic ($cPLA_2$, 85-kDa). The structures and functions of these two forms are quite different. (Lin et al., 1993). 1) $sPLA_2$, in platelets, is localized in alpha granules of resting platelets, and released to the extracellular medium when platelets are activated by thrombin (Mounier, et al., 1993). $cPLA_2$ is generally considered to be free in the cytosol and translocates to the membrane where its substrate is located following activation (Clark et al., 1991). 2) $sPLA_2$ activity requires extracellular millimolar concentration of Ca^{2+}, but $cPLA_2$ activity only requires intracellular micromolar concentration of Ca^{2+} (Lin, et al., 1993) 3) $sPLA_2$ shows nonselectivity of hydrolyzing the sn-2 fatty acyl chain of phospholipids, but $cPLA_2$ shows a high selectivity for arachidonic acid (Kim et al., 1988). It is generally agreed that $cPLA_2$, but not $sPLA_2$, mediates the rapid intracellular production of arachidonic acid (Bartoli et al., 1994). $cPLA_2$ is composed of two functional domains: a regulatory Ca^{2+}-dependent lipid-binding domain which facilitates $cPLA_2$ association with phospholipids, and a Ca^{2+}-independent catalytic domain responsible for hydrolysis of arachidonic acid from phospholipids (Nalefski et al., 1994). It has been demonstrated that $cPLA_2$ activity in stimulated platelets can be regulated by several mechanisms. (1) Increased intracellular Ca^{2+} concentration in platelet receptor-mediated responses is required to trigger activation of $cPLA_2$ (Rittenhouse et al., 1984). (2) Blocking Na^+/H^+ exchange will prevent intracellular alkalinization and arachidonic acid release induced by weak agonists such as epinephrine and ADP (Banga et al., 1986). (3) Change in the metabolic pools of diacylglycerol, phosphoinositides and phosphatidic acid can possibly alter local membrane structure thus

facilitating $cPLA_2$ hydrolysis of phospholipids (Kramer et al., 1987) as well as activating $cPLA_2$ by mediating calcium flux and protein kinase activation. This hypothesis suggests that the PLC pathway can participate in the activation of $cPLA_2$. (4) Phosphorylation of $cPLA_2$ may be important for its activity. In platelets stimulated by thrombin, increased $cPLA_2$ activity corresponds to the extent of phosphorylation of $cPLA_2$ (Kramer et al., 1993). Either protein kinase C or mitogen-activated protein (MAP) may be involved in phosphorylation of $cPLA_2$ in platelets (Halenda, et al., 1989; Lin et al., 1993). (5) Finally, activation of G_i or G_q proteins causes increased cytosolic Ca^{++} (through PLC activation) which indirectly activates $cPLA_2$ by mediating its association with the phospholipid membrane. It has also been shown that a G_i mediated process is required for $cPLA_2$ activity independent of calcium flux by an unknown mechanism (Kim et al., 1989).

Several important platelet receptors, such as those for TxA_2, PAF, thrombin, PGE_1, and PGI_2 have been demonstrated to be coupled with G proteins. G proteins are heterotrimeric structure consisting of α, β, and γ subunits, with the α subunit containing the GTP/GDP binding site. Binding of agonist to the receptor coupled with G proteins results in the conformational change of the G proteins, in which GDP dissociates from α subunit, and α subunit binding to GTP then dissociates it from the $\beta\gamma$ subunits. G_α and $G_{\beta\gamma}$ may both be involved in upregulating some enzyme activities (Brass et al., 1993). There are at least nine forms of α subunits identified so far in human platelets. G_s and G_i are involved in regulating cAMP formation in platelets. G_s coupled receptors stimulate adenylyl cyclase which converts adenosine triphosphate (ATP) to cyclic-adenosine monophosphate (cAMP). PGI_2, through its G_s coupled receptor events,

elevates cytosolic cyclic AMP, which blocks and reverses cytosolic calcium flux, cytoskeleton assembly and GPIIb/IIIa mediated aggregation (Carroll et al 1982). Thrombin, ADP and epinephrine inhibit adenylate cyclase by stimulation of their G_i protein-coupled receptors. G protein regulation of PLC has been shown to be through PLC_β. G_q (linked to TxA_2 receptor), which is not sensitive to pertussis toxin, and G_i family members such as G_{i1}, G_{i2}, and G_{i3}, which are sensitive to pertussis toxin, could mediate PLC activity in stimulated platelets. Regulation of $cPLA_2$ activation by G protein involves two possible mechanisms, increased calcium and diacylglycerol activation of PKC through G_i and G_q mediated PLC pathways, and a G_i-mediated $cPLA_2$ activation by an unknown mechanism required in addition to calcium elevation and PLA_2 phosphorylation (Brass et al., 1993; Winitz et al., 1994).

Tyrosine phosphorylation in platelets is very important in the induction of cellular response to extracellular stimuli. Many agonists including thrombin, ADP, epinephrine, collagen, and vasopressin can induce different types of tyrosine phosphorylation in platelet activation (Nalamura et al., 1989, Golden et al., 1989, Golden, et al., 1990, Granot, et al., 1990). In platelets stimulated by thrombin, three waves of tyrosine phosphorylation taking place at 5-20 s, 1-3 min, 3-5 min, have been detected (Ferrell et al., 1988). Tyrosine phosphorylation of intracellular proteins corresponds to shape change, aggregation and granule secretion of thrombin-stimulated platelets (Shattil et al., 1991; Pumiglia et al. 1993). The important nonreceptor tyrosine kinases involved in platelet activation are part of the Src family. One of these, pp60src, represents 0.2%-0.4% of total platelet protein (Golden et al.,1990). Others related to src family in stimulated platelets

are fyn, lyn and yes. (Cichowski et al., 1992). The function of many of the proteins phosphorylated by the these enzymes is not yet clear.

MAP kinase, an important protein in signal transduction, has been implicated in regulating mitosis and proliferation in nucleated cells (Blenis, 1991). MAP kinase can be stimulated by G protein coupled receptor or receptor tyrosine kinase pathways (Blenis 1993; Lange-Carter et al. 1993). MAP kinase can also directly induce activation and phosphorylation of $cPLA_2$ at serine residues, as detected by site-directed mutagenesis of the $cPLA_2$ in Chinese Hamster Ovary (CHO) cells (Lin, et al 1993). In platelets, MAP kinase showed enhanced phosphorylation on serine, threonine, and tyrosine residues and increased activation by thrombin stimulation, which was not reduced by blocking aggregation mediated by GPIIb/IIIa using RGDS peptides (Papkoff et al 1994). The function of MAP kinase in platelets, not yet elucidated, could be related to $cPLA_2$ activation and modulating the function of cytoskeletal proteins, connected to platelet secretion and adhesion. Nakashima et al. reported that tyrosine phosphorylation of MAP kinase, in thrombin-stimulated platelets, corresponded to a late phase $cPLA_2$ activation and release of arachidonic acid (Nakashima et al 1994; Papkoff et al 1994).

Recently, GPIIb/IIIa-mediated aggregation or GPIa/IIa-mediated adhesion to collagen has been linked to tyrosine phosphorylation of 101, 105-kDa peptides and $p125^{FAK}$ (focal adhesion kinase which is involved in cellular adhesion such as at the focal contacts of cells with substratum) (Haimovich et al 1993). This tyrosine phosphorylation was subsequently shown to require both integrin receptor occupancy and agonist-mediated activation (Shattil et al., 1994). Seufferlein et al. (1994) reported that 1-oleoyl-

lysophosphatidic acid (LPA) induces tyrosine phosphorylation of $p125^{FAK}$ and other proteins in Swiss T fibroblasts. This was independent of PKC activation and intracellular calcium flux since inhibition of PKC and depletion of intracellular calcium did not block tyrosine phosphorylation of these proteins. However, integrity of the actin cytoskeleton was showed to be required for LPA-stimulated tyrosine phosphorylation of $p125^{FAK}$ since it was blocked by cytochalasin treatment to disrupt actin filament assembly (Seufferlein et al. 1994).

In platelet activation, there is a dynamic balance between tyrosine phosphorylation and dephosphorylation, mediated by protein tyrosine phosphatase (PTPase). PTP-IB, a tyrosine phosphatase, has been identified in human platelets. Cleavage of PTP-1B and subcellular relocation from the cytosolic face of the endoplasmic reticulum to the cytosol, which correlates with increased activity of PTP-IB, can be induced by the engagement of GPIIb/IIIa in stimulated platelets (Frangioni et al., 1993). The substrates of PTP-IB are not known.

Significance of This Study

Platelet aggregation has a central role in excess thrombi formation and occlusion of vessels in pathogenic conditions such as atherosclerosis. Ro 44-9883 a platelet GPIIb/IIIa antagonist was chosen for our research purpose due to its greater efficiency and selectivity than other platelet inhibitors to block platelet aggregation. Anti-GPIIb/IIIa monoclonal antibodies are more expensive, and also can evoke an immune response as a side effect, thus such compounds as Ro 44-9883 are more desirable as clinical

therapeutic agents. Such compounds are superior to aspirin which not only inhibits platelets, but also inhibits the production of prostaglandins in other tissues including prostacyclin which is antithrombotic. The objective of our current study is to use a biochemical approach to detect the effects of GPIIb/IIIa antagonist Ro 44-9883 by blocking aggregation on platelet activation, phospholipid metabolism, granule secretion, and signal transduction pathways, especially in PLA_2 pathway. In particular, one important part of this study is to determine the role of integrin mediated aggregation in regulating PLA_2 activity in order to more fully understand the effects of aggregation inhibitors in clinical trials. The results of this study may help to interpret clinical findings and possibly provide valuable information for designing better therapeutic strategies.

PART II

MATERIALS AND METHODS

Materials

Ro 44-9883 and TRAP (synthetic SFLLRNPNDKYEPF peptide) were both obtained from Hoffman La Roche. Arachidonic acid was purchased from Nuchek Prep, Elysian, MN. Peroxidase-conjugated Goat Anti-Rabbit lgG was purchased from Jackson ImmunoResearch Laboratories, Inc. Phosphatidic acid and diacylglycerol (dioleoyl) were obtained from Avanti Polar Lipids, Birmingham, AL. Silica gel 60A, 250 um, K6 TLC plates were manufactured by Whatman Internal., LTD, Maidstone, England. ADP, lysophosphatidic acid (oleoyl), Thrombin, α-D(+)Glucose, Trizma base, phenylmethylsulphonyl fluoride, leupeptin, bovine serum albumin, EGTA, molybdic acid, beta-mercaptoethanol, protein A sepharose, sepharose CL-2B, EDTA, bromophenol blue, Triton X-100, TEMED, β-glycerophosphate, sodium orthovanadate, dithiothreitol, and prestained molecular weight standard were purchased from Sigma Chemicals, St. Louis, MO. Potassium chloride, calcium chloride, HEPES, sodium chloride, glycerol, acetic acid, methyl alcohol, and isopropanol were purchased from Mallinckroft, Paris, KY. Acrylamide, ammonium persulfate, bis-acrylamide, glycine, Tween-20 and the nitrocellulose membranes were purchased from BioRad, Richmond, CA. The sodium doecyl sulfate was purchased from BDH Chemical, Pooie, England. Sodium citrate, magnesium chloride was purchased from Fisher Scientific, Fair Lawn, NJ. Normal goat serum was purchased from Pelfreeze Biological, Roger, AZ.

Methods

Platelet Preparation

Blood was collected from volunteers giving informed consent and who denied taking drugs for at least two weeks prior to donation. Blood was drawn from the antecubital vein into one-tenth volume of CCD (100 mM sodium citrate and 130 mM glucose, pH 6.5). Platelet rich plasma (PRP) was prepared by centrifugation at room temperature for 3 minutes at 1000g. Platelets were labeled with ^{14}C-serotonin (0.05 uCi/ml, Amersham, Arlington Heights, IL), for 45 minutes at 37^{0}C before experimentation. Blood was also acquired from individuals with Glanzmann's thrombasthenia (GT), after obtaining informed consent, both in the Coagulation Laboratory, Christiana Hospital, Newark, DE (shipped to Knoxville overnight at ambient temperature as CCD anticoagulated whole blood along with control blood samples, courtesy of Dr. Robert Abel) and in CRTS laboratories, Strasbourg, FRANCE, which were assayed the same day in Dr. Lanza' laboratory. PRP was used without adjusting platelet counts which ranged from 3 to 6 x 10^{8} /ml. After PRP was collected, it was cooled on ice for 15 minutes and mixed with one half volume of 4^{0}C CCD buffer. The platelet pellet was prepared by centrifugation of the mixed PRP at 4^{0}C for 10 minutes at 900g and decanting the supernatant. The platelet pellet was resuspended in a Tangen-HEPES-BSA buffer (145 mM sodium chloride, 5 mM potassium chloride, 0.1 mM magnesium chloride, 0.05 mM calcium chloride, 5.5 mM glucose, 15 mM HEPES, and 1mg/ml bovine serum albumin). The platelet suspension was warmed at 37^{0}C for 45 minutes and then cooled on ice for 10 minutes. Gel-filtered platelets (GFP) was obtained by filtering the platelet

suspension on a Sepharose-2B column (Sigma) equilibrated and eluted with a Tangen-HEPES-BSA buffer and warmed to 37°C for 45 minutes before experimentation.

Aggregation of Platelets

Aliquots (0.5 ml) of PRP or GFP were pretreated 3 minutes with varying concentrations of Ro 44-9883 (obtained from Hoffman La Roche), and then activated with indicated agonist for an additional 5 minute continuous stirring at 37°C. Aggregation of platelets in PRP or gel-filtered platelets was carried out at 37°C with constant stirring in siliconized glass cuvettes monitored with a Payton dual channel aggregometer (Payton Scientific. Inc, Buffalo NY). Light transmittance was calibrated to 0% using the platelet suspension and at 100% using plasma for PRP and Tangen-HEPES-BSA buffer for GFP samples.

Serotonin Secretion

Triplicate aliquots (0.1 ml) of PRP pre-labeled with ^{14}C serotonin were pretreated 3 minutes with or without Ro 44-9883, and then activated with ADP or TRAP. After an additional 5 minute continuous stirring at 37°C, the secretion was stopped by immediately mixing the aliquots into 0.9 ml of ice cold phosphate-buffered saline (PBS) with 10 mM EGTA and 0.2% glutaraldehyde. The platelets were removed by centrifugation for 3 minutes at 10,000g and percent release was evaluated by scintillation counting of 0.5 ml aliquots.

d-Myo-Inositol 1-, 4-, 5-Triphosphate (IP_3) Assays

0.5 ml GFP (5 x 10^8/ml) were sampled at the indicated time points after adding 20 uM TRAP either without or with pretreatment with 1 uM Ro 44-9883 with continuous stirring at 37°C in a siliconized aggregometer cuvette. Then 0.3 ml aliquots were immediately mixed with 0.06 ml of 100% trichloroacetic acid (TCA) to stop the reactions, and IP_3 was extracted. IP_3 was assayed by a competitive calf brain receptor binding assay after TCA in the samples had been removed by solvent extraction per manufacture's directions (Dupont-NEN, Boston, MA).

Fluorescence Assays of Calcium and pH

PRP labeled with either the calcium indicator fura-2/AM (2uM) or the pH indicator BCECF/AM (10 uM) (from Molecular Probes, Eugene, OR) at 37°C for 45 minutes prior to gel-filtration were activated with continuous stirring at 37°C by 20 uM TRAP following pretreatment with nothing or 1 uM Ro 44-9883. Fluorescent assays were carried out in plastic cuvettes (UV type) using a Shimadzu RF5000 Spectrofluorometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) with stirring at 37°C, which was discontinued 30 seconds following addition of an agonist for the BCECF-labeled platelets to avoid uneven fluorescent signal due to the formation of large aggregates. This discontinuous stirring was not required for the fura-2 fluorescence, which was evaluated by signal ratio of two excitation wavelengths. Responses over time are shown as the ratio of fluorescence sensitive to $[Ca]_i$ at 510 nm with excitation at 335 or 360 nm or as the fluorescence changes sensitive to pH_i at 530 emission with 495 excitation.

Detection of Phosphatidic Acid

GFP were labeled by ^{32}P-orthophosphate (0.5 mCi/ml, ICN, Costa Mesa, CA) at 37^{0}C for 45 minutes before experimentation. 0.5 ml aliquots of ^{32}P-labeled GFP (5 x 10^{8}/ml) were activated with 20 uM TRAP for indicated time points with continuous stirring at 37^{0}C either with or without 1 uM Ro 44-9883, and were extracted with water saturated n-butanol to isolate total phospholipids. The samples were concentrated to dryness by evaporation under a stream of nitrogen gas, dissolved in 20 ul of chloroform:methanol (2:1), and analyzed on TLC plates by two-dimensional solvent development. The plates were air dried and phospholipids were visualized by iodine vapor or sulfuric acid charring. ^{32}P-label was measured by autoradiography/densitometry. The position of phosphatidic acid was confirmed by co-development of a pure phosphatidic acid standard.

Phosphorylation of Myosin Light Chain and p47

^{32}P-labeled GFP (5 x 10^{8}/ml) were activated at 37^{0}C with continuous stirring with 20 uM TRAP for indicated time points with or without pretreatment by 1 uM Ro 44 9883. Aliquots (60 ul) removed at the indicated times, were immediately added to 30 ul of 3 fold reducing denaturing solution (6% SDS, 6% β-mercaptoethanol, 1 mM EGTA, 3 mM EDTA, 30% glycerol, 0.03% bromophenol blue, 150 mM Tris-HCI, pH 6.8) to stop the reaction and to denature proteins. After SDS-PAGE of these aliquots, phosphorylated 20-kDa myosin light chain (p20) and the 47 kDa (p47) substrate of protein kinase C were visualized on autoradiograghs and quantitated by densitometry.

Enzyme Immunoassay (EIA) for TxA_2

Paired aliquots (0.5 ml) of PRP were pretreated for 3 minutes with Ro 44-9883, and then activated with TRAP or ADP. After an additional 5 minute continuous stirring at 37°C, the paired samples were then diluted into 4.5 ml of ice cold distilled water containing 20 ug/ml indomethacin to stop the platelets from further synthesis of thromboxane. After freeze-thawing, the samples were centrifuged at 3000g for 10 minutes at 4°C to remove the lysed platelets. The level of TxB_2, a stable metabolite of TxA_2, was determined on these platelet lysates by EIA per manufacture's instructions (Cayman Chemical Co., Ann Arbor, MI). Data is expressed as TxA_2.

In vitro Assays for Phospholipase A_2 Activity

Equal aliquots (1 ml) of gel-filtered platelets (5 x 10^8/ml) were activated with continuous stirring at 37°C for 6 minutes with 20 uM TRAP, or pretreated with 1 uM Ro 44-9883 before addition of 20 uM TRAP. Controls without treatment were included. The 1 ml aliquots were then mixed with a freshly-made 20 fold concentrated stock solution (2 mM EGTA, 0.2 mM leupeptin, 0.1 mM phenylmethylsulfonylfluoride, 0.4 mM sodium vanadate, 2 mM dithiothreitol, 1 mM beta-glycerophosphate, 0.4 mM sodium molybdate, with or without 1 mg/ml DNase I as indicated), and sonication of the samples was carried out at 0°C for 45 seconds. Platelet cytosol fractions were obtained by centrifugation at 150,000g for 45 minutes at 4°C. Cytosolic PLA_2 activity was determined at 37°C with triplicate 20 ul aliquots of the cytosolic fractions containing equal amounts of proteins mixed with 200 ul of pH 7.4 assay buffer containing 150 mM sodium chloride, 50 mM

HEPES, 1mg/ml bovine serum albumin, 1 mM calcium chloride, 1 mM dithiothreitol, 0.6 nM phosphatidylcholine, L-alpha-1-palmitoyl-2-arachidonyl (0.02 uCi arachidonyl-1-[^{14}C], Dupont-NEN, Boston, MA), plus diacylglycerol (dioleoyl) (2:1). Released ^{14}C-arachidonic acid was separated from the phosphatidylcholine by silica gel absorption and heptane solvent extraction, and CPM was determined by scintillation counting in triplicates.

Western Blotting

Platelet samples treated under the above conditions to obtain the cytosolic fraction, and unfractionated samples, with or without DNase I in the lysis buffer, were subjected to western blotting analysis. 200 ul of the cytosol fraction or the unfractionated sample were separately added to 100 ul of 3 fold reducing-denaturing solution (as described previously). The mixture was heated at 90^{0}C for 5 minutes before electrophoreses. 90 ul of the cytosol and the unfractionated samples were run on 6%-17.5% gradient SDS-PAGE. After being transferred to nitrocellulose overnight by standard protocols, the proteins were probed with rabbit anti-cPLA$_2$ polyclonal antibody obtained from Dr. Kramer (Eli Lilly), then reprobed with mouse anti-phosphotyrosine monoclonal antibody (mAb) (PY-20 from ICN, Costa Mesa, CA) and mouse anti-focal adhesion kinase (p125^{FAK}) monoclonal antibody (from Transduction Laboratories, Lexington, WI) following stripping of the membrane per manufacture direction (Enhanced Chemiluminescence ECL Kit, purchased from Amersham) Bound antibodies were detected by ECL using a horseradish peroxidase-conjugated anti-rabbit immunoglobulin, and quantitated by densitometry. Different exposure times were quantitated to assure that

values were in the linear range of exposure time. Percent distribution of $cPLA_2$ based on the densitometry units of the cytosolic fractions divided by the average total platelet content of $cPLA_2$ was determined.

^{32}P-labeled $cPLA_2$ Immunoprecipitation (IPPT)

^{32}P-labeled GFP were activated with 20uM TRAP with or without 1 uM Ro 44-9883 preadded. Unactivated controls were included. Total platelet extracts were prepared by mixing and sonicating platelet samples with an equal volume of 2% Triton X-100 buffer containing proteases and phosphatase inhibitors as described previously and 2 mg/ml DNase I (to help depolymerize actin filaments). Identically treated platelet sample were fractionated by sonication without Triton X-100 and centrifugation as described above. The cytosolic fraction was mixed with same volume of 2% Triton X-100 extraction buffer containing 2 mg/ml DNase I, and the membrane fraction was mixed with 1 % Triton X-100 extraction buffer containing one fold concentration of protease and phosphatase inhibitors, plus 1mg/ml DNase I. The total platelet, cytosolic fraction, and membrane fraction samples are diluted to equivalent volumes in terms of the original platelet volume. Extracts were then recentrifuged to remove insolubles, and the supernatants were incubated one hour with an excess (as determined in a previous titration) of rabbit anti-PLA_2 antibody (provided by Dr Kramer, R.M.). Immune complexes were collected by protein A-sepharose, and washed with extraction buffer three times before they were denatured and loaded on SDS-PAGE. Autoradiograms were analyzed by video densitometry (Applied Imaging). Insoluble membrane fraction and

post-immuno-precipitation supernatant were also subject to SDS-PAGE to detect if any $cPLA_2$ was present in post-immuno-precipitation supernatants or insoluble extraction residues.

Gel-Shift Assay for Phosphorylated $cPLA_2$

Aliquots of GFP (1.5 ml) samples were either activated with 20uM TRAP for 6 minutes or left unactivated. The platelet samples were mixed with 20 X protease and phosphatase inhibitors before sonication. The membrane fractions were obtained by centrifugation at 150,000g for 45 minutes at 4^0C. The unfractionated samples were denatured and reduced as previously described, and pellets of platelet membrane were dissolved in 400 ul reducing-denaturing solution by sonication. 90 ul of the unfractionated samples and 30 ul of membrane fraction samples (equivalent loads relative to the original volume of GFP) were run on 10% acrylamide SDS-PAGE, followed by western blot analysis with rabbit anti-$cPLA_2$ Ab, and detection with ECL Kit.

$cPLA_2$ Co-IPPT with Membrane Proteins

Aliquots of GFP (1 ml) were not treated or activated with 20uM TRAP with or without 1 uM Ro 44-9883 preadded, or activated with 0.2 U/ml thrombin, before $cPLA_2$ co-IPPT with anti-GPIb antibody. Aliquots of GFP (1.5 ml) were unactivated for $cPLA_2$ co-IPPT with other anti-membrane antibodies.

The membrane fractions were prepared by sonication and ultracentrifugation. Membranes were then extracted with 1% Triton X-100 buffer and 1 X protease and

phosphatase inhibitors following by ultracentrifugation to remove insolubles. The extracts were then treated with 14 ug of GPIb mAb (clone AN51 from Dako-Patts), 15 ug of CD9 mAb (SYB-1, IgG1 subclass from Dr. J. Breard in Villejuif, France), 10 ug of β2-microglobulin mAb (B2.62.2, IgG1 described by Liabeuf et al., 1981), 16 ug of CD32 mAb (IV-3, IgG_{2b} subclass from Drs. C. Anderson, in Columbus, Ohio), and 20 ug of CD62 mAb (CLB-thromb/6 from SANBIO, Uden, Holland). Immune complexes were collected and run on SDS-PAGE. Western blotting with rabbit anti-$cPLA_2$ and detection by ECL system were then carried out by procedures described above.

Statistics and Calculations

Percent release of granules was calculated by the following formula:

(Sample - zero time) / (total - zero time) x 100.

The percent inhibition was normalized for different donor results by the following formula:

[1 - (Ro 44-9883 pretreated % response / % response with agonist alone)] x 100.

Statistical comparisons (SigmaStat software, Jandel Scientific Software, San Rafael, CA) of % inhibition data used the standard students t-test with significance set at $P < 0.05$. Third and fourth order regression curve fitting was done with SigmaPlot software (Jandel Scientific Software).

PART III

RESULTS

Effects of Ro 44-9883 on Aggregation and Serotonin Secretion

Platelets Aggregation and Serotonin Secretion

Figure 1 shows that Ro 44-9883 inhibits 20 uM TRAP induced aggregation and serotonin secretion of platelets in a concentration-dependent manner. Interestingly, TRAP-induced dense-granule was abolished more readily than was aggregation (IC_{50} of 10 versus 25 nM). The secretion of serotonin induced by weak agonists is dependent on platelet aggregation and subsequent generation of TxA_2 which will be determined later.

Serotonin Secretion Compared with Normal and Glanzmann's Thrombasthenia (GT) Platelets

GT patient's platelets were pretreated with 1 uM Ro 44-9883 to determine if its effects were mediated solely through the GPIIb/IIIa complex. Figure 2 shows a representative result of such a study comparing the effects of pretreatment of GT and normal platelets with Ro 44-9883 on the serotonin secretion responses to low (20 uM) and high (80 uM) concentration of TRAP. The inhibition by Ro 44-9883 of serotonin secretion at low doses of TRAP correlated with inhibition of TxA_2 production which was overcome at the high dose of TRAP (TxA_2 formation in normal platelets: 20 uM TRAP with no pretreatment = 131 ng/ml versus 1 uM Ro 44-9883 = 21 ng/ml, 80 uM TRAP with no pretreatment = 229 ng/ml versus 1 uM Ro 44-9883 = 152 ng/ml). In comparison to normal platelets the GT platelets had less of a response to a low dose of TRAP in

terms of serotonin secretion and there was no significant additional inhibition by the GPIIb/IIIa antagonist. This was also the case with the Strasbourg I variant GT platelets which have near normal amount of a non-functional GPIIb/IIIa. It was consistently noted that the GT platelets had a greater response to low dose TRAP in terms of serotonin secretion than normal platelets pretreated with Ro 44-9883. This may reflect a compensation by other pathways in response to the aggregation deficit.

Effects of Ro 44-9883 on the Phospholipase C Pathway

IP_3 and Phosphatidic Acid Formation

To determine if Ro 44-9883 also affected the PLC pathway and generation of IP_3 or diacylglycerol, we measured these metabolites. Figure 3 shows the combined results of four time courses of IP_3 formation induced by 20 uM TRAP with or without pretreatment with 1 uM Ro 44-9883. Although there was considerable variation in fold increases in IP_3, the combined data with and without pretreatment can be fitted to a single curve with no significant inhibition by Ro 44-9883. The lack of effect of Ro 44-9883 on the PLC pathway was also evident in the ^{32}P-labeling of phosphatidic acid (Figure 4) which reflects diacylglycerol formation (Nozawa et al., 1991). Again, the data with or without pretreatment can be fitted to a single curve within 95% confidence limits, and no significant inhibition by Ro 44-9883 was found.

Calcium and pH Responses

More direct parameters of platelet stimulus-response coupling include calcium flux and pH changes within the cytosol. These were evaluated in GFP loaded with fura-2 to measure calcium and with BCECF to measure pH changes. In separate experiments we confirmed that serotonin secretion in GFP remained sensitive to Ro 44-9883 mediated inhibition. At 20 uM TRAP the secretion was reduced from 18 $\pm$ 1% to 2 $\pm$ 1% by 1uM Ro 44-9883. Shown in Figure 5A is the lack of effect of 1 uM Ro 44-9883 on the basal levels or increase of calcium induced by 20 uM TRAP. Ro 44-9883 also had no effect on the basal cytosolic pH or the alkalinization induced by 20 uM TRAP (Figure 5B).

Protein Kinase C and Myosin Kinase Activity

The above data suggested that many of the stimulus-response coupling pathways remained unaffected by blocking aggregation. ^{32}P-autoradiograms have proven a convenient way to monitor platelet PKC and myosin kinase responses to agonists. Such data are shown in figure 6A & B to evaluate the effect of Ro 44-9883 on 20 uM TRAP activation over a 60 second time course. The initial phosphorylation of the P47 substrate of PKC or myosin light chain (MLC), the 20 kDa substrate of myosin kinase, were not significantly affected by blocking aggregation.

Effects of Ro 44-9883 on the Phospholipase A_2 Pathway

TxA_2 Formation

It is well known that the secretion induced by weak agonists is dependent on platelet aggregation and subsequent generation of TxA_2. To determine if the inhibition of secretion by Ro 44-9883 correlated with an inhibition of TxA_2 production we measured its formation by EIA (Figure 7). TxA_2 formation induced by 100 uM ADP was inhibited by Ro 44-9883 in a concentration dependent manner. It can be seen that there was a maximal inhibition of TxA_2 formation and serotonin secretion, which occurred near the IC_{50} for aggregation. Similar data has been obtained with 20 uM TRAP as the agonist (data not shown).

Both TxA_2 and LPA are Required to Overcome the inhibition of Serotonin Secretion

The data above suggested an aspirin-like effect of Ro 44-9883. However, unlike aspirin, Ro 44-9883 did not block TxA_2 formation from exogenously added 100 ug/ml arachidonic acid (no pretreatment = 1390 ± 156 ng/ml versus 1 uM Ro 44-9883 pretreatment = 1785 ± 266 ng/ml versus 100 uM aspirin pretreatment = 2.49 ± 0.2 ng/ml). When the TxA_2 mimetic, U46619, was titrated into either control, 100 uM aspirin, or 1 uM Ro 44-9883 pretreated PRP, there was a clear deference in the ability of U46619 to induce serotonin secretion (Figure 8). An optimal concentration of 10 uM U46619 to overcome the aspirin inhibition by only partially overcomes the inhibition by Ro 44-9883. These results let us to consider if other possible phospholipid metabolites of the PLA_2 pathway were required to completely restore serotonin secretion.

Lysophosphatidic acid (LPA) is known to be a platelet activator. Exogenously added 100 uM LPA induced serotonin secretion comparable to 100 uM ADP or exogenously added arachidonate (Figure 9). As with ADP the secretion induced by LPA was almost completely inhibited by an excess of Ro 44-9883 to block aggregation. However the combination of ADP, arachidonate, and LPA overcame the inhibitory effects of Ro 44-9883 on serotonin secretion, as shown in Figure 9, but not on aggregation which remained completely inhibited (not shown). Inhibition by Ro 44-9883 is significant ($P < 0.05$) for all agonists except for the last combination of ADP, arachidonic acid (AA), and LPA.

In vitro Assays of PLA_2 Activity

The results above suggested that Ro 44-9883 inhibition was specific for the PLA_2 pathway. To determine if this was a direct effect on PLA_2 activation we assayed its activity in platelet cytosol fractions before and after 20 uM TRAP activation with or without 1 uM Ro 44-9883 as shown in figure 10. Although the fold increase in PLA_2 activity was variable between donors, there was no apparent effect of blocking aggregation with Ro 44-9883 on the fold increase of PLA_2 activity in any of the experiments. No significant difference is observed with or without 1 uM Ro 44-9883 pretreatment.

Distribution of Cytosolic Phospholipase A_2 Between the Cytosol and Membrane

Distribution of Phosphorylated $cPLA_2$

To determine if Ro 44-9883 plays a role in the phosphorylation of $cPLA_2$ in stimulated platelets by TRAP and how phosphorylated $cPLA_2$ is distributed, ^{32}P-labeled $cPLA_2$ IPPT and gel-shift assays for phosphorylated $cPLA_2$ were carried out in 20 uM TRAP-activated platelets without or with 1 uM Ro 44-9883 pretreatment and in control platelets. The results from ^{32}P-labeled $cPLA_2$ IPPT (Figure 11) showed that phosphorylation of $cPLA_2$ was markedly increased (10 fold increase quantified by densitometry) in TRAP-stimulated total platelet samples. In cytosolic fractions phosphorylation of $cPLA_2$ accounted for a part (40% - 50%) of the label observed in total platelet extracts, while in membrane fractions no labeled phosphorylated $cPLA_2$ was detected. By western blotting with anti-$cPLA_2$ Ab no $cPLA_2$ was detected in the post-immuno-precipitation supernatant, showing that $cPLA_2$ was almost completely solubilized from the membrane fraction by Triton X-100 and DNase I extraction. These results suggested that Ro 44-9883 had no effect on the phosphorylation of $cPLA_2$ in total stimulated platelet samples or the presence of phosphorylated $cPLA_2$ in the cytosol. In gel-shift assay, the phosphorylated $cPLA_2$ in TRAP-activated platelets (lane 2 & 4) over control (lane 1 and 3) was shifted to a slower mobility relative to the unphosphorylated $cPLA_2$ (Figure 12), which is clearly seen in unfractionated platelet. Additionally a trace amount of shifted and presumably phosphorylated $cPLA_2$ in the membrane fraction can be detected. This result suggests that the gel-shift assay may be more sensitive than ^{32}P-

labeling phosphorylation and that a small amount of phosphorylated $cPLA_2$ may remain associated with membrane fraction.

Effect of DNase I on Distribution of $cPLA_2$

To determine if $cPLA_2$ membrane association and redistribution was associated with the platelet cytoskeleton, DNase I was used to depolymerize actin filaments in conjunction with sonication to prepare the cytosolic fractions. The results from western blotting for $cPLA_2$ distribution (Figure 13) showed that 20 uM TRAP increased the amount of $cPLA_2$ in the cytosolic fractions. However, when TRAP-stimulated samples were treated with DNase I, a considerably larger portion of $cPLA_2$ was released into the cytosolic fractions. The increase of $cPLA_2$ in cytosol fraction by DNase I treatment was statistically significant ($P < 0.05$). Ro 44-9883 does not have any significant effects on the distribution of $cPLA_2$.

In vitro Assays of Phospholipase A_2 Activity (+/- DNase I)

The results (Figure 14) showed that $cPLA_2$ activity was increased in 20 uM TRAP-stimulated platelets with or without blocking aggregation by 1 uM Ro 44-9883. After DNase I was added, $cPLA_2$ activity was increased nearly two fold in TRAP-stimulated platelets but not in unstimulated platelets compared with no DNase I treatment. This increase of $cPLA_2$ activity in the cytosolic fractions by DNase I treatment was consistent with the increase in the amount of $cPLA_2$ found in the cytosol fractions in DNase I treated samples. Ro 44-9883 pre-treatment had no effect on the increased $cPLA_2$ activity

in TRAP-stimulated platelets and in TRAP-stimulated, DNase I-treated platelets. Difference in $cPLA_2$ activity between the samples with or without DNase I treatment was significant ($P < 0.05$).

$cPLA_2$ Co-IPPT with Membrane Proteins

To demonstrate the possible association of $cPLA_2$ in unactivated and activated platelets with transmembrane proteins, a variety of antibodies were used to immunoprecipitate their corresponding membrane proteins. The samples from immunoprecipitation were loaded on SDS-PAGE for separation, and the $cPLA_2$ was detected with western blotting as described previously. These results (Figure 15A&B) demonstrated that $cPLA_2$ was associated with a variety of membrane proteins. Panel A shows that $cPLA_2$ was associated with GPIb and this association was not interrupted after platelet samples were activated with TRAP without or with Ro 44-9883 pretreatment (lane 2 & 3), thrombin (lane 4) and unactivated (lane 1). In panel B, using unactivated platelet membrane extracts, $cPLA_2$ was associated with CD9 (lane 1), β_2-m (lane 2) and CD32 (lane 3), but not with CD62 (lane 4). Lane 5 is a negative control, and lane 6 is $cPLA_2$ standard. In panel B the slight difference in migration of $cPLA_2$ among the sample lanes might be a protein migration artifact, or might reflect a difference in phosphorylation of $cPLA_2$ associated with the membrane proteins in the unactivated platelets. DNase I treatment doesn't remove this coprecipitated $cPLA_2$ suggesting actin is not involved in this association (data not shown).

Effect of Ro 44-9883 on Tyrosine Phosphorylation

Tyrosine Kinase Activation

Tyrosine kinases have been implicated in platelet activation, and in particular associated with GPIIb/IIIa ligand binding and aggregation. To evaluate tyrosine phosphorylation, total platelet proteins resolved on SDS-PAGE were western blotted with anti-FAK antibody and then reprobed, following stripping of the membranes, with anti-phosphotyrosine Ab. The results (Figure 16) showed the total amount of FAK in control, 20 uM TRAP-stimulated with or without 1 uM Ro 44-9883 pretreatment samples was similar, and that Ro 44-9883 did not cause degradation of FAK in TRAP-stimulated total platelet samples. Tyrosine phosphorylation of FAK in TRAP-stimulated platelets was increased nearly three fold (quantitated by densitometry) compared with control samples, but inhibition of aggregation by Ro 44-9883 totally abolished the increased tyrosine phosphorylation of FAK induced by TRAP. This inhibition of FAK tyrosine phosphorylation by Ro 44-9883 was not overcome by higher doses of TRAP (data not shown).

PART IV

DISCUSSION

GPIIb/IIIa antagonists are likely to become useful tools to treat thrombotic episodes in unstable angina (Theroux et al., 1994) and prevent restenosis of arteries following angioplasty (Weller et al., 1994). To understand the in vivo effects of these compounds it is important to evaluate any effects on the basal platelet state and on the platelet responses to different agonists. This study revealed that Ro 44-9883 had no effect on basal, or agonist-stimulated, levels of cytosolic calcium, cytosolic pH changes, protein kinase C or myosin kinase activities.

Despite an apparent lack of effect on signal transduction, serotonin secretion in response to a weak agonist, ADP, and low doses of TRAP, was severely curtailed by the inhibition of aggregation induced by Ro 44-9883, and this correlated with reduced TxA_2 production. The effect of blocking aggregation on weak agonists such ADP or epinephrine in terms of reduced serotonin secretion and TxA_2 formation has been known for several years. This inhibition has been attributed to a role of platelet aggregation mediated by GPIIb/IIIa in promoting calcium influx (Lecompte et al., 1990; Ware et al., 1991). However, the precise functional role of GPIIb/IIIa in stimulus response coupling remains controversial. It has been suggested that the activated form of GPIIb/IIIa can act as a calcium channel to elevate cytosolic calcium levels and initiate TxA_2 formation and serotonin secretion induced by weak agonists such as ADP or epinephrine by several study groups (Powling et al., 1985; Yamaguchi et al., 1990; Lecompte et al., 1990; Rybak et al., 1992). But in other studies the ability of ligand occupied GPIIb/IIIa to serve as

a calcium channel has been disputed since reduced TxA_2 and serotonin secretion in dysfunctional GPIIb/IIIa platelets does not correspond to reduced calcium flux (Lages et al., 1994). More recent studies using inhibitory monoclonal antibodies to GPIIb/IIIa or other potent inhibitors based on the RGDS sequence to block the fibrinogen binding site also showed an inhibition of dense granule secretion in response to weak agonists without specifically addressing the mechanism (Coller et al., 1983; Foster et al., 1994). In our studies, the TxA_2 mimetic, U46619, at levels which completely overcome aspirin inhibition of serotonin secretion, still did not induce full serotonin secretion when aggregation was blocked by Ro 44-9883.

This study demonstrated that, in addition to TxA_2, full secretion in the absence of aggregation required LPA. A role for LPA in platelet activation was also suggested many years ago (Gerrard et al., 1979). Originally LPA was suggested to have direct calcium ionophore activity but more recent studies suggested a receptor-mediated activation process (Watson et al., 1985). LPA, through a G-protein coupled receptor, leads to activation of PLC and PLD pathways and inhibits adenylate cyclase to evoke its effects (van der Bend et al., 1992). LPA formation has previously been reported to be dependent on either platelet aggregation or calcium influx in a study using EGTA to block aggregation (Watson et al., 1985; Eichholtz et al., 1993). However, the visualization methods (charring or iodine vapor) we employed did not detect formation of LPA either with or without aggregation. Neither did we observe any ^{32}P-labeled material co-incident with the migration of pure LPA standard. This may reflect that LPA is formed in the platelet from unlabeled pools or alternatively is not the major lysophospholipid species.

In this connection it has been reported that PE or PC may serve as the primary sources for released arachidonic acid (Kim et al., 1988). Whichever the endogenous species, our findings suggest a more direct role of lysophospholipids in dense granule secretion independent of calcium flux. Since lysophospholipids are well known membrane fusogenic agents we suggest this as a possible mechanism by which they promote dense-granule secretion.

Our findings suggests a greater requirement for aggregation-dependent formation of the products of the $cPLA_2$ pathway in dense-granule release. However, it was unclear what role the aggregation-dependent $cPLA_2$ pathway plays since all of the signal transduction parameters of regulating $cPLA_2$ activity, such as calcium flux and its phosphorylation by kinase, appear to be unaltered by blocking aggregation.

Our results demonstrate that a lack of $cPLA_2$ activation in response to weak agonists when aggregation was blocked by a GPIIb/IIIa inhibitor was not due to a direct effect on the $cPLA_2$ since its activity in platelet cytosol (2-3 fold increase over controls) was only slightly reduced. Platelet $cPLA_2$ was suggested to be mainly regulated by cytosolic calcium flux and phosphorylation (Kramer et al., 1993). The kinases involved are probably either PKC and /or mitogen-activated protein kinase (MAP kinase). Recent studies suggest that calcium primarily functions in mediating lipid binding through a calcium-dependent domain separate from the catalytic domain (Nalefski et al., 1994). Studies using mutant G-protein α_i subunit transfected CHO continuous cell line presented a complicated picture of $cPLA_2$ activation and release of arachidonic acid (Winitz et al., 1994). That paper demonstrated major roles for both α_i and PKC-mediated pathways

independent of cytosolic calcium and MAP kinase activation of the $cPLA_2$. The inhibitory effect of mutated α_i on release of arachidonic acid from membranes in situ was overcome by direct PKC activation with phorbol ester. However, in our experiments, phorbol, 12-myristate, 13-acetate, (PMA) was not effective in reversing the inhibition of serotonin release by Ro 44-9883, and endogenous activation of PKC by low dose of TRAP was not inhibited by Ro 44-9883.

Papkoff et al. (1994) reported that blocking platelet aggregation by RGDS peptide does not reduce the phosphorylation and activation of MAP kinase in thrombin stimulated platelets. Since neither MAP kinase nor PKC require aggregation for their activation, this could explain the lack of an effect by Ro 44-9883 on $cPLA_2$ phosphorylation.

We postulated that additional components regulating $cPLA_2$ activity could be accessibility of $cPLA_2$ substrates or the interaction of $cPLA_2$ with the membrane or cytoskeleton.

The function of platelet cytoskeleton in localizing signal molecules has been suggested in recent years. That the membrane skeleton is also considered to be involved in regulation of signaling events since tyrosine kinases such as $pp60^{c\text{-}src}$ and $pp62^{c\text{-}yes}$, found in association with membrane skeleton during platelet activation, have been implicated in the tyrosine phosphorylation of some cytoskeletal proteins. (Fox et al., 1993). Phosphatidyliositol 3-kinase, PKC and $p125^{FAK}$, translocated to cytoskeleton following platelet aggregation, is also considered to be involved in regulating cytoskeleton reorganization (Zhang et al., 1992). These observation may fit in with our findings which

reveal a complex interaction of $cPLA_2$ with the unactivated and activated platelet membrane and cytoskeleton.

Unactivated platelets have a major portion of $cPLA_2$ associated with the membrane, which is not mediated by actin and calcium, and possibly mediated by interaction through an unknown mechanism with several membrane proteins. In an attempt to determine the association of the $cPLA_2$ in resting platelets with membrane proteins, $cPLA_2$ co-IPPT with different antibodies such as against GPIb, CD9, β2-m, CD32 and CD62 membrane proteins were carried out. The results demonstrated $cPLA_2$ was associated with several surface membrane proteins such as GPIb, CD9, β2-m and CD32 but not CD62. The possible explanation for no detectable $cPLA_2$ associated with CD62, is that CD62, which is also called P-selectin, is expressed on the membranes of α granules in resting platelets, and only appears on the platelet surface following stimulation and degranulation.

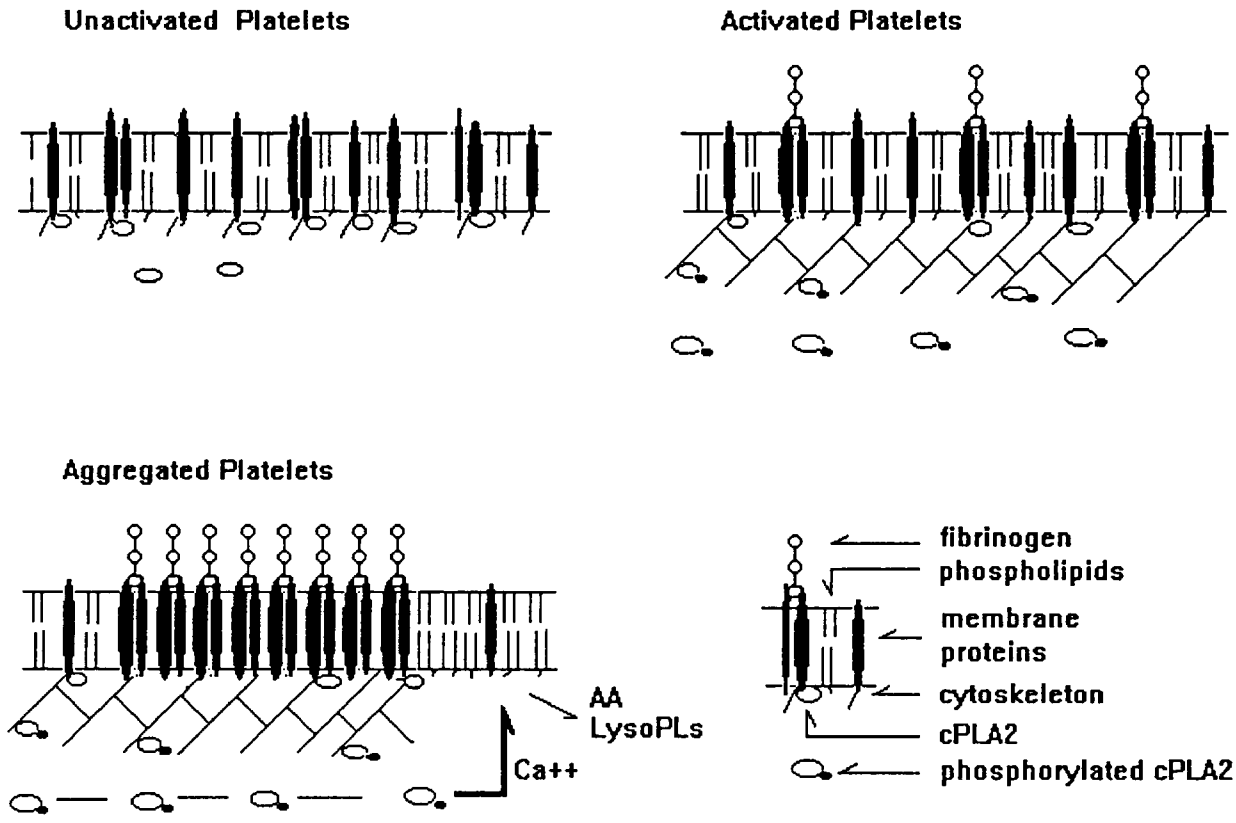
The redistribution of $cPLA_2$ occurring upon platelet activation is from a mostly non-calcium based membrane association of $cPLA_2$ to a mostly free cytosolic $cPLA_2$ and cytoskeletal actin based association of $cPLA_2$ since sonication in the presence of DNase I (which depolymerizes actin by binding the actin monomer thus shifting the equilibrium away from filamentous actin) releases considerably more $cPLA_2$ into the cytosol after platelet activation. Even in the activated platelets a substantial amount of $cPLA_2$ remains associated with the membrane even after sonication in the presence of DNase I. The results from measuring cytosolic PLA_2 activity in vitro are consistent with these $cPLA_2$ distribution assays plus or minus added DNase I. The more $cPLA_2$ in the cytosol, and

the more PLA_2 activity is detected. Our results also do not agree with current published hypotheses about $cPLA_2$ distribution, that all $cPLA_2$ exists in the cytosol, and only associates with the membrane through calcium mediated binding.

The association of $cPLA_2$ with the membrane fraction platelets may have a regulatory role in mediating its activation and/or interaction with the phospholipid substrates on the membrane. This role could be either negative or positive. To determine if the membrane-associated $cPLA_2$ was activated, its phosphorylation state relative to the cytosolic $cPLA_2$ was determined. The results showed that 40%-50% of the phosphorylated $cPLA_2$ is recovered in the cytosolic compartment indicating that either the phosphorylation of $cPLA_2$ releases it from the membrane fraction or that only the cytosolic $cPLA_2$ is available for phosphorylation. The results also suggest that the membrane-associated $cPLA_2$ is not highly activated since phosphorylation has been shown to upregulate $cPLA_2$ activity, and $cPLA_2$ in membrane fractions did not have any detectable phosphorylation by ^{32}P-labeling and only trace amounts by the gel shift assay. Blocking aggregation by Ro 44-9883 did not effect the distribution of $cPLA_2$ or phosphorylated $cPLA_2$ in TRAP-stimulated platelets.

A summary of our findings reveals a complex interaction of $cPLA_2$ with cytoskeleton (see the diagram below). Our hypothesis is that a part of the $cPLA_2$ in activated platelets is sequestered by membrane protein interaction and is partially released during the actin and membrane protein rearrangements associated with activation. Unactivated $cPLA_2$ is mostly associated with membrane, in non-calcium and non-actin based association. Following platelet aggregation $cPLA_2$ is redistributed with a small part

of the $cPLA_2$ remaining in a non-calcium and non-actin based membrane association, combined with a larger portion of free cytosolic $cPLA_2$ and actin filament associated $cPLA_2$. After platelet aggregation, the membrane proteins are reorganized into clusters, which causes $cPLA_2$ substrates to be exposed and facilitates the interaction between $cPLA_2$ and phospholipids. In the presence of calcium, activated $cPLA_2$ releases arachidonic acid and lysophospholipids from the membrane phospholipids.



Schematic Diagram of $cPLA_2$ Distribution in Platelets

Previous attempts have been made to study $cPLA_2$ redistribution following cellular activation. Durstin et al. (1994) reported that a western blot analysis of cytosol and membrane fractions (a similar approach as in our studies) indicated a translocation of $cPLA_2$ to the membrane fraction during neutrophil activation by growth factor and fMLP peptide. This translocation was enhanced and sustained by pretreatment with growth factor which activates MAP-kinase. Based on mobility shifts associated with $cPLA_2$ phosphorylation, these authors suggested that the activated phosphorylated $cPLA_2$ was translocated to the membrane. A more direct determination of $cPLA_2$ phosphorylation state in the cytosol versus membrane compartments wasn't carried out, nor was the percentage relative to total $cPLA_2$ determined.

Sa et al. (1995) recently reported that the results of $cPLA_2$ activity assay in vitro and gel-shift assay for phosphorylated $cPLA_2$ indicated a translocation of activated $cPLA_2$ to the membrane from the cytosol during endothelial cell activation by basic fibroblast growth factor. The increased $cPLA_2$ activity and phosphorylated $cPLA_2$ in the membrane fraction was correlated with decreases in the cytosol following stimulation, but they also didn't determine the percentage relative to total $cPLA_2$. They also demonstrated $cPLA_2$ activation was upregulated by phosphorylation with MAP-kinase, and required micromolar Ca^{2+}. So far no direct evidence has been presented to indicate subcellular translocation of activated $cPLA_2$ in stimulated platelets.

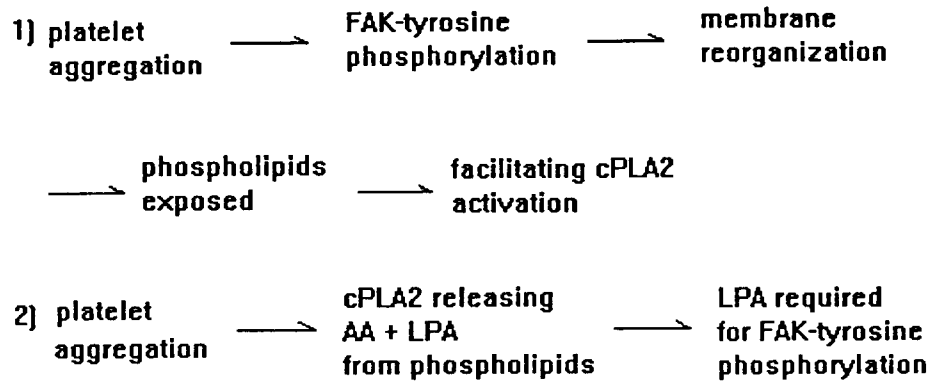
The state of phospholipid ordering has been suggested to be a mechanism involved in regulating intracellular activity of $cPLA_2$. Many studies have supported the concept that the activity of $cPLA_2$ against native and artificial membranes is sensitive to the

phospholipid composition, added detergents, and method of native membrane preparation (Nozawa et al.,1991). Our results reveal a possibility that the $cPLA_2$ activity in platelets stimulated with a weak agonist may be dependent on aggregation which in turn may regulate membrane phospholipid availability through receptor redistribution. This must be a selective process for $cPLA_2$ since the activity of PLC, reflected by IP_3 formation and ^{32}P-labeling of diacylglycerol to form phosphatidic acid, is unaffected by blocking aggregation.

The only other facet of signal transduction that was affected by Ro 44-9883 was tyrosine phosphorylation of $p125^{FAK}$, with the nearly 3 fold increased over the controls after 6 minutes exposure to 20 uM TRAP, being completely abolished by blocking aggregation. This was in agreement with previous findings (Haimovich et al., 1993; Shattil et al., 1994) suggesting a requirement for both integrin receptor-mediated aggregation or adhesion and agonist-mediated stimulation for increased $p125^{FAK}$ tyrosine phosphorylation. The membrane reorganization caused by platelet aggregation is possibly necessary for $cPLA_2$ substrates to be exposed. Tyrosine phosphorylation of $p125^{FAK}$ may be upstream or downstream regulation of $cPLA_2$ to release arachidonic acid and LPA.

Our experimental results suggest two hypotheses (see scheme below) for platelet aggregation finally leading to FAK-tyrosine phosphorylation and the $cPLA_2$ activation. In support of the second scheme, FAK tyrosine phosphorylation induced by LPA has been confirmed in Swiss 3T3 cells (Seufferlein et al. 1994). This may not fit in with the results from platelets because the high doses of TRAP in the absence of aggregation still causes TxA_2 formation, but not FAK tyrosine phosphorylation. Even addition of

exogenous LPA did not restore FAK tyrosine phosphorylation in the absence of aggregation in our experiments. Meanwhile the first scheme doesn't fit the results with the high doses of TRAP since this overcomes the inhibition of TxA_2 production but not FAK tyrosine phosphorylation. However high doses of TRAP may bypass the requirement for aggregation and FAK tyrosine phosphorylation, and directly affect the membrane reorganization to facilitate $cPLA_2$ activation. The third possibility is not shown that FAK tyrosine phosphorylation may not be related to $cPLA_2$ activation.



There is perhaps a physiological basis for such a dependency on aggregation for generation of PLA_2 metabolites and dense granule secretion in response to weak agonists. This would insure that only platelets recruited into the hemostatic plug will release dense granule contents or form TxA_2 to promote further platelet recruitment to the growing hemostatic plug. Such a mechanism would limit downstream platelet activation and formation of emboli, as well as vasoconstriction.

REFERENCES

Alig, L., Edenhofer, A., Hadvary, P., Hurzeler, M., Knopp, D., Muller, M., Beat, S., Trzeciak, A., and Weller, T. (1992) Low molecular weight, non-peptide fibrinogen receptor antagonists. J Med Chem 35:4393-4407

Banga, H.S., Simons, E.R., Brass, L.F. & Rittengouse, S.E. (1986) Activation of phospholipase A and C in human platelets exposed to epinephrine: role of glycoproteins IIb/IIIa and dual role of epinephrine. Proc Natl Acad Sci USA 83: 9197-9201

Bartoli, F., Lin, H., Ghomashchi, F., Gelb, M.H., Jain, M.K. & Apitz-Castro, R. (1994) Tight binding inhibitors of 85-kDa phospholipase A_2 but not 14-kDa phospholipase A_2 inhibit release of free arachidonate in thrombin-stimulated human platelets. J Biol Chem 269:15625-15630

Blenis, J. (1991) Growth regulated signal transduction by the MAP kinases and RSKs. Cancer Cells 3:445-449

Blenis, J. (1993) Signal transduction via the MAP kinase; proceed at your own RSK. Proc Natl Acad Sci USA 90:5889-5892

Bode, A.P., Sandberg, H., Dombrose, F.A., Lentz, B.R. (1985) Association of factor V activity with membranous vesicles released from human plateletes: Requirement for platelet stimulation. Thromb Res 39:49

Brace, L.D., Venton, D.L. and Le Breton, G.C. (1985) Thromboxane A_2/prostaglandin H_2 mobilizes calcium in human blood platelets. Am J Physiol 249:H1

Bress, L.F., Hoxie, J.A. and Manning, D.R. (1993) Signaling through G proteins and G protein-coupled receptors during platelet activation. Thromb and Haemost 70 (1) 217-223

Broekman, M.J.(1986) Stimulated platelets release equivalent amounts of arachidonate from phosphatidylcholine, phosphatidylethanolamine, and inositides. J Lipid Res 27:884-891

Carroll, R.C., Butler, R.G., Morris, P.A. and Gerrard, J.M. (1982) Separable assembly of platelet pseudopodal and contractile cytoskeletons. Cell 30:385-395

Carroll, R.C., Worthington, R. E. and Boucheix, C. (1990) Stimulus-response coupling in human platelets activated by monoclonal antibodies to the CD9 antigen, a 24 kDa surface-membrane glycoprotein. Biochem J 266:527-535

Chang, J., Musser, J. H. & Mcgregor, H. (1987) Phospholipase A_2: function and pharmacological regulation. Biochem Pharmacol 36:2429-2436

Cichowski, K., McCormick, F. and Brugge, J.S. (1992) p21rasGAP association with Fyn, Lyn, and Yes in thrombin-activated platelets. J Biol Chem 267(8):5025-5028

Clark, J.D., Lin, L.L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milona, N. & Knopf, J.L. (1991) A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. Cell 65: 1043-1051

Coller, B.S., Peerschke, E.I.B., Scudder, L.E. and Sullivan, C.A. (1983) A murine monoclonal antibody that completely blocks the blinding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoprotein IIb and/or IIIa. J Clin Invest 72:325-338

Coller, B.S. (1985) Platelet-von Willebrand factor interactions, in Platelet Glycoproteins, edited by George, J., Phillips, D. and Nurden, A., 215-244

Cox, A.C., Carroll, R.C. White, J.G. and Rao, G.H.R. (1984) Recycling of platelet phosphorylation and cytoskeletal assembly. J Cell Biol 98:8-15

Detwiler, T.C. and Huang, E.M. (1980) The interrelationship of platelet aggregation, secretion and prostaglandin synthesis, in The Regulation of Coagulation, edited by Mann, K.G. and Taylor, F.B. Elsevier/North-Holland, New York. 377-383

Deuel, T.F., Huang,S.S. and Huang, S.J. (1986) Platelet derived growth factor: Purification, characterization and role in normal and abnormal cell growth, in Biochemistry of Platelets, edited by Phillips, D.R. and Shuman, M.A. 347-375

Durstin, M., Durstin, S., Molski, T.F.P., Becker, E.L. and Sha'afi, R.I. (1994) Cytoplasmic phospholipase A_2 translocates to membrane fraction in human neutrophils activated by stimuli that phosphorylate mitogen-activated protein kinase. Proc Natl Acad Sci U S A 91:3142-3146

Eichholtz, T., Jalink, K., Fahrenfort, I. and Moolenaar, W.H. (1993) The bioactive phospholipid lysophosphatidic acid is released from activated platelets. Biochem J 291:677-680

Fenton, J.W., (1988) Regulation of thrombin generation and functions. II Semin. Thromb Haemost 14:234-240

Ferrell, J.E. and Martin, G.S. (1988) Platelet tyrosine-specific protein phosphorylation is regulated by thrombin. Mol Cell Biol 8:3603-3610

Fisher, T.H., Gatling, M.N., McCormick, F., Duffy, C.M. and White, G.C. (1994) Incorporation of Rap 1b into the platelet cytoskeleton is dependent on thrombin activation and extracellular calcium. J Biol Chem 269:17257-17261

Foster, M.R., Hornby, E.J., Brown, S., Hann, M., Kitchin, J., Pike, N. and Ward, P. (1994) Inhibition of human platelet aggregation by GR91669, a prototype fibrinogen receptor antagonist. Thromb Res 75:269-284

Fox, J.E.B., Boyles, J.K., Reynolds, C.C. and Philips, D.R. (1984) Actin filament content and organization in unstimulated platelets. J Cell Biol 98:1985-91

Fox, J.E.B., (1993) The platelet cytoskeleton. Thromb Haemost 70 (6) 884-893

Fox, J.E.B., Lipfert, L., Clark, E.A., Reynolds, C.C., Austin, C.D. and Brugge, J.S. (1993) On the role of GP IIb/IIIa, $pp60^{c\text{-}src}$, $pp62^{c\text{-}yes}$, and $p21^{ras}$ GTPase-activating protein (GAP) with the membrane skeleton. J Biol Chem 189: 131-136

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

Gartner, T.K. and Bennett, J.S. (1985) The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregation and fibrinogen binding to activated platelets. J Biol Chem 260:11891-11894

Gerrard, J.M., Kindom, S.E., Peterson, D.A., Peller, J., Krantz, K.E. and White, J.G. (1979) Lysophosphatidic acids. Influence on platelet aggregation and intracellular calcium flux. Am J Pathol 96:423-438

Gerrard, J.M. (1988) Platelet aggregation: cellular regulation and physiologic role. Hosp Pract 23:89-108

Golden, A. and Brugge, J.S. (1989) Thrombin treatment induces rapid changes in tyrosine phosphorylation in platelets. Proc Natl Acad Sci U S A 86:901-905

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

Granot, Y., Van, P.V. and Schrier, R.W. (1990) Vasopressin dependent tyrosine phosphorylation of a 38 kDa protein in human platelets. Biochem Biophys Res Commun 168:566-573

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

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

Halenda, S.P., Banga, H.S., Zavoico, G.B., Lau, L. and Feinstein, M.B. (1989) Synergistic release of arachidonic acid from platelets by activators of protein kinase C and Ca^{2+} ionophores. Evidence for the role of protein phosphorylation in the activation of phospholipase A_2 and independence from the Na^+/H^+ exchanger. Biochemistry 28:7356-7363

Jennings, L.K. and Fox, J.E.B., Edwards, H.H., Philips, D.R. (1981) Changes in the cytoskeletal structure of human platelets following thrombin activation. J Biol Chem 256:6927-6932

Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K. and Nishizuka, Y. (1980) Phospholipid turnover as a possible transmembrane signal for protein phosphorylation during human platelet activation by thrombin. Biochem Biophys Res Commun 93:309

Kim, D.K., Kudo, I. and Inoue, K. (1988) Detection in human platelets of phospholipase A_2 activity which preferentially hydrolyzes an arachidonoyl residue. J Biochem (Tokyo) 104:492-494

Kim, D., Lewis, D.L., Graziadei, L., Neer, E.J., Bar-Sagi, D. and Clapham, D.E (1989) G-protein beta gamma-subunits activate the cardiac muscarinic K^+-channel via phospholipase A_2. Nature 337:557-560

King, G.L. and Buchwald, S. (1984) Characterization and partial purification of an endothelial cell growth factor from human platelets. J Clin Invest 73:392

Kramer, R.M.,Checani, C.C., & Deykin, D. (1987) Stimulation of Ca^{2+}-activated human platelet phospholipase A_2 by diacylglycerol. Biochem J 248:779-783

Kramer, R.M., Roberts, E.F., Manetta, J.V. Hyslop, P.A. and Jakubowski, J.A. (1993) Thrombin-induced phosphorylation and activation of Ca^{2+}-sensitive cytosolic phospholipase A_2 in human platelets. J Biol Chem 268:26796-26804

Kunicki, T.J., Pidard, D., Rosa, J.P. and Nurden, A.T. (1981) The formation of calcium-dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. Blood 58:268

Kunicki, T.J., Nugent, D.J., Staats, S.J., Orchekowski, R.P., Wayner, E.A. and Carter, W.G. (1988) The human fibroblast class II extracellular matrix receptor mediates platelet adhesion to collagen and is identical to the platelet glycoprotein Ia-IIa complex. J Biol Chem 263:4516

Lages, B. and Weiss, H.J. (1994) Evidence for a role of glycoprotein IIb-IIIa, distinct from its ability to support aggregation, in platelet activation by ionophores in the presence of extracellular divalent cations. Blood 83:2549-2559

Lange-Carter, C.A., Pleiman, C.M., Gardner, A.M., Blumer, K.J. and Johnson, G.L. (1993) A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260:315-319

Lecompte, T., Potevin, F., Champeix, P., Morel, M.C., Favier, R., Hurtaud, M.F., Schlegel, N., Samama, M. and Kaplan, C. (1990) Aequorin-detected calcium changes in stimulated thrombasthenic platelets - aggregation-dependent calcium movement in response to ADP. Thromb Res 58:561-570

Lin, L.-L., Wartman, M., Lin, A.Y., Knopf, J.L., Sem, A. & Davis, R.J. (1993) $cPLA_2$ is phosphorylated and activated by MAP kinase. Cell 72: 269-278

Mounier, C., Faili,. A., Vargaftig, B. B., Bon, C. and Hatmi, M. (1993) Secretory phospholipase A_2 is not required for arachidonic acid liberation during platelet activation. Eur J Biochem 216:169-175

Nakashima, S., Chatani, Y., Nakamura, M., Miyoshi, N., Kohno, M. and Nozawa, Y. (1994) Biochem Biophys Res Commun 198:497-503

Nalamura, S. and Yamamura, H. (1989) Thrombin and collagen induce rapid phosphorylation of a common set of cellular proteins on tyrosine in human platelets. J Biol Chem 264:7084-7091

Nalefski, E.A., Sultzman, L.A., Martin, D.M., Kriz, R.W., Towler, P., Knopf, J.L. and Clark, J.D. (1994) Delineation of two functionally distinct domains of cytosolic phospholipase A_2, a regulatory Ca^{2+}-dependent lipid-binding domain and a Ca^{2+}-independent catalytic domain. J Biol Chem 269:18239-18249

Nozawa, Y., Nakashima, S. and Nagata, K. (1991) Phospholipid-mediated signaling in receptor activation of human platelets. Biochim Biophys Acta 1082:219-238

Oates, J.A., Fitzgerald, G.A., Branch, R.A., Jackson, E.K., Knapp, H.R. and Roberts, L.J. (1988) Clinical implication of prostaglandin and thromboxane A formation. N Engl J Med 319:689-698

Papkofe, J., Chen, R., Blenis, J. and Forsman, J. (1994) p42 mitogen-activated protein kinase and p90 ribosomal S6 kinase are selectively phosphorylated and activated during thrombin-induced platelet activation and aggregation Mol Cell Biol 463-472

Phillips, D.R., Charo, I.F., Parise, L.V., and Fitzgerald, L.A. (1988) The platelet membrane glycoprotein IIb-IIIa complex. Blood 71:831-843

Picot, D., Loll, P.J. and Garavito, R.M. (1994) The X-ray crystal structure of the membrane protein prostaglandin H_2 synthase-1. Nature 367:243

Pidard, D., Didry, D., Kunicki, T.J. and Nurden A.T. (1986) Temperature-dependent effects of EGTA on the membrane glycoprotein IIb-IIIa complex and platelet aggregability. Blood 67:604-611

Plow, E.F., McEver, R.P., Coller, B.C. et al. (1985) Related binding mechanisms for fibrinogen, fibronectin, von Willebrand factor and thrombospondin on thrombin-stimulated human platelets. Blood 66:724

Powling, M.J. and Hardisty, R.M. (1985) Glycoprotein IIb-IIIa complex and Ca^{2+} influx into stimulated platelets. Blood 66:731-734

Pumiglia, K.M. and Feinstein, M.B. (1993) Thrombin and thrombin receptor agonist peptide induce tyrosine phosphorylation and tyrosine kinases in the platelet cytoskeleton. Translocation of $pp60^{c\text{-}src}$ and integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) is not required for aggregation, but is dependent on formation of large aggregate structure. Biochem J 294:253-260

Rifkin, D.B., Kojima, S., Abe, M. and Harpel, J.G. (1993) TFG-β: Structure, function, formation. Thromb Haemost 70:177

Rittenhouse, S.E., & Horne, W.C. (1984) Ionomycin can elevate intraplatelet Ca^{2+} and activate phospholipase A without activating phospholipase C. Biochem Biophys Res Commun 123:393-397

Rubinstein, E., Boucheix, C., Urso, I. and Carroll, R.C. (1991) Fcγ receptor-mediated interplatelet activation by a monoclonal antibody against β2 microglobulin. J Immunol 147:3040-3046

Rybak, M.E.M. and Renzulli, L.A. (1992) Effect of calcium channel blockers on platelet GPIIb-IIIa as a calcium channel in liposomes: Comparison with effects on the intact platelet. Thromb Haemost 67:131-136

Sa, G., Murugesan, G., Jaye, M., Ivashchenko, Y. and Fox, P.L. (1995) Activation of cytosolic phospholipase A_2 by basic fibroblast growth factor via a p42 mitogen-activated protein kinase-dependent phosphorylation pathway in endothelial cells. J Biol Chem 270:2360-2366

Scharf, R.E., and Harker, L. A. (1987) Thrombosis and atherosclerosis: regulatory role of interactions among blood components and endothelium. Blut 55(3):131-144

Schick, P.K. (1994) Megakaryocyte and platelet lipids, in Hemostasis and Thrombosis: Basic Principles and Clinical Practice, 3d ed, edited by Colman, R.W., Hirsh, J., Marder, V.J., and Salzman, E.W. 574-589

Seufferlein, T. and Rozengurt, E. (1994) Lysophosphatidic acid stimulates tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130. J Biol Chem 269:9345-9351

Shattil, S.J. and Bruggge, J.S. (1991) Protein tyrosine phosphorylation and the adhesive functions of platelets. Curr Opin Cell Biol 3:869

Shattil, S.J., Haimovich, B., Cunningham, M., Lipfert, L., Parsons, J.T., Gigsberg, M.H. and Brugge, J.S. (1994) Tyrosine phosphorylation of $pp125^{FAK}$ in platelets requires coordinated signaling through integrin and agonist receptors. J Biol Chem 269: 14738-14745

Shebuski, R.J., Berry, D.E., Bennett, D.B., Romoff, T., Storer, B.L., Ali, F. and Samanen, J. (1989) Demonstration of Ac-Arg-Gly-Asp-Ser-NH_2 as an antiaggregatory agent in the dog by intracoronary administration. Thromb Haemost 61:183-188

Sixma, J.J. (1994) Interaction of blood platelets with the vessel wall, in Hemostasis and Thrombosis, 3d ed. edited by Bloon, A.L., Forbes, C.D., Thomas, D.P. and Tuddenham, E.G.D. 259-285

Theroux, P.S., Kouz, M.L., Knudtson, C., Kells, J., Nasmith, L., Roy, S., Dalle Ave, B., Steiner, Z.X., and Rapold, H.J. (1994) A randomized double-blind controlled trial with the non-peptidic platelet GPIIb/IIIa antagonist Ro 44-9883 in unstable angina. Circulation 90:1243 (Abstr.)

Tohyama, Y., Yanagi, S., Sada, K. and Yamamura, H. (1994) Translocation of $p72^{syk}$ to the cytoskeleton in thrombin-stimulated platelets. J Biol Chem 269:32796-32799

Torti, M., Ramaschi, G., Sinigaglia, F., Lapetina, E.G. and Balduini, C. (1994) Glycoprotein IIb-IIIa and the translocation of Rap2B to the platelet cytoskeleton. Proc Natl Acad Sci USA 91:4239-4243

Turitto, V.T., Weiss, H.J. and Baumagrtner H.R. (1983) Decreased platelet adhesion on vessel segments in von Willebrand's disease: a defect in initial platelet attachment. J Lab Clin Med 102:551-564

van der Bend, R.L., de Widt, J., van Corven, E.J., Mookenaar, W.H. and van Blitterswijk, W.J. (1992) The biologically active phospholipid, lysophosphatidic acid, induces

phosphatidylcholine breakdown in fibroblasts via activation of phospholipase D. Comparison with the response to endothilin. Biochem J 285:235-240

Walsh, P.N. and Schaier, A.H. (1994) Platelet-coagulant protein interactions, in Hemostasis and Thrombosis: Basic Principles and Clinical Practice, 3d ed, edited by Colman, R.W., Hirsh, J., Marder, V.J., and Salzman, E.W. 629-651

Ware, S.A., Decenzo, M.T., Smith, M. and Saitoh, M. (1991) Calcium mobilization and glycoprotein-IIb-IIIa complex ligands in epinephrine-stimulated platelets. Am J Physiol 260:H1619-H1624

Ware, J.A. and Coller, B.S. (1995) Platelet morphology, biochemistry, and function, in Williams Hematology, 5th ed, edited by Beutler, E., Lichtman, M.A., Coller, B.S. and Kipps, T.J. 1161-1163

Watson, S.P., Mcconnell, R.T. and Lapetina, E.G. (1985) Decanoyl lysophosphatidic acid induces platelet aggregation through an extracellular action. Biochem J 232:61-66

Weller, T., Alig, L., Hurzeler-Muller, M., Kouns, W.C. and Steiner, B. (1994) Fibrinogen receptor antagonists-a novel class of promising antithrombotics. Drugs of the Future 19:461-476

White, J.G. (1974) Electron microscopic studies of platelet secretion. Prog Hemost Thromb 2:49

White, J.G. (1994) Anatomy and structural organization of the platelet, in Hemostasis and Thrombosis: Basic Principles and Clinical Practice, 3d ed, edited by Colman, R.W., Hirsh, J., Marder, V.J., and Salzman, E.W. 397-413

Winitz, S., Gupta, S.K., Qian, N., Heasley, L.E., Nemenoff, R.A. and Johnson, G.L. (1994) Expression of a mutant G_{i2} A subunit inhibits ATP and thrombin stimulation of cytoplasmic phospholipase A_2-mediated arachidonic acid release independent of Ca^{2+} and mitogen-activated protein kinase regulation. J Biol Chem 269:1889-1895

Yamaguchi, A., Tanoue, K. and Yanazaki, H. (1990) Secondary signals mediated by GPIIb/IIIa in thrombin-activated platelets. Biochem Biophys Acta 1054:8-13

Zhang, J., Fry, M.J. and Waterfield, M.D. (1992) Activated phosphoinositide 3-kinase associates with membrane skeleton in thrombin-exposed platelets. J Biol Chem 267:4686

Zucker, M.B. (1980) Observations on the release reaction, in The Regulation of Coagulation, edited by Mann, K.G. and Taylor, F.B. Elsevier/North-Holland, New York. 385-391

APPENDIX

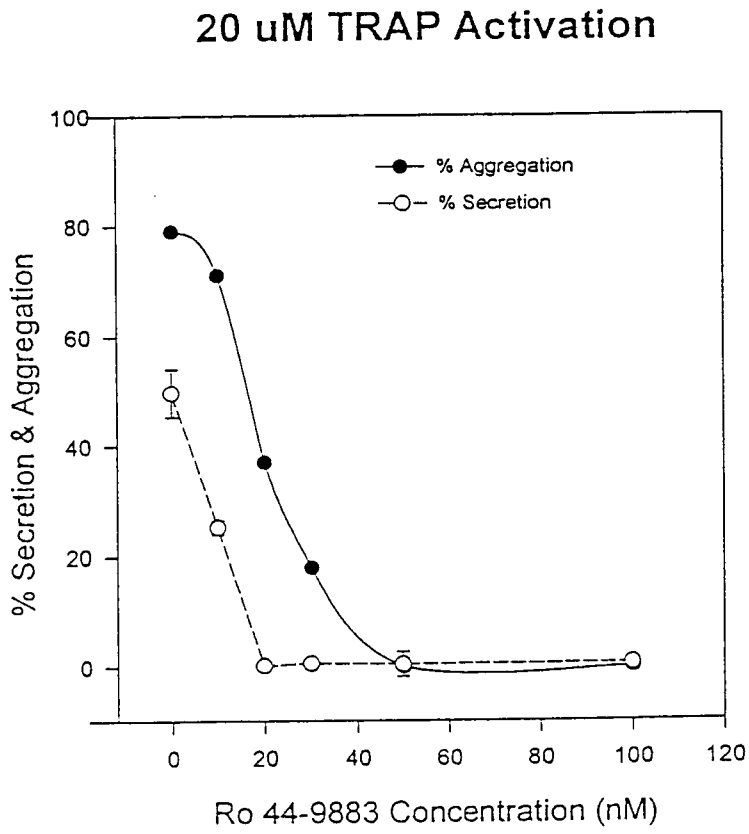


Figure 1. **Effects of Ro 44-9883 on Aggregation and Serotonin Secretion.** Platelet aggregation (closed circles) and serotonin release (open circles) were determined in triplicate aliquots of PRP pretreated for 3 minutes with the indicated concentration of Ro 44-9883 and then activated with 20 uM TRAP. After an additional 5 minutes continuous stirring at 37°C, the triplicate samples were assayed as described in Methods for serotonin release.

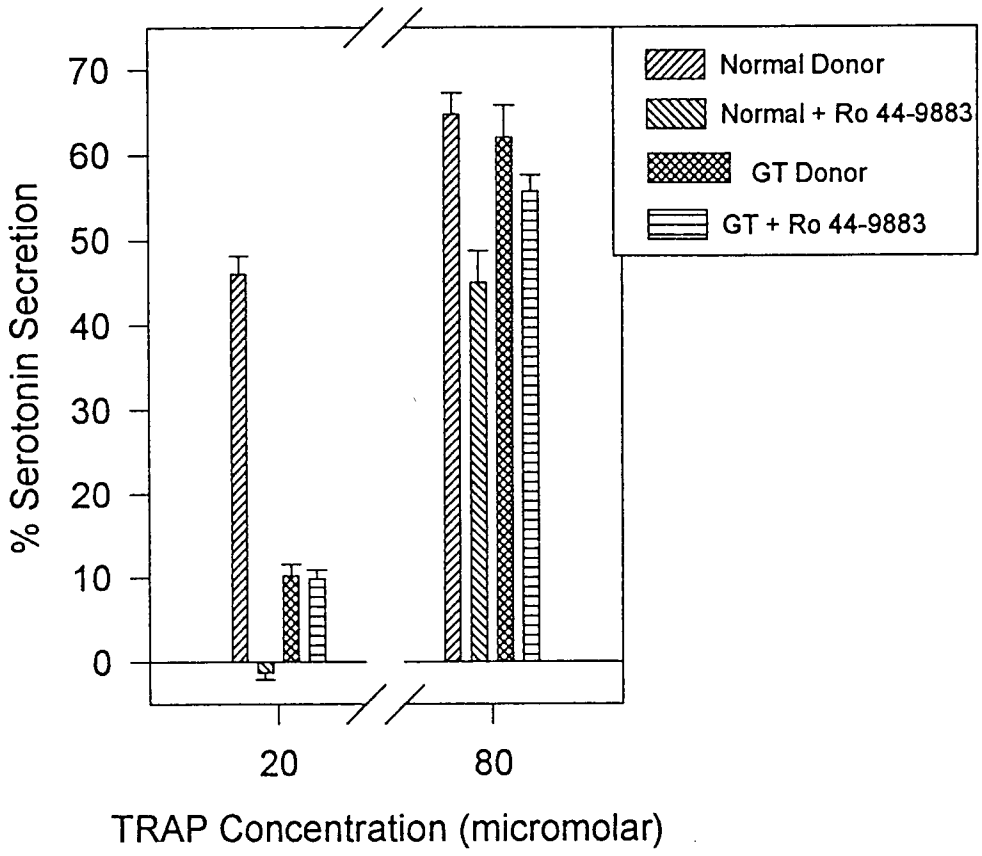


Figure 2. **Effects of Ro 44-9883 on Serotonin Secretion in normal and GT platelets.** Triplicate separate aliquots of PRP (from normal donor and GT donor) were assayed at the same day as described in Methods for serotonin secretion in response to 20 or 80 uM TRAP either without pretreatment or with 3 minutes pretreatment with 1 uM Ro 44-9883, with continuous stirring at 37°C (see figure insert for bar legend). Results are representative of 3 different GT patients).

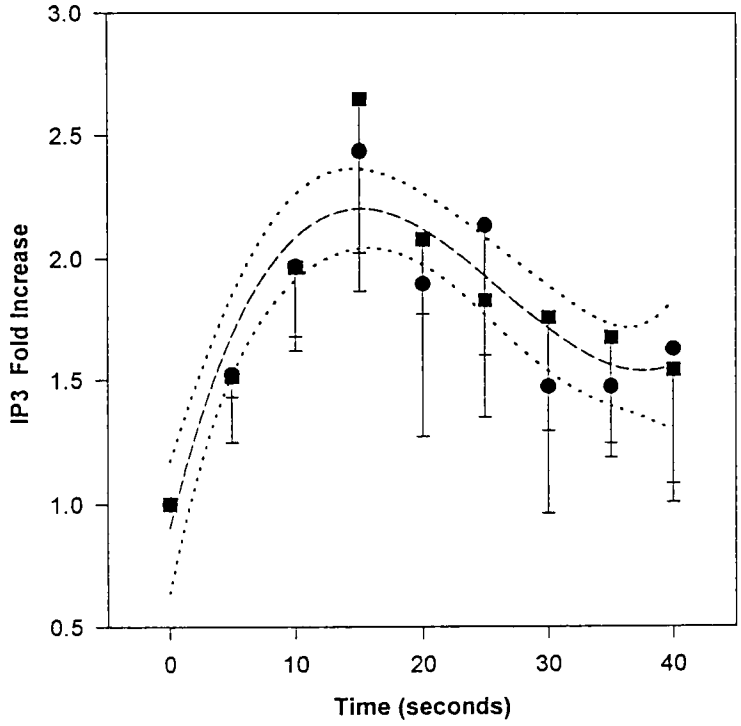


Figure 3. **Effects of Ro 44-9883 on IP_3 Formation**. Gel-filtered platelets (0.5 ml aliquots at 5 x 10^8) were sampled at the indicated times after adding 20 uM TRAP either without (circles) or with (squares) pretreatment with 1 uM Ro 44-9883. The IP_3 was extracted and measured as described in Methods. Data from 4 separate experiments is expressed as fold increase over basal levels (typically 0.91 ± 0.02 pmol/10^8 platelets). Data, with and without pretreatment, was fitted using SigmaPlot software to a single curve (dashed line) using 3rd order regression with confidence intervals of 95% (dotted lines). No significant difference is observed with or without 1 uM Ro 44-9883 pretreatment.

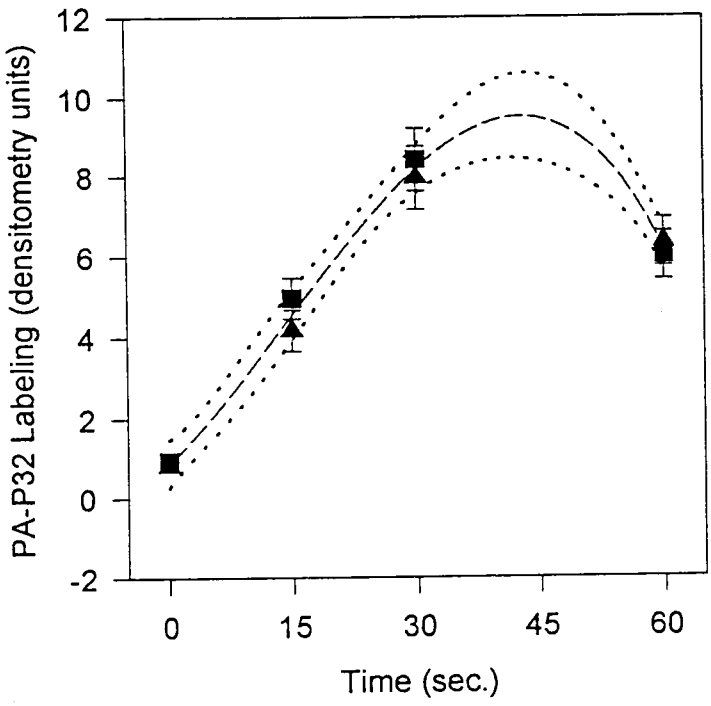


Figure 4. **Effects of Ro 44-9883 on ^{32}P-Labeled Phosphatidic Acid Formation.** ^{32}P-labeled GFP were activated with 20 uM TRAP for the indicated time points with continuous stirring at 37°C either without (squares) or with (triangles) 1 uM Ro 44-9883. Determinations were done for triplicate runs with the same platelets and the densitometry of TLC autoradiography expressed as arbitrary peak area units. Data was fitted, as in Figure 2, to a single curve (dashed line) with 3rd order regression and 95% confidence limits (dotted lines). Data is representative of 3 separate experiments with different donors. No significant difference is observed with or without 1 uM Ro 44-9883 pretreatment.

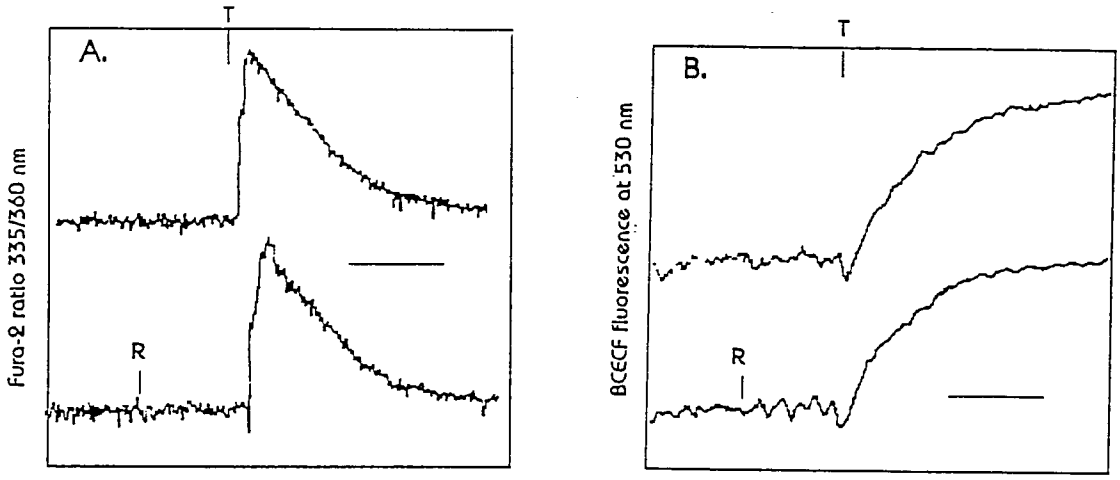


Figure 5A&B. **Effects of Ro 44-9883 on Calcium and pH Responses.** Gel-filtered platelets labeled with the calcium indicator fura-2 (panel A) or the pH indicator BCECF (panel B) were activated with continuous stirring at 37°C by 20 uM TRAP (addition indicated by line labeled T) after the indicated pretreatment with nothing (top trace) or 1 uM Ro 44-9883 (bottom trace, addition indicated by line labeled R). Data shown is representative of 4 different experiments with different donors. The horizontal line in each panel indicates a 1 minute time period. In these experiments, basal $[Ca]_i$ was 67 ± 5 nanomolar, peaking at 334 ± 18 nanomolar. Cytosolic pH was not calibrated in these experiments. No significant difference is observed with or without 1 uM Ro 44-9883 pretreatment.

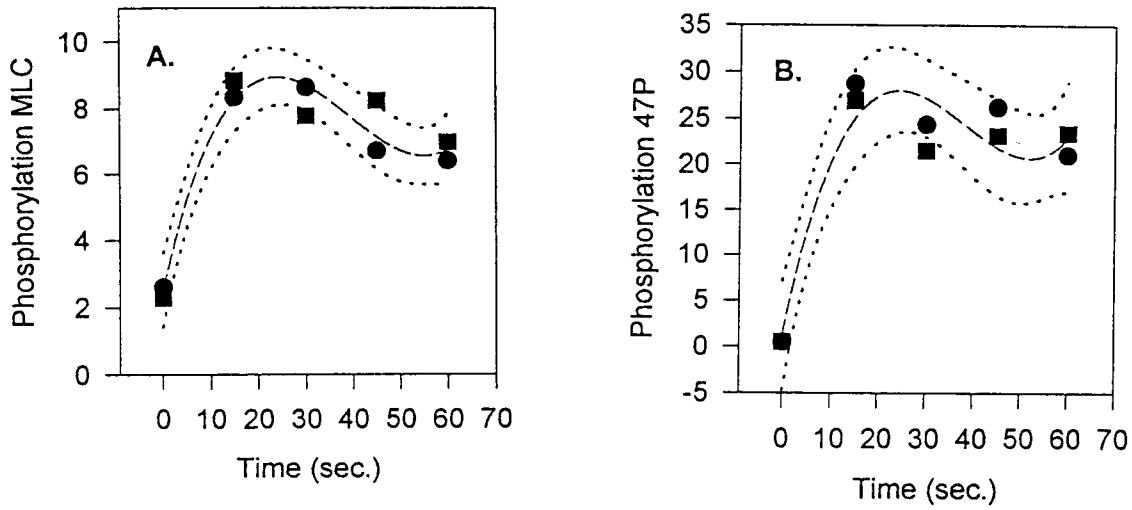


Figure 6A&B. **Effects of Ro 44-9883 on ^{32}P-Labeled Phosphorylation of Myosin Light Chain and p47.** ^{32}P-labeled GFP were activated with 20 uM TRAP without pretreatment (circles) or pretreatment with 1 uM Ro 44-9883 (squares) at the indicated times. After SDS-PAGE, phosphorylation of MLC (panel A) or p47 (panel B) was evaluated by densitometry of autoradiography. Units are expressed as arbitrary peak areas corresponding to MLC or p47. Similar data was obtained in 3 separate experiments with different donors. Data was fitted, as in Figure 2, to a single curve (dashed line) with 3rd order regression with 95% confidence limits (dotted lines). No significant difference is observed with or without 1 uM Ro 44-9883 pretreatment.

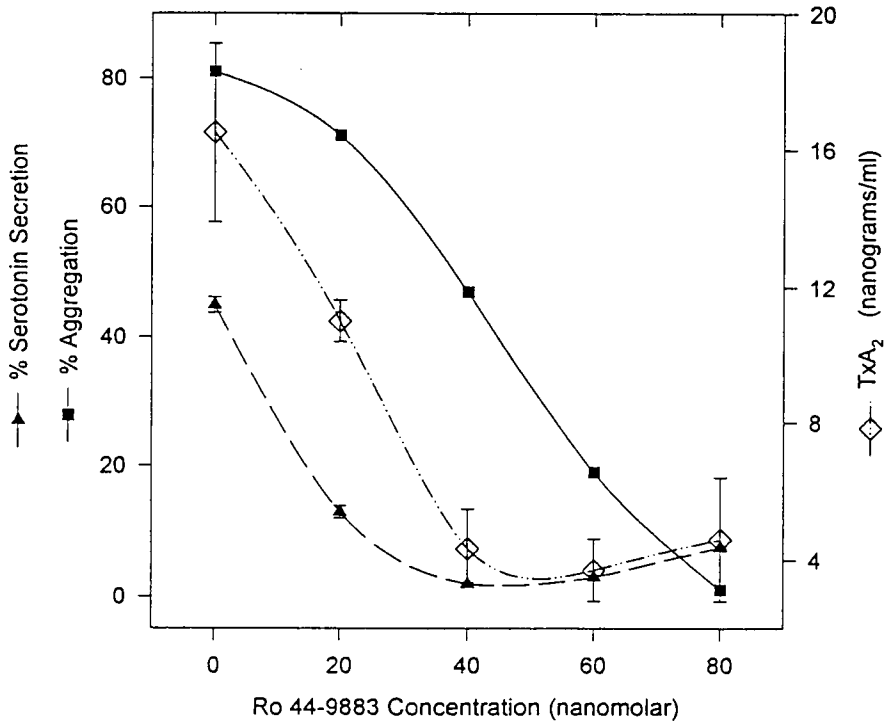


Figure 7. **Effects of Ro 44-9883 on TxA_2 Formation.** Platelet aggregation (closed squares), serotonin release (closed triangles), and TxA_2 formation (open diamonds) were determined in paired aliquots of PRP pretreated with the indicated concentration of Ro 44-9883 and then activated with 100 uM ADP. After an additional 5 minutes continuous stirring at 37°C the paired samples were assayed for either serotonin release or TxA_2. Aggregation and secretion data are expressed without normalization to facilitate comparison to TxA_2 formation.

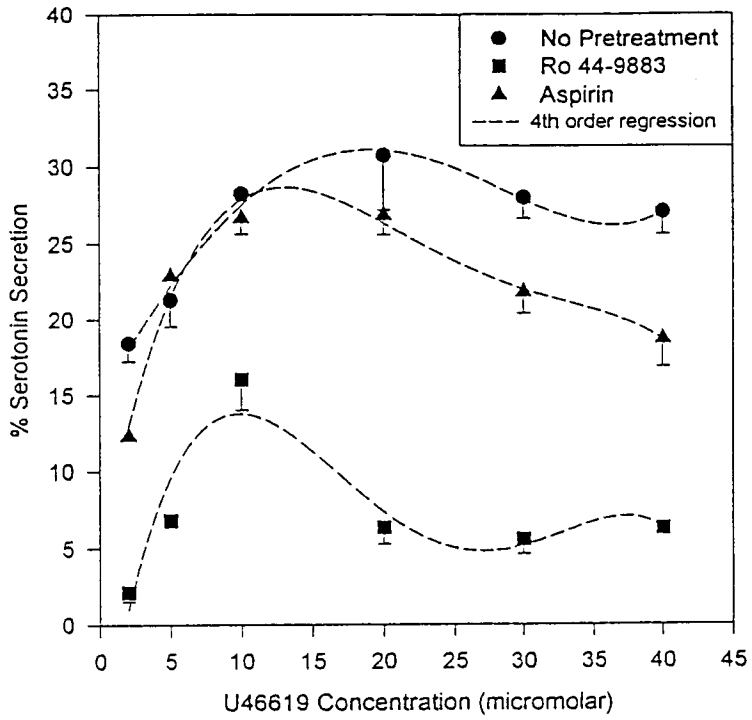


Figure 8. **Effects of U46619 on the Inhibited Serotonin Secretion.** PRP was not pretreated (circles), or pretreated 3 minutes with 1 uM Ro 44-9883 (squares), or 10 minutes with 100 uM aspirin (triangles). Aliquots from these pretreated samples were then activated with the indicated concentration of U46619 at 37°C with continuous stirring for 5 minutes and serotonin secretion measured as described in Methods. Curves are fitted using 4th order regression as previously described in figure 2.

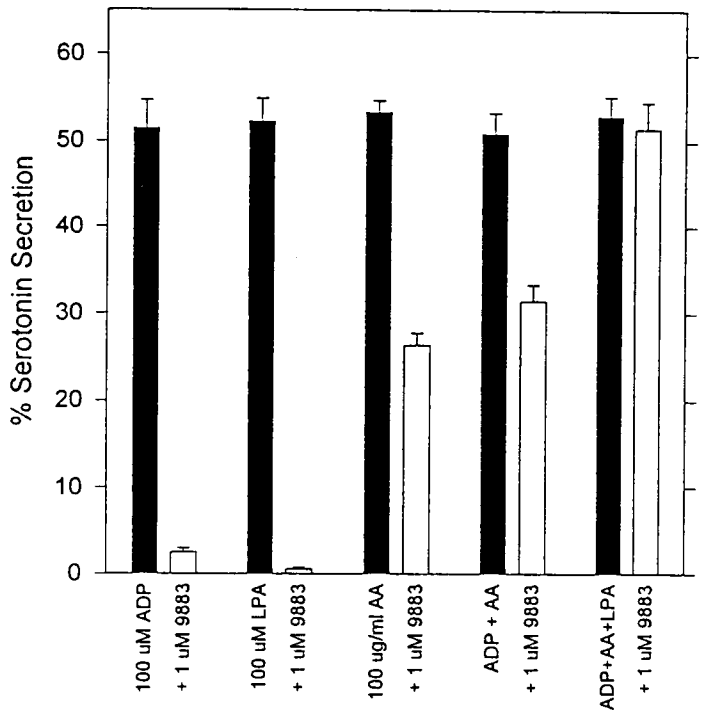


Figure 9. **Both TxA_2 and LPA are Required to Overcome the Inhibitory Effects on Serotonin Secretion.** PRP pre-labeled with C-14 serotonin was activated at 37°C with the indicated agonists, without (solid bars) or with (open bars) pretreatment with 1 uM Ro 44-9883. The indicated combination of activators was added simultaneously as freshly made mixtures and serotonin secretion determined on triplicate aliquots after 5 minutes continuous stirring. Data shown is from a single experiment representative of 4 different donors. Inhibition by Ro 44-9883 is significant ($p < 0.05$) for all agonists except for the last combination of ADP, arachidonic acid (AA), and LPA.

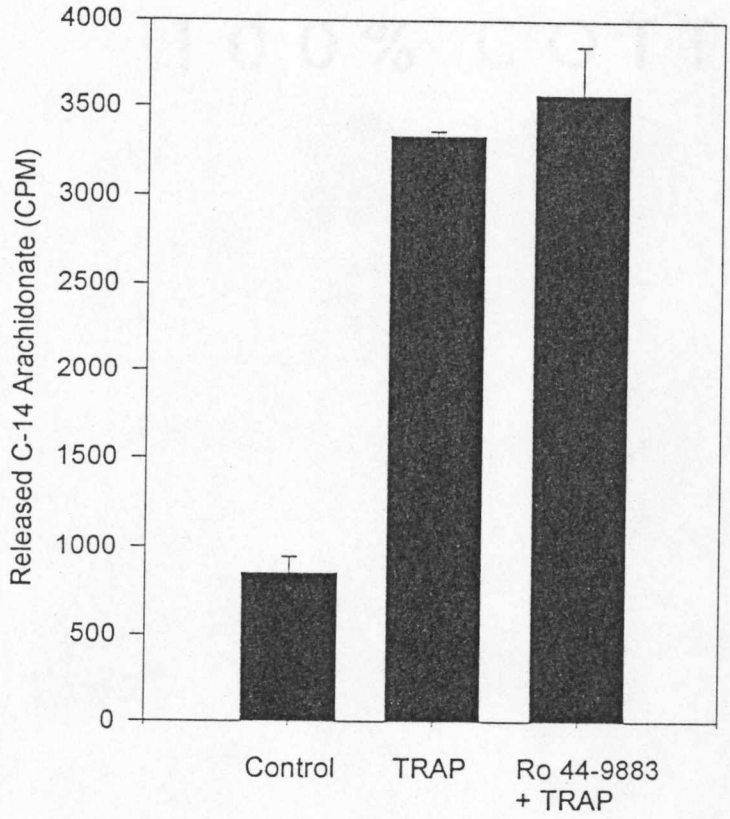


Figure 10. **In Vitro Assays of Phospholipase A_2 Activity.** Aliquots of GFP were not activated (bar labeled-Control), activated with 20 uM TRAP (bar labeled-TRAP), or pretreated with 1 uM Ro 44-9883 before activation with 20 uM TRAP (bar labeled-Ro 44-9883 + TRAP). Cytosolic PLA_2 activity was determined as described in Methods. Released ^{14}C arachidonic acid CPM was determined on triplicate assay samples by scintillation counting. We have verified a linear release of label from these samples over this time course. This is a representative result from 4 different donors showing no significant effect of 1 uM Ro 44-9883.

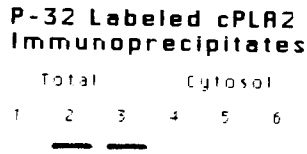


Figure 11. **^{32}P-Labeled cPLA$_2$ IPPT for Phosphorylated cPLA$_2$.** ^{32}P-labeled GFP were activated with 20 uM TRAP without (lane 2 & 5) or with 1 uM Ro 44-9883 pretreatment (lane 3 & 6). Lane 1 and 4 were controls. The samples of unfractionated (lane 1, 2 & 3), cytosol fractions (lane 4, 5 & 6) and membrane fractions (not shown) were mixed with Triton X-100 extraction buffer with protease and phosphatase inhibitors as described in Methods. The soluble extracts obtained by centrifugation were incubated with rabbit anti-cPLA$_2$ antibody. Immune complexes collected by protein A-sepharose were subject to SDS-PAGE and autoradiograms. The membrane fractions did not show any detectable phosphorylation of cPLA$_2$. Results shown are representative of 3 different donors.

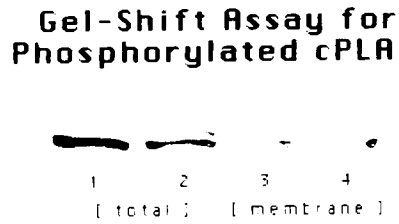


Figure 12. **Gel-Shift Assay for Phosphorylated $cPLA_2$.** Equal aliquots of GFP were activated with 20 uM TRAP (lane 2 & 4). Lane 1 and 3 were controls. The protein samples of unfractionated (labeled total), and membrane fractions (labeled membrane) were subjected to 10% SDS-PAGE and then immunoblotted with rabbit anti-$cPLA_2$, and $cPLA_2$ was detected by the ECL Kit.

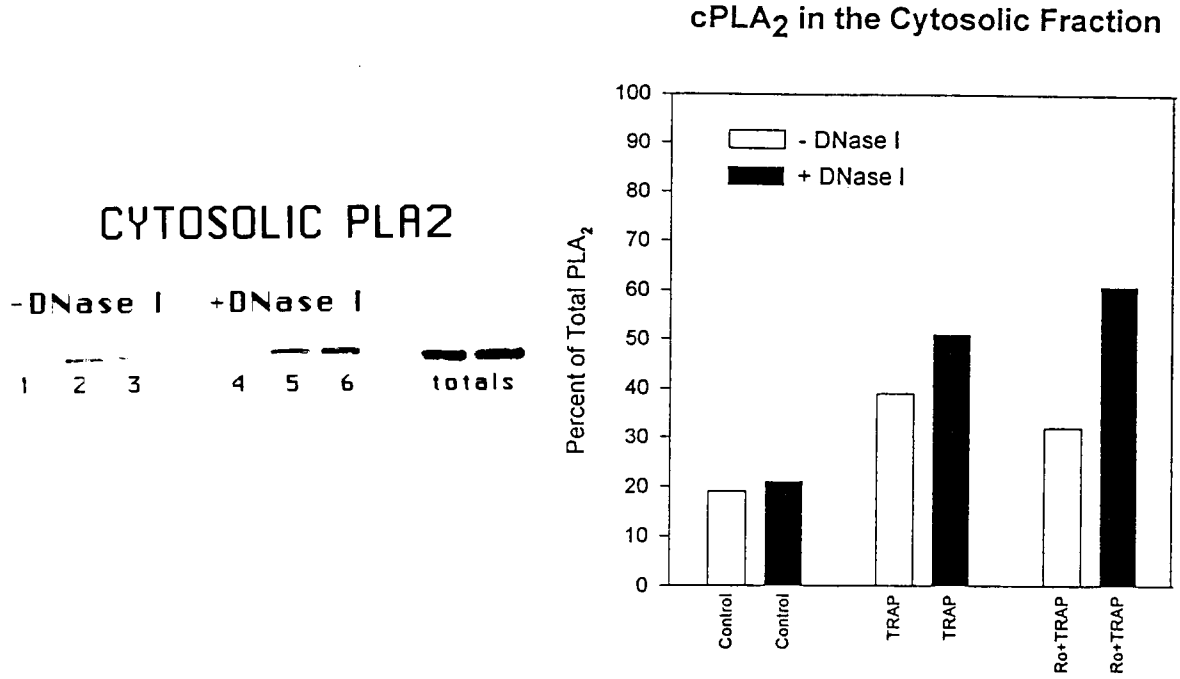


Figure 13. **Effect of DNase I on Distribution of $cPLA_2$** Equal aliquots of GFP were activated with 20 uM TRAP for 6 minutes (TRAP, lanes 2 & 5), or pretreated with 1 uM Ro 44-9883 before activation with 20 uM TRAP (Ro + TRAP, lanes 3 & 6). Lane 1 and 4 were controls. Before samples were lysed by sonication, the protease and phosphatase inhibiting buffer (described in Material and Methods) without or with 1 mg/ml DNase I was added. The unfractionated and cytosolic fraction samples were denatured and reduced (see Material and Methods), run on SDS-PAGE, and immunoblotted with rabbit anti-$cPLA_2$. $cPLA_2$ was detected by the ECL system and quantitated by densitometry. Similar data was obtained in three independent experiments with different donors. The increase of $cPLA_2$ in TRAP-stimulated platelets with DNase I treatment over that without DNAase I treatment is statistically significant ($P < 0.05$).

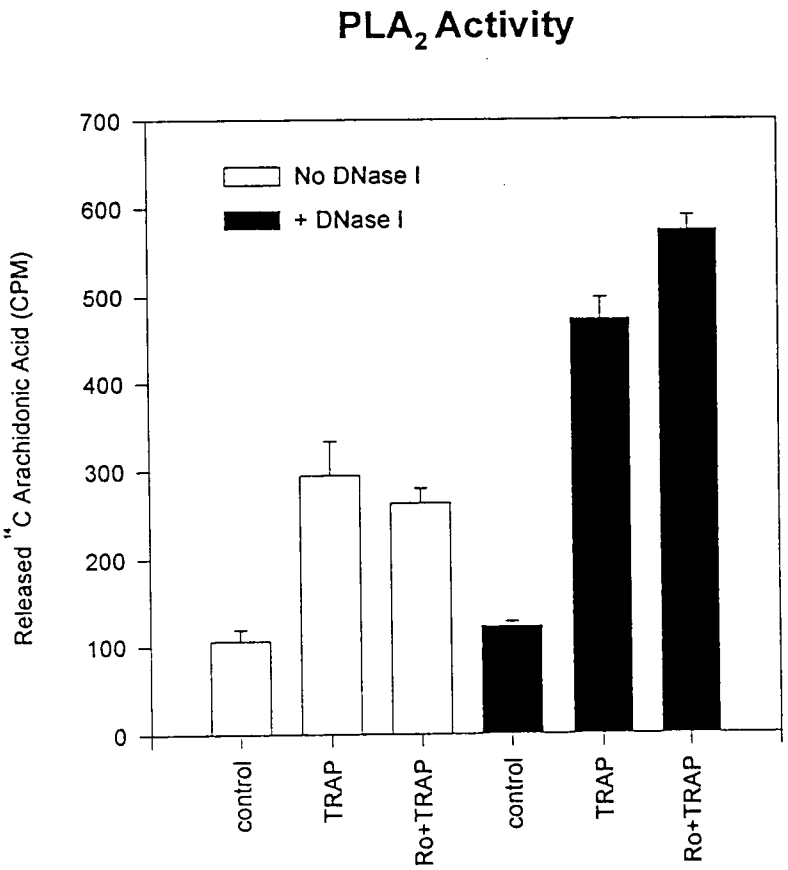
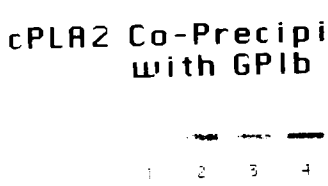


Figure 14. **<u>In Vitro</u> Assays of Phospholipase A_2 Activity (+/- DNase I).** Equal aliquots of GFP were not activated (labeled-control), or activated with 20 uM TRAP for 6 minutes (labeled-TRAP), or pretreated with 1 uM Ro 44-9883 before activation with 20 uM TRAP (labeled-Ro + TRAP). Cytosolic fractions were treated with or without 1 mg/ml DNase I, and cytosolic PLA_2 activity assays were carried out as described in Materials and Methods. Similar data was obtained in three independent experiments with different donors. 1 mg/ml DNase I significantly increased PLA_2 activity in stimulated samples ($P < 0.05$), but not in controls.

A.



B.

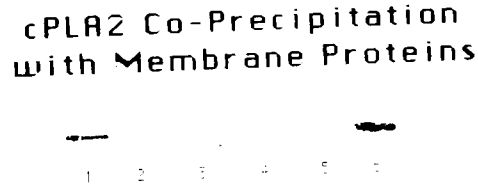


Figure 15A&B. **cPLA$_2$ Co-IPPT with Membrane Proteins.** In panel A equal aliquots of GFP (1 ml) were activated by TRAP with or without Ro 44-9883 preadded (lane 2 & 3), thrombin (lane 4), and unactivated (lane 1) for cPLA$_2$ co-IPPT with anti-GPIb antibody. In panel B equal aliquots of GFP (1.5 ml) was unactivated for cPLA$_2$ co-IPPT with other different anti-membrane antibodies. The membrane fractions from aliquots of GFP were prepared. Following treatment with 1% Triton X-100 buffer, the sample was then ultracentrifuged to remove insolubles. The extracts were separately incubated with GPIb mAb in panel A, and in panel B CD9 mAb (lane 1), β_2-microglobulin mAb (lane 2), CD32 mAb (lane 3), CD62 mAb (lane 4), and murine IgG2a as a control (lane 5). Immune complexes were collected and loaded on SDS-PAGE (lane 7, cPLA$_2$ standard), and a western blot with rabbit anti-cPLA$_2$ was performed.

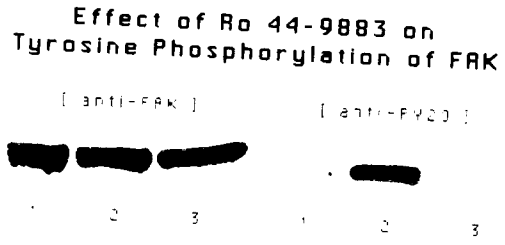


Figure 16. **Effect of Ro 44-9883 on Tyrosine Phosphorylation of Focal Adhesion Kinase.** Equal aliquots of GFP were activated with 20 uM TRAP for 6 minutes (lanes 2), or pretreated with 1 uM Ro 44-9883 before activation with 20 uM TRAP (lanes 3). Lane 1 was controls. Before samples were lysed by sonication, the buffer (described in Material and Methods) was added. The total platelet samples denatured by reducing denature solution (see Material and Methods) were run on SDS-PAGE and immunoblotted with anti-FAK and anti-phosphotyrosine (PY20) monoclonal antibodies separately. The protein, FAK and tyrosine phosphorylated FAK were detected by the ECL system. Similar data was obtained in three independent experiments with different donors.

VITA

Xiao-Fen Wang was born in Shanghai, The People's Republic of China on February 26, 1957. She attended primary, middle and high schools in Shanghai, where she graduated from the Shanghai 12th high school in 1974. She was assigned to the Shanghai Mechanical School to study for two years, and then worked as mechanical worker for one year in the Shanghai Mechanical Factory. She entered the Shanghai Second Medical University in 1978 where she received the Bachelor of Medicine degree in December 1982. From 1982 to 1990 she was in the Shanghai Children Hospital as a pediatrician following residency training. In July of 1990, she came to The United States of America to join her husband. She was accepted into the Comparative and Experimental Medicine Graduate Program as a Master's degree student under the supervision of Dr. Roger C. Carroll in the Department of Medical Biology of the University of Tennessee in 1993, and received a master degree of science at the University of Tennessee in May of 1996.