HUMAN PLATELET FIBRINOGEN RECEPTOR, GLYCOPROTEIN IIb-IIIa, REDISTRIBUTION AFTER PLATELET ACTIVATION

By

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ABSTRACT

HUMAN PLATELET FIBRINOGEN RECEPTOR, GLYCOPROTEIN IIb-IIIa, REDISTRIBUTION AFTER PLATELET ACTIVATION

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The platelet plays a key role in hemostasis and thrombosis. Normal function requires activation of the platelet and is dependent on specific membrane glycoproteins that act as receptors for both agonists and specific plasma adhesive proteins. One of the most abundant glycoproteins in the platelet is Glycoprotein IIb-IIIa (GPIIb-IIIa). It is responsible for binding fibrinogen, an adhesive protein critical for platelet-platelet cohesion. Examination of this receptor is therefore important in the understanding of platelet plug formation.

The participation of the GPIIb-IIIa receptor in both aggregation and adhesion events was examined using particulate (fibrinogen-gold) and soluble (biotinylated fibrinogen) probes to localize the receptor following platelet activation. The primary goal was to determine what effects, if any, the form of the fibrinogen probe had on GPIIb-IIIa receptor reorganization. Platelets were exposed to various stimuli, which included weak (ADP) and strong (thrombin) agonists, in both stirred and unstirred systems, as well as contact-induced activation to determine what effect each of these micro-environmental variables had on the GPIIb-IIIa receptor. Finally, to answer questions related to receptor internalization, bovine platelets, which contain no open canalicular system, were used to

evaluate endocytic vesicle formation.

From these manipulations, it was determined that, 1) the stirring of platelets was enough of a platelet activator to enable GPIIb-IIIa competent to bind ligand, and to enhance agonist-induced platelet stimulation, 2) low dose thrombin activation did not resemble that of a weak (ADP) agonist, 3) ligand type had an effect on platelet activation and function, 4) probe solubility had an effect on both the biological activity of the fibrinogen, and on the platelet handling of the ligand-receptor complex, and 5) bovine platelets handled particulate probes in a manner different than human platelets.

These results confirm that GPIIb-IIIa is critical in platelet activation and function. With a better understanding of this major platelet receptor, control of unwanted thrombosis, at the cellular level, may become a possibility.

DEDICATION

To my parents...I couldn't have done it without their help

To Karen...there isn't a better role model, or friend, or psychotherapist, or sewing buddy, or just about anything else...you truly made this possible

And to my friends...you were there when I needed decompression, you gave me drink, a comfortable place to be, a hazy mind, and memories that will always make me smile.

Thanks

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LIST OF ABBREVIATIONS AND TERMS

Adhesion	platelet interaction with non-platelet surface
ADP	adenosine diphosphate
ADP/F-Au	ADP-stimulated, F-Au incubated
ADP/F-Biotin	ADP-stimulated, F-Biotin incubated
ADP/PEG-Au	ADP-stimulated, PEG-Au incubated
AP-2	monoclonal antibody specific for GPIIb-IIIa complex
CB	cacadylate buffer
Cohesion	platelet-platelet interaction
dH_2O	distilled water
EDTA	ethylenediamine tetraacetic acid
F-Au	fibrinogen-colliodal gold complex
F-Biotin	biotinylated fibrinogen
GPIIb-IIIa	Glycoprotein IIb-IIIa
HABA	4'-hydroxyazobenzene-2-carboxylic acid
HEPES	hydroxyethylpiperazine ethane sulphonic acid
MoAb-Au	monoclonal antibody-colloidal gold complex
OCS	open canalicular system
PBS	phosphate buffered saline
PEG	polyethylene glycol
PEG-Au	polyethylene glycol-colloidal gold complex
PGE_1	prostaglandin E_1
PRP	platelet rich plasma
RT	room temperature
TB	Tyrode's buffer
TB+Ca^{++}	Tyrode's buffer with 1mM Calcium
TEM	transmission electron microscopy
Thrombin/F-Au	thrombin-stimulated, F-Au incubated
Thrombin/F-Biotin	thrombin-stimulated, F-Biotin incubated
Thrombin/PEG-Au	thrombin-stimulated, PEG-Au incubated

INTRODUCTION

Adhesion, spreading and recruitment of platelets are critical events in hemostatic plug formation. The mechanisms involved include specific membrane glycoproteins, ligand-induced transmembrane signaling events and subcellular changes in platelet cytoskeleton. This work focuses on the platelet receptor, Glycoprotein IIb-IIIa, and its redistribution in response to activation and subsequent ligand binding.

To date, most ultrastructural studies designed to evaluate receptor mobility have centered around the use of particulate probes. Evidence suggests that the platelet responds differently to particulate probes than it does to soluble phase ligands. In order to further evaluate these findings, protein biotinylation techniques were used in order to trace the path of receptor reorganization in platelets activated in suspension, as well as via contact-activation. In addition, the variable contribution of the platelet open canalicular system and endocytotic vesicle formation to receptor redistribution was assessed.

Specific receptor/ligand interactions are key in membrane coupled cellular responses. As an example, Isenberg (1987) has demonstrated that ADP stimulation, of and by itself, is

not sufficient to induce platelet aggregation. It has been suggested that fibrinogen binding to the GPIIb-IIIa receptor may be a critical event in subsequent initiation of subcellular biochemical events, and therefore, mandatory for ADP activation. However, stimulation with thrombin results in platelet activation independent of a specific requirement for fibrinogen binding. Therefore, not only is ligand/agonist binding important, but the specific nature and characteristics of the binding are equally important and in fact may determine the subsequent cellular responses.

These studies were designed to more precisely determine the nature of the specific ligand-receptor interactions in response to agonist stimulation that result in either aggregation or platelet adhesion and spreading.

LITERATURE REVIEW

Morphology, Function and Regulation of the Platelet Glycoprotein IIb-IIIa Receptor in Health and Disease

The platelet circulates in the blood as a metabolically active cell fragment, that is responsible for the leak-free maintenance of the vasculature. Under normal conditions, the platelet does not interact with the vessel wall, nor other platelets. It is when the vessel becomes damaged, or the platelet is affected by some pathological condition, that the platelet undergoes activation allowing it to perform its hemostatic function. This activation, results in both morphological and biochemical alterations facilitating platelet attachment and spreading on the vessel surface, and the recruitment of passing platelets to form a platelet plug.

Adhesion does not require prior activation, though, through adhesion, the platelet becomes activated (George, and Shattil, 1991). Contact-induced activation is mediated by platelet membrane receptors interacting with specific vessel wall components, such as collagen, von Willebrand factor, fibronectin and laminin (Ginsberg, et al., 1988). Platelet aggregation, in contrast, requires agonist-induced cell activation, with the conversion of

membrane glycoprotein IIb-IIIa (GPIIb-IIIa) to a competent fibrinogen/adhesive protein receptor. These adhesive proteins, primarily fibrinogen and to some degree, von Willebrand factor, act as bridges between adjacent platelet receptors, and facilitate aggregation (Gralnick, et al., 1991).

The platelet plasma membrane is an integral component of platelet activation. On the plasma membrane surface, there are a number of glycoprotein receptors involved in agonist and adhesive protein recognition. Intimately associated with the plasma membrane, is the platelet cytoskeletal system which includes actin and actin-binding proteins (Wencel-Drake, et al., 1991; Fox, J.E.B., 1986). In addition to the plasma membrane, the open canalicular system (OCS), which is a surface connected system that spans the platelet edge to edge, provides significant additional membrane for activation-induced conformational changes (Escolar, et al., 1989). Though these separate systems have been studied in depth, there is still much to be understood regarding the relationship between these platelet membrane elements, and receptor reorganization.

Of the glycoprotein receptors found on the membrane surface, Glycoprotein IIb-IIIa (GPIIb-IIIa) is the most abundant, with approximately 50,000 copies, and comprising 1% to 2% of the total platelet protein (Kieffer and Phillips, 1990). It is responsible for the binding of adhesive proteins, and, as a result, is instrumental in the formation of platelet aggregates (Plow, et al., 1984).

I. Physical Properties

The GPIIb-IIIa complex is composed of two components, glycoprotein IIb (GP IIb) and glycoprotein IIIa (GP IIIa). The components are associated in a calcium-dependent, complex that requires platelet activation, prior to being able to bind plasma adhesive proteins. This heterodiamer consists of a globular region and two projecting, tail-like structures that anchor it in the plasma membrane (Carrell, et al, 1985). In the presence of EDTA (a calcium chelator) and high pH, this structure is dissociated into separate GP IIb and GP IIIa components (Kunicki, et al., 1981; Gogstad, et al., 1982). This phenomenon was first demonstrated by isolating the GPIIb-IIIa complex, and incorporating it into lipid vesicles (Solum, N.O., 1985). One of the most useful tools in the characterization of GPIIb-IIIa, has been receptor specific monoclonal antibodies. Antibodies specific for each portion of the heterodiamer (Calvete, et al., 1986; Santoso, et al, 1986), as well as the activated complex (Pidard, D., 1986; Ruggeri, Z.M., 1986) have been utilized. Of interest, was that the activated complex expressed an antigenic site that was not found in the non-activated complex. This suggested a conformational change in one or both of the component glycoproteins following platelet activation. This modification of GPIIb-IIIa could result from: 1) membrane reorganization, 2) a new calcium-dependent conformation of one of the glycoproteins following activation, or 3) the impact of one glycoprotein on the molecular structure of the other, (Shattil, S.J., 1988).

To maintain the GPIIb-IIIa complex, a minimum calcium concentration of 1 x 10^{-5}M is required (Steiner, et al., 1991). However, complex dissociation is favored with elevated pH, increased temperature and low ionic strength (Rivas and González-Rodríguez, 1991). The dissociation of the complex, through the removal of calcium, can result in polymer formation of the component parts. Once polymerized, the addition of calcium does not reverse this effect, and the complex remains dissociated (Fitzgerald and Phillips, 1985). The calcium interaction with the GPIIb-IIIa complex is further illustrated by the presence of calcium-binding sites on the molecule. These binding sites have been shown to be located extracellularly, though the actual number of sites have not yet been clearly determined (Brass and Shattil, 1984).

The GPIIb-IIIa complex has also shown cation specificity. Studies report that either calcium or magnesium can be used to prevent dissociation of the complex (Gogstad, et al., 1982), and are required for complex association (Fitzgerald and Phillips, 1985), where manganese and other divalent cations are not. The effect of calcium on the GPIIb-IIIa receptor is, therefore, significant for three reasons. First, calcium binding to the receptor is required for association of the complex. Second, this association in turn allows agonist-induced activation/exposure of the complex and, therefore, binding of native adhesive proteins to augment platelet aggregation. Third, considering the fact that the platelet requires calcium for a number of physiological functions, the GPIIb-IIIa complex may act as a calcium channel or source of calcium for other platelet mechanisms (Davies, et al., 1989; Kroll and Schafer, 1989).

II. Receptor Function

The importance of the GPIIb-IIIa receptor on platelet function has been approached in three general ways: by the use of monoclonal antibodies to inhibit ligand binding (Calvete, et al., 1988), reconstitution of isolated complexes into phospholipid vesicles (D'Souza, et al., 1988), and the use of thrombasthetic platelets that lack or have modified GPIIb-IIIa receptors (Ginsberg, et al., 1986). These studies have demonstrated the following key concepts. The primary function of the GPIIb-IIIa complex is the binding of adhesive proteins. These proteins, are responsible for both platelet cohesion and adhesion to a non-platelet surface. The receptor binds a variety of proteins, including fibronectin, von Willebrand factor, and most importantly, fibrinogen (Plow, et al., 1986). All of these adhesive proteins contain a common recognition sequence determined to be Arg-Gly-Asp (RGD) (Pierschbacher and Ruoslahti, 1984). In addition, GPIIb-IIIa-like proteins have been located in the membranes of other cell types, such as endothelial cells (Sugiyama, et al., 1990; Cheresh, et al., 1989; Thiagarajan, et al., 1985), making it a prominent adhesive protein receptor in the vascular system.

The binding of ligand to the GPIIb-IIIa receptor can be divided into two general types. Those that are instrumental in platelet adhesion to non-platelet surfaces, and those that are involved in platelet-platelet cohesion. All adhesive ligands have been classified as proteins that contain the RGD sequence. This sequence is found in a large variety of proteins, including collagen, thrombospondin, laminin and vitronectin (Ruoslahti and

Pierschbacher, 1987), all associated with the vasculature. The binding of proteins involved with platelet aggregation or cohesion, also contain the RGD recognition sequence (Steiner, et al., 1989). It has been reported that fibrinogen, the primary ligand involved with activated platelet cohesion, has an additional recognition sequence. This peptide region is 12 amino acids long and is located at the carboxyl-terminal end of the gamma chain of the fibrinogen molecule. It is referred to as the dodecapeptide. No other adhesive protein contains this sequence (Lam, et al., 1987; Kloczewiak, et al., 1984). In both the adhesive and cohesive ligands, peptides containing the recognition sequence can inhibit ligand binding, and inhibit adhesion and aggregation of platelets (Nicholson, et al., 1991).

As stated earlier, in unstimulated platelets, the GPIIb-IIIa receptor is not capable of binding native ligand. Adhesive proteins only bind following platelet activation. Two basic theories have been proposed to explain this phenomenon. First, is a conformational change in the receptor that exposes the RGD-binding sequence (De Christofaro, et al., 1989; Parise, et al., 1987). And second, activation causes the removal of an inhibitory protein, exposing a hidden binding site (Polley, et al., 1981). Studies to determine the mechanism responsible for the "activation" of the receptor have primarily used receptor-specific monoclonal antibodies in the characterization. Antibodies specific for the ligand-competent binding site of the receptor, bind in much higher numbers to activated platelets (Pidard, D., 1986), thus demonstrating an activation-dependent alteration of the GPIIb-IIIa receptor. Other antibodies, do not require activation to bind, nor inhibit ligand binding

(Ruggeri and Hodson, 1989), thus illustrating two states in which the receptor can exist.

The GPIIb-IIIa receptor has also been shown to bind fibrinogen following treatment with alpha chymotrypsin (Pidard, et al., 1991). This treatment, in the absence of platelet activation and/or secretion, resulted in modification of the GP IIb molecule heavy chain, that allowed ligand binding and aggregation. The proteolytic degradation is both time and dose dependent, where an excess of either results in the loss the receptor binding ability.

Agonist-induced activation of the platelet, and subsequent conformational alteration of the GP IIb-IIIa receptor, is dependent on a number of factors specific to the agonist used. These factors include, agonist potency, their requirement for calcium for platelet activation, the metabolic pathways activated, and the responses initiated (ie. receptor activation, shape change, degranulation) (Shattil, S.J., 1988). For example, adenosine diphosphate (ADP) stimulation is enough to "expose" surface GPIIb-IIIa receptors (Isenberg, et al., 1989), while thrombin, can release internal stores of the receptor that are competent to bind ligand (Wencel-Drake, et al., 1986; Woods, et al., 1986).

The platelet plasma membrane is associated with the actin cytoskeleton (Suzuki, et al., 1991; Taylor, et al., 1975; Zucker-Franklin and Grutzy, 1972), thus providing the necessary medchanisms important to shape change, pseudopodia formation and clot retraction. The platelet contains a number of surface glycoproteins that are available to

be involved in this interaction, but only three have been implicated in this role, they are, glycoproteins Ib (Fox, et al., 1983; Solum, et al., 1983), IIb and IIIa (Painter and Ginsberg, 1982; Phillips, et al., 1980) . The discussion here, will only deal with the latter two.

The GPIIb-IIIa complex is considered an integral membrane glycoprotein, because removal from the plasma membrane requires disruption of the lipid bilayer with detergents (Jennings and Phillips, 1982). The complex, in the inactivated state is not believed to be associated with the platelet cytoskeleton. Detergent solubilization of thrombin stimulated platelets, or aggregated platelets, resulted in the GPIIb-IIIa complex being isolated along with the insoluble actin filaments isolated from these preparations (Phillips, et al., 1980). Since fibrinogen is the major ligand responsible for platelet aggregation, the association of the receptor with the actin network is likely central to platelet clot retraction. This theory of clot retraction is based on skeletal muscle models of actin-myosin interaction (Fox, J.E.B., 1985). If this model holds true, the myosin would pull actin filaments into the body of the activated platelet, and the actin-receptor interaction would provide retraction of the fibrin clot (Cohen, I., 1985). The association with the actin cytoskeleton may also explain the relative distribution of the GPIIb-IIIa receptor in stimulated and unstimulated platelets. In unstimulated platelets, the receptor is evenly dispersed along the plasma membrane (Isenberg, et al., 1989). Following activation, the receptor demonstrates clustering or patching (Santoso, et al., 1986). It has been reported, that this translocation is not due to the cytoplasmic actin network, but to

shorter actin filaments, present in the plasma membrane cytoskeleton (White and Escolar, 1990). In addition, the link between the receptor and the actin network has still not been identified whether it is a direct link or through the interaction of another protein.

III. GPIIb-IIIa Dysfunction

Glanzmann's thrombasthenia is an inherited abnormality of the platelet that involves the GPIIb-IIIa receptor. In this disorder, the platelet receptor is either reduced in number or lacking entirely (Phillips and Agin, 1977), or has reduced ability to bind ligand (Nurden and Caen, 1975). The primary clinical manifestation of this disease is reduced aggregation responses and clot retraction (Caen, et al., 1966), resulting in a spectrum of bleeding abnormalities ranging from mild bruising to hemorrhage (George and Nurden, 1987). These patients have normal platelet counts, normal shape change with agonist stimulation, and normal platelet secretion (Rendu and Dupuy, 1991). Glanzmann's patients can also undergo initial adhesion to collagen fibers and the subendothelium (Caen and Michel, 1972), suggesting that the receptor is not as critical in platelet adhesion as it is in platelet cohesion.

The binding of ligand, primarily fibrinogen, to these platelets has been examined in detail. Thrombasthenic platelets show a decreased level of internal stores of fibrinogen even with normal plasma concentrations (Gostad, et al., 1981). It has been shown that plasma fibrinogen is actively taken up and stored internally in the platelet alpha granules, either

during megakaryocyte maturation or as a circulating cell (George and Nurden, 1987). Upon strong enough activation, the platelet secretes these stores of fibrinogen along with other secretory proteins to facilitate platelet aggregation. This reduction in fibrinogen stores may be involved with the reduced ability of the platelets to aggregate (Gralnick,et al., 1991; Lewis, et al., 1990).

In addition to fibrinogen, the GPIIb-IIIa receptor can also bind the other major adhesion proteins, fibronectin, von Willebrand factor and thrombospondin. As demonstrated with the binding of thrombospondin, another possible mechanism for the binding defect in thrombasthenic platelets has been suggested (George, et al., 1984). In normal platelets, thrombospondin binds to the GPIIb-IIIa receptor in a partially calcium-dependent manner. Thrombasthenic patients lack the calcium-dependent site. Since GPIIb-IIIa is the major calcium binding site on the surface of unactivated platelets, and all of the aforementioned adhesive proteins require some level of calcium binding for receptor interaction, it is possible then, that calcium binding through the GPIIb-IIIa receptor is involved with the platelet dysfunction.

Pathological alteration of the GPIIb-IIIa may also occur as a result of circulating immune complexes and platelet antibodies induced by a number of diseases, including hemophilia, immune and immunodeficiency virus-related thrombocytopenic purpura, and systemic lupus erythematosus (Kamiyama, et al., 1991; Moake, J.L., 1990). These serum factors modify receptor activity by binding to, and inhibiting, native ligand interactions. It is

therefore apparent, that the GPIIb-IIIa receptor is critical for the normal maintenance of the vasculature, and therefore, modification or inhibition of the receptor in disease states can have a profound effect.

CHAPTER 1

VARIABILITY IN GLYCOPROTEIN IIb-IIIa MEMBRANE REORGANIZATION DEPENDENT ON MODE OF PLATELET ACTIVATION

INTRODUCTION

Blood platelets are instrumental in maintaining an intact vasculature by attachment to exposed subendothelium, and subsequent aggregation following vascular injury. Receptors that mediate the attachment to these substrates are found on the surface of the platelet. Of particular interest in this work, is the Glycoprotein IIb-IIIa (GPIIb-IIIa) receptor. Glycoprotein IIb-IIIa serves as the receptor for a variety of adhesive proteins found circulating in the plasma. This receptor is a primary mediator of platelet to platelet cohesion. It has been demonstrated *in vivo* (Coller, et al. 1989) and *in vitro* (Pidard, D., 1986) that if the receptor was blocked with monoclonal antibodies directed against the receptor, aggregation was prevented. In addition, in Glanzmann's thrombasthenia, in which the GPIIb-IIIa receptor is either absent, reduced in number, or is impaired in function, aggregation of platelets is also reduced (George, et al., 1990). It is known that the receptor requires activation, as well as a calcium source, before it is competent to bind ligand (Apitz-Castro, et al., 1991; Nurden, et al., 1986; Clemetson, K.J., 1985). In contrast, short synthetic peptides containing the recognition sequence Arg-Gly-Asp (RGD) (Du, et al., 1991), as well as monoclonal antibodies that recognize the GPIIb-IIIa receptor in either the complexed or non-complexed state (Pidard, D., 1986; Ruggeri, Z.M., 1986)

can bind directly.

The GPIIb-IIIa complex is the most prevalent glycoprotein in the platelet membrane, consisting of an estimated 50,000 copies per platelet (Kieffer and Phillips, 1990). An internal store of these receptors also exist. This additional source of GPIIb-IIIa receptors is centrally located, associated with alpha-granule membranes and seems to be transported to the platelet surface after stimulation with a strong agonist, such as thrombin. (Wencel-Drake, et al., 1986; Woods, et al., 1986)

Of the ligands that bind to the GPIIb-IIIa receptor, fibrinogen is the key protein for platelet aggregation. In situations where the activation of the platelet is induced by weak agonists such as ADP or epinephrine, fibrinogen is required to be present in the aggregation media (Mustard, et al., 1978). In the case of a strong agonist, where degranulation occurs, fibrinogen can be supplied for aggregation via internal stores within the platelet (Legrand, et al., 1989; Shattil, S.J., 1988).

The redistribution of the GPIIb-IIIa receptor after platelet activation has been studied by a variety of methods. The direct conjugation of colloidal gold to fibrinogen (Escolar, et al., 1989; White, et al., 1990)), or an anti-GPIIb-IIIa antibody (Isenberg, et al., 1987) provided an electron dense probe to label the receptor at the ultrastructural level. The secondary labelling of fibrinogen or GPIIb-IIIa primary antibodies with Protein A conjugated to colloidal gold has also been used (Isenberg, et al., 1987). Much of this

previous work has been with human platelet suspensions subjected to some level of activation, either through an agonist or stirring, followed by examination of thin sections.

However, the differential effects of weak or strong agonists combined with the specific micro-environment (unstirred or stirred) of the platelet itself, have not been separated to determine their individual effects. In this study, GPIIb-IIIa, was examined in the context of ligand-receptor activated redistribution, using fibrinogen directly coupled to a colloidal gold probe. Both strong and weak agonists, in stirred and unstirred preparations of human platelet suspensions were evaluated to determine what, if any differences, these variables had on GPIIb-IIIa redistribution.

MATERIALS AND METHODS

Platelet Isolation: Donor blood was drawn into a 30ml syringe charged with 3.8% sodium citrate to give a final anticoagulant concentration of 0.38%. The sample was then spun at 1000 rpm for 10 minutes to obtain platelet rich plasma (PRP). The PRP was carefully removed and transferred to a new plastic test tube where 1μM Prostaglandin E_1 (PGE_1; final reaction concentration) was added. This suspension was allowed to rest, in a covered tube for 10 minutes before it was spun to concentrate the platelets (2000 rpm, 10 min.). The resulting pellet was resuspended in 1ml Tyrode's Buffer without calcium (TB; 0.2% bovine serum albumin, 130mM NaCl, 2.6mM KCl, 10mM HEPES, 5.5mM glucose, 2mM $MgCl_2$, pH 7.4), and additional PGE_1 (to 1 μM final concentration) was

added. The concentrated platelet suspension was covered, and allowed to rest 10 minutes before gel filtration.

Gel Filtration: A 10ml column was prepared using Sepharose 4B-200, equilibrated with TB. The 1ml platelet suspension was layered onto the surface and gel filtered to remove any residual plasma proteins. The first 25 cloudy drops (approx. 1.25mls) were pooled, the platelets counted, and the concentrate diluted with Tyrode's buffer with calcium ($TB+Ca^{++}$; 1mM $CaCl_2$) to give a final platelet count of $2x10^8$ cells/ml. This suspension was allowed to rest (30 min.) before being used for experimental protocols.

Colloidal Gold Probes: Fifteen to 18nm colloidal gold was prepared according to the methods of Bendayan (1984) and Frens (1973), and was conjugated with fibrinogen and polyethylene glycol (PEG) as follows:

Fibrinogen - One hundred and thirty milligrams of lyophilized human fibrinogen (KABI) was dissolved in 3mls 5mM NaCl and desalted on a 20ml Sephadex G-25 column (to remove contaminating citrate and reduce the NaCl concentration) equilibrated with 5mM NaCl. The 280nm absorbance of the fractions were determined, and the 3 tubes with the highest readings were pooled and filtered to remove macro-aggregates. A Markwell protein assay was performed to determine the protein concentration. The fibrinogen was conjugated to the colloid at pH 6.4 after a minimum protecting protein concentration was determined.

PEG - To determine if the platelets would internalize a non-specific gold probe

in the course of activation, a control gold probe was made. The control was produced simultaneously with F-Au, substituting polyethylene glycol for fibrinogen. This probe was used as a control in all treatments to parallel fibrinogen gold labelling. The minimum protecting concentration of 1% PEG in 5mM NaCl was established, and the PEG was conjugated to the colloidal gold at pH 6.0 to provide a non-specific binding control for the gold labelling.

Agonists: Thrombin from human plasma (Sigma, T-8885) was diluted to provide a final reaction concentration of 0.1U/ml. Adenosine diphosphate (ADP) was made as a 2.4 x 10^{-4} stock, and 200μl aliquots were stored at -60°C until use. The ADP was diluted to a final reaction concentration of 10μM.

Unstirred Platelet Treatments: Five hundred microliters of the platelet suspension was aliquoted into 1500μl microfuge tubes. To each of these tubes, 100μl of gold probe or TB+Ca^{++} was added, the tube inverted to mix, then allowed to incubate at 37°C for 3 minutes. At the end of the initial incubation 20μl of agonist or TB+Ca^{++} was added, inverted to mix and incubated an additional 5 minutes at 37°C. See Figure 1.1 for particular experimental variables. The reaction was terminated with the addition of 600μl of 0.1% glutaraldehyde in 0.1M cacodylate buffer (CB), pH 7.4. After 30 minutes, the platelet suspensions were pelleted at 12,800 rpm for 10 seconds.

Stirred Platelet Treatments: Five hundred microliters of platelet suspension was placed

in an aggregation cuvette (with stirbar) along with 100μl of probe or TB+Ca^{++}. This mixture was covered and incubated 2 minutes at 37°C. After incubation, the cuvette was placed in a Bio/Data Platelet Aggregometer and a baseline trace was taken for 1 minute.

	PROBE	AGONIST
	(100μl, 3 min, 37°C)	(20μl, 5 min, 37°C)
1	TB+Ca^{++}	TB+Ca^{++}
2	TB+Ca^{++}	10μM ADP
3	TB+Ca^{++}	0.1U/ml Thrombin
4	PEG-Au	TB+Ca^{++}
5	PEG-Au	10μM ADP
6	PEG-Au	0.1U/ml Thrombin
7	Fibrinogen-Au	TB+Ca^{++}
8	Fibrinogen-Au	10μM ADP
9	Fibrinogen-Au	0.1U/ml Thrombin

Figure 1.1. Platelet Treatment Combinations

Agonist or TB+Ca^{++} was added, and the aggregation response was monitored until aggregation was complete or the pattern had stabilized (approx. 5 minutes) (Figure 1.1). The reaction was stopped by the addition of 600μl of 0.1% glutaraldehyde in CB, and the mixtures carefully transferred to microfuge tubes. Fixation was allowed to continue for 30 minutes before centrifugation at 12,800 rpm for 10 seconds.

Labelling Specificity Using AP-2: To determine F-Au labelling specificity, the monoclonal antibody AP-2 was used (generous gift of T.J. Kunicki, Blood Center of

Southeastern Wisconsin). The concentration of AP-2 chosen for these experiments was based on the amount that gave a maximum inhibition of a PRP aggregation using 10μM ADP. Platelet suspensions were pre-incubated with this inhibiting concentration, which was found to be 0.05mgs of the monoclonal antibody / $2x10^8$ cells/ml , for 20 minutes at 37°C. then reacted as before using ADP stimulation in both stirred and unstirred systems.

Transmission Electron Microscopy: After centrifugation, the supernatant was removed and fresh, 2.5% glutaraldehyde in 0.1M CB was added. The pellet was broken up into smaller pieces, and allowed to fix for an additional hour. The samples were centrifuged at 12,000 rpm for 1 minute, then the pellets were removed to be suspended in 1% agarose in water. The solidified agarose was cut into pieces, fixed in 2.5% glutaraldehyde in CB (1 hour), post-fixed in 1% aqueous osmium tetroxide, water washed, then dehydrated through a graded series of ethanol. Infiltration in Spurrs-Mollenhauer resin (Klomparens, et al. 1986) was through 3:1, 1:1 and 1:3 (ethanol:resin) for 2 hours each, and 100% resin overnight. Blocks were polymerized at 65°C. for 48 hours, ultrathin sectioned, and stained with 2% aqueous uranyl acetate and Reynold's lead citrate (1963) before examination in a JEOL 100CXII TEM.

All data based on at least two experimental repetitions, and the observation of at least 20 platelets per experimental treatment.

RESULTS

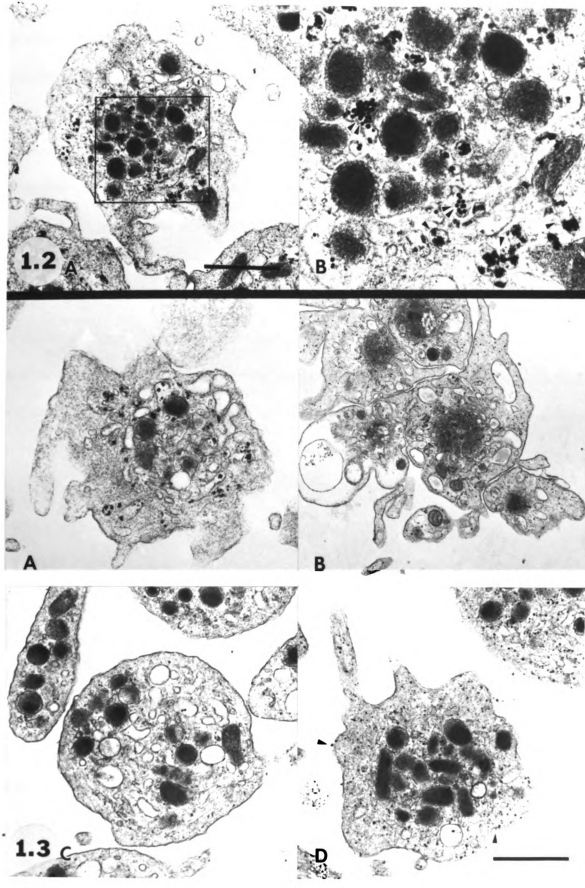
General Morphology: Except for the presence of probe, there was no morphological difference between the unlabelled controls and the gold labelled treatments. In all platelet preparations, a structure of approximately 16nm diameter was present within the confines of membrane-bound vesicles, that resembled the star-like structure of glycogen. This inclusion decreased following platelet activation, and was not found in vesicles which contained the gold probe (Figure 1.2).

Specificity of Fibrinogen-Gold Probe: No significant gold labelling was seen in either platelets labelled with PEG-Au, or those pre-incubated with AP-2. In addition, there was no evidence of PEG-Au trapping between thrombin-aggregated platelets (Figures 1.3a and 1.3b). In AP-2 pre-treated samples, F-Au labelling was either absent or significantly reduced in both stirred and unstirred preparations stimulated with ADP (Figures 1.3c and 1.3d).

Unstirred Suspension Treatments: In this system, platelets remained monodispersed under all conditions. Fibrinogen-gold labelling without the addition of agonist resulted in little to no labelling of the cells (Figure 1.4a). Treatment of the platelets with ADP resulted primarily in, external labelling that was randomly dispersed in small clusters along the plasma membrane (Figure 1.4c). In approximately 7.3 ± 0.4% of the ADP-stimulated platelets, internalized probe could be found.

Figure 1.2 Colloidal Gold Probe versus Glycogen-like Particles. Bar = 1μM. A) ADP-stimulated platelet. B) Inset enlargement of 1.2A. Glycogen-like particles (single arrowheads) and colloidal gold probe (double arrowheads). Both located in membrane bound vesicles.

Figure 1.3 Platelet Labelling Controls. Bar = 1μM. A) Unstirred, thrombin-stimulated, PEG-Au incubated. No labelling internally or externally. B) Stirred, thrombin-stimulated, PEG-Au incubated. Aggregate shows no labelling, internally or externally, and no trapping of F-Au between cells. C) Unstirred, AP-2 pre-treated, ADP-stimulated, F-Au incubated. No labelling internally or externally. D) Stirred, AP-2 pretreated, ADP-stimulated, F-Au incubated. No internal labelling. External labelling (arrowheads) minimal.


1.2
A
B
A
B
1.3
C
D

In contrast, thrombin stimulation produced a marked internalization of the probe which was found to be centralized around the granulomere region. In these sections, the gold label was packed into membrane bound vesicles that resembled the open canalicular system (OCS). Internal membranes were obvious, and the number of secretory granules were decreased, but still present. External labelling was minimal, as was labelling in the peripheral margins of the cell (Figure 1.4e).

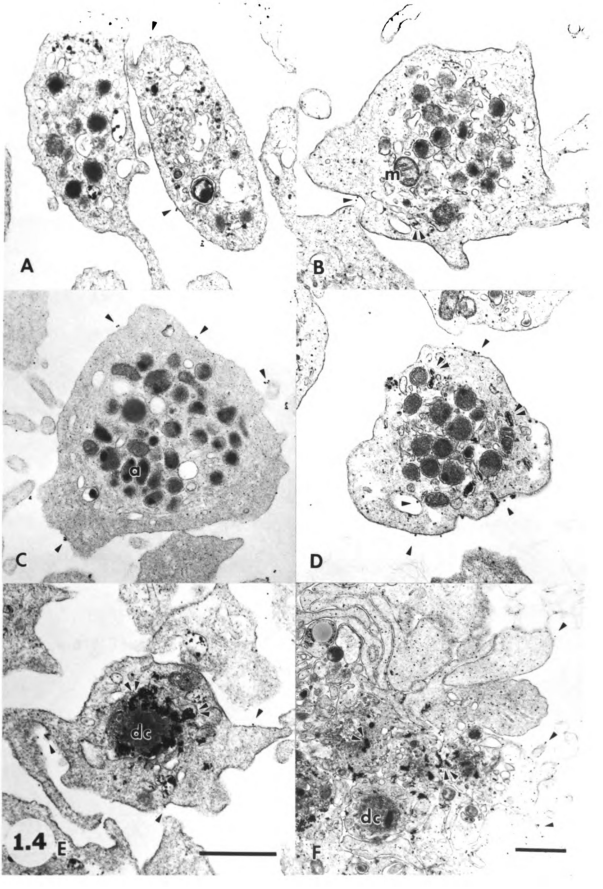
Stirred Suspension Treatments: In the stirred preparations, blunt pseudopodia formation could be seen in the controls and was increased with agonist addition (Figure 1.4). This is in contrast to the unstimulated platelet treatments in which pseudopodia formation was not seen until agonist addition. Interestingly, stirred platelets treated with F-Au with no agonist addition, gave slightly increased external labelling over that seen in an unstirred suspension (Figure 1.4b).

Stimulation with ADP resulted in an increase in external labelling compared to the unstirred preparation. Like the unstirred sample, this labelling was also clustered. Internalization of the F-Au labelling in the ADP preparation could be found in all platelets. All internal labelling was contained in membrane bound vesicles (Figure 1.4d).

Treatment with thrombin resulted in centralized gold labelling, around a dense, virtually non-granulated platelet core. Internal membranes were less distinct, as were the membranes surrounding the gold label (Figure 1.4f).

Figure 1.4 Unstirred versus Stirred Platelet Treatments. Bar = 1μM.

A) Unstirred, F-Au incubated, no agonist. No internal labelling. External labelling (arrowheads) found that is single and infrequent. B) Stirred, F-Au incubated, no agonist. Minimal internal labelling (double arrowheads) contained in membrane-bound vesicles. External labelling (arrowhead) infrequent. Mitochondria (m) present. C) Unstirred, ADP-stimulated, F-Au incubated. Minimal internal labelling, not seen in this micrograph. External label (arrowhead) primarily clustered. D) Stirred, ADP-stimulated, F-Au incubated. Internal labelling (double arrowheads). External labelling (arrowheads) both single and clustered. E) Unstirred, thrombin-stimulated, F-Au incubated. Concentrated internal labelling (double arrowheads) surrounding dense core (dc). External labelling (arrowheads) both single and clustered. F) Stirred, thrombin-stimulated, F-Au incubated. Aggregate shows marked internal labelling (double arrowheads) and dense core (dc). External labelling (arrowheads) both single and clumped, found at sites of platelet-platelet interaction.



1.4

A comparison of the thrombin-stimulated, non-aggregated and aggregated platelets revealed some major differences. Extensive probe labelling was found between platelet aggregates. Interestingly, there was a lack of F-Au probe in totally degranulated platelets found at the margins of large aggregates. There was also an apparent decrease in the level of F-Au internalization in platelet micro-aggregates when compared to single platelets (Figure 1.5).

Aggregation traces of the buffer control, PEG-Au only and F-Au only samples produced increased light transmission due to dilutional effects (an overall drop in baseline trace) (Figure 1.6a). Adenosine diphosphate resulted in platelet shape change, for all treated samples, and primary aggregation in the F-Au incubated sample (Figure 1.6b). Thrombin stimulation gave a normal aggregation response, resulting in a secondary wave of aggregation. The rate of aggregation was slightly reduced from the thrombin control, to thrombin/F-Au and thrombin/PEG-Au, respectively (Figure 1.6c), but very little differences in total percent aggregation was noted.

Figure 1.5 Comparison of Micro- and Macro- Aggregates. Thrombin-stimulated, stirred suspensions of human platelets. Bar = 1μM. A) Micro-aggregate of three platelets. F-Au labelling internally and between platelets. B) Large aggregate of platelets. Labelling seen in central platelets. Labelling absent in peripherally located and degranulated platelets.

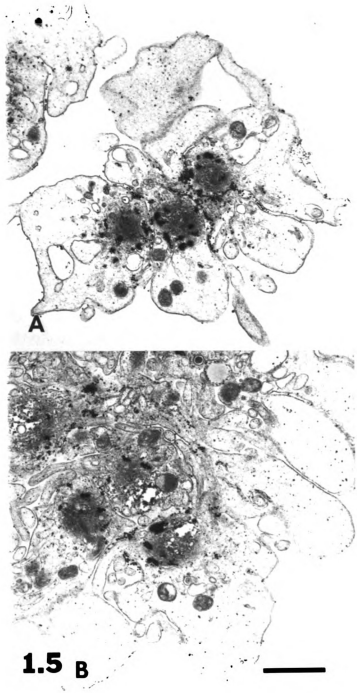

A
1.5 B

Figure 1.6 Aggregation Traces. Graphs are based on the change in light absorbance (Y-axis) over time (X-axis). A) F-Au, PEG-Au or buffer incubation. No agonist addition. Increased light transmission due to dilutional effect. B) ADP-stimulated: PEG-Au or buffer incubation resulted in shape change alone that returned to baseline levels. F-Au incubation resulted in shape change with primary aggregation, but no granule release. C) Thrombin-stimulated: Buffer incubation gave secondary aggregation and granule release. PEG-Au incubation had a slightly reduced rate of shape change onset, though secondary aggregation onset was equivalent to the buffer control. F-Au incubation resulted in a control rate shape change, with delayed secondary aggregation.

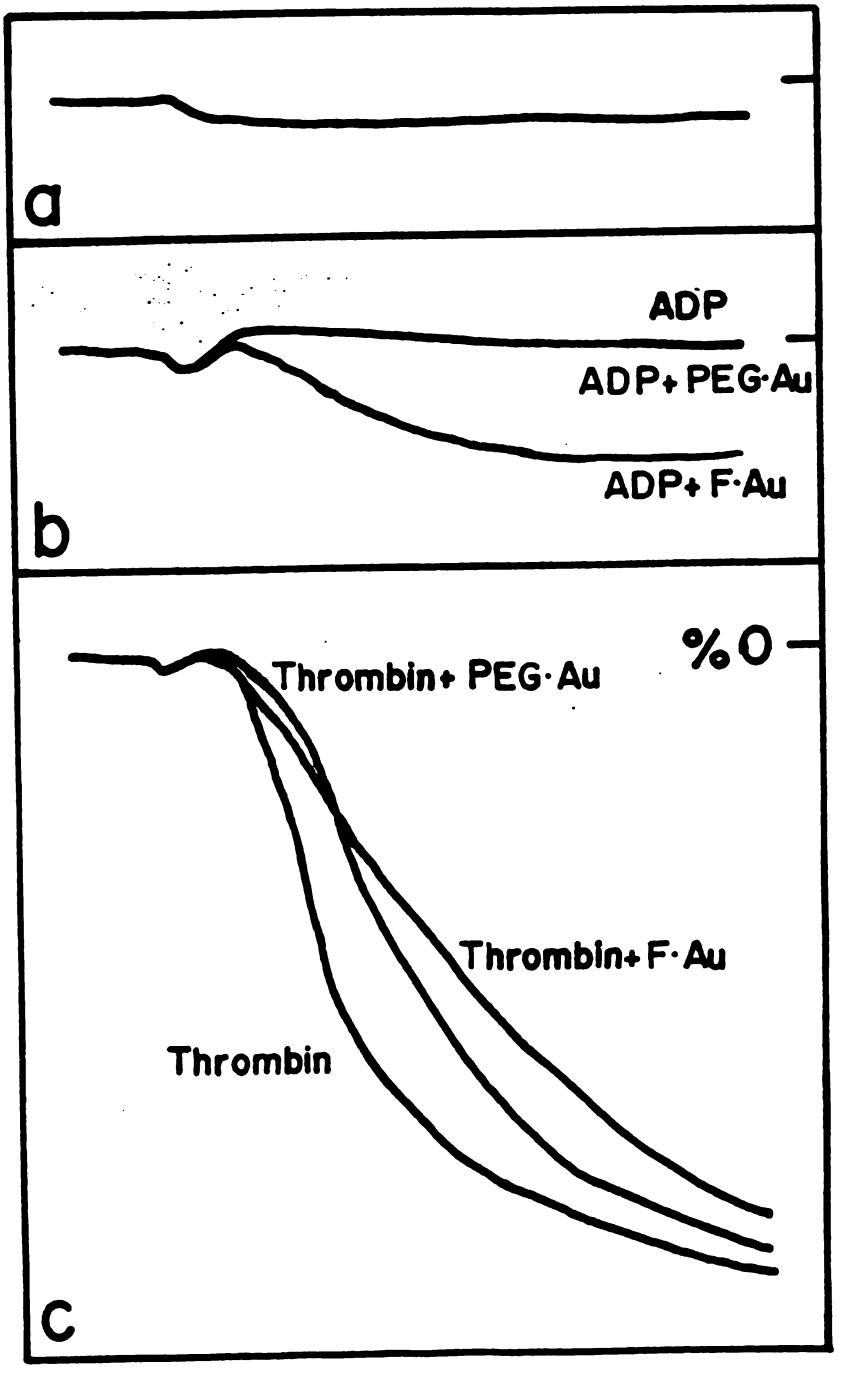


Figure 1.6 Aggregation Traces

DISCUSSION

In this study, the redistribution of the GPIIb-IIIa receptor, following platelet activation was examined using fibrinogen directly linked to a colloidal gold probe. The effect of differing micro-environments (stirred and unstirred) and agonist strengths on receptor redistribution, confirmed and extended existing data.

The majority of the work on the GPIIb-IIIa receptor during platelet activation, has used indirect labelling methods to characterize the receptor redistribution. The use of a direct labelling method helped to eliminate possible errors known to be associated with secondary labelling of internalized tracers. These errors include, the loss of antigenicity in post-embedding labelling (Bendayan, M., 1984), loss of availability of label to secondary probe after internalization, reactivity of endogenous platelet immunoglobulins when Protein-A is used as the secondary probe (George, J., 1989) and partial activation of the receptor by non-physiological tags (Du, et al., 1991). By using fibrinogen complexed directly to the gold probe, I was able to eliminate these disadvantages. One disadvantage, however of using a particulate probe conjugated to fibrinogen, was the suspected alteration in the biological activity of the fibrinogen molecule. This was evidenced by the fact that F-Au does not support aggregation to the same extent as the native ligand. This modification to the normal ligand/receptor interaction, may be important to the interpretation of the receptor redistribution.

The effect of the platelet micro-environment during presentation of ligand-receptor binding, has been shown to influence the degree of platelet activation. Longmire and Frojmovic (1990) demonstrated that in platelet rich plasma, the ADP concentrations required in an unstirred suspension of platelets were 3-8 times higher to produce a similar level of aggregation than those in a stirred preparation. For this reason, the platelet micro-environment, in terms of stirred vs unstirred, in relation to agonist activation, was examined for its role in receptor reorganization.

The relative potency of the agonist used for platelet activation also has profound influence on the interaction of the ligand, fibrinogen, with the GPIIb-IIIa receptor. In a stirred system using platelet rich plasma and 10μM ADP, platelets respond with secretory granule release and full aggregation. In these experiments, 10μM ADP combined with gel-filtered platelets resulted in shape change alone in the absence of exogenous fibrinogen, and only primary aggregation (reversible) in the presence of F-Au. This concentration of ADP did not result in release of the secretory granules, and little or no label centralization under either stirred or unstirred conditions. This demonstrated the importance of normal receptor crosslinking and subsequent platelet-platelet interaction to platelet activation (Frojmovic, et al., 1990). These points would indicate that in the absence of fibrinogen or following receptor occupancy without platelet cohesion, the platelet is unable to release in response to an agonist concentration that would normally cause complete aggregation. Platelet-platelet interaction is, therefore, an important second step in platelet aggregation. The thrombin concentration (0.1U/ml) used did not cause aggregation or striking

degranulation in an unstirred suspension, however, full aggregation was observed in the stirred treatments with or without the addition of exogenous fibrinogen. The decreased rate of aggregation onset seen between platelets stimulated with thrombin alone and thrombin/F-Au, may be due to F-Au blocking of receptors with an altered ligand, or the induction of ligand/receptor internalization as a result of the particulate probe binding.

Comparison of the labelling characteristics between stirred and unstirred systems demonstrated that the turbulence of stirring induces a low level of activation. This level of activation, when combined with ADP as the agonist, had a marked effect on receptor redistribution. In platelets incubated with F-Au alone, the level of labelling was minimal compared to agonist-stimulated samples in both the stirred and unstirred preparations, though a slight increase was noted in the stirred platelets. In the case of ADP stimulation, the differences between the two micro-environments were more striking. Labelling of the unstirred platelets was restricted to the external membrane in a majority of the platelets, and demonstrated small clusters, that were evenly distributed on the platelet membrane, and internalization in approximately 7% of the platelets. In the stirred preparation, the label was also found externally, but in addition, there was internalization of probe into the OCS in 100% of the platelets examined. Thrombin stimulation caused internalization in both systems, and the formation of a dense core in the center of most platelets. In the unstirred system, the internalized probe surrounded the granulomere region in obvious channels. In the stirred system, few intact secretory granules remained in the platelets. And in the area surrounding the dense core, the majority of the

sequestered probe could be found.

One possible explanation for an increase in F-Au binding in stirred platelets, is the cell to cell collisions inherent in a stirred system. In a stirred platelet system, there was formation of short pseudopodia even in unstimulated platelets, indicating some level of activation. This level of activation may have been sufficient to expose the GPIIb-IIIa binding sites, thus, increasing probe binding. These findings appear to correlate with the observations of O'Brien (1990) who proposed that high (40mm Hg) shearing forces alone, could activate a unique domain on the GPIIb-IIIa receptor, specific for von Willebrand's factor. He also suggested that the degree of shear determines the degree of GPIIb-IIIa exposure. It is possible, therefore, that at lower shearing forces, there may also be exposure of the receptor to fibrinogen that leads to some level of activation, without subsequent platelet aggregation. This would explain my observations of increased F-Au labelling in the non-stimulated and ADP-stimulated platelets in the stirred system.

In the case of thrombin stimulated platelets, the increase in internalization of the probe may be due to the exposure of an internal store of GPIIb-IIIa receptors. It was demonstrated that after surface dissociation of the GPIIb-IIIa receptor, thrombin stimulation exposed a new set of receptors previously sequestered in internal compartments (Woods, et al., 1986; Wencel-Drake, et al., 1986). These receptors would then be available to bind the F-Au, and to internalize it.

The use of gold-coupled fibrinogen allowed the observation of the ligand/receptor complex with a minimum of manipulation of the platelets. This method provided direct labelling of the receptor during internalization, and did not depend on diffusion of the secondary label to the binding site. By comparing the effects of the micro-environment on platelet treatments, I reported that in a stirred system, there was an inherent level of activation. This activation resulted in enhanced binding of the ligand, fibrinogen, to GPIIb-IIIa, and caused an overall increase in the effects of ADP and thrombin stimulation. Most importantly, agonist strength was the key to successful platelet aggregation. This was based on the possible release of both, internal stores of the GPIIb-IIIa receptor, as well as endogenous adhesive proteins, upon agonist-induced platelet degranulation.

It has been demonstrated that there is a differential effect on GPIIb-IIIa receptor redistribution based on agonist strength. I postulated that part of this difference may be related to exposure of internal receptors following thrombin-induced platelet activation. These experiments raised two important questions. First, is there a threshold level of thrombin that is necessary for GPIIb-IIIa activation and redistribution? Second, what is the overall contribution of internal stores of GPIIb-IIIa to ligand binding, and is it effected by agonist concentration? These questions are examined in the following chapter.

BIBLIOGRAPHY

Apitz-Castro, R., Jain, M.K., Bartoli, F., Ledezma, E., Ruiz, M-C. and Salis, R. 1991 Evidence for the Direct Coupling of Primary Agonist-receptor Interaction to the Exposure of Functional IIb-IIIa Complexes in Human Blood Platelets. Results from Studies with the Antiplatelet Compound Ajoene. Biochim. Biophys. Acta 1094:269-280.

Bendeyan, M. 1984 Protein-A Gold Electron Microscopic Immunocytochemistry: Methods, Applications, and Limitations. J. Electron Microsc. Tech. 1:243-270.

Clemetson, K.J. 1985 Glycoproteins of the Platelet Plasma Membrane. in Platelet Membrane Glycoproteins. George, J.N., Nurden, A.T., Phillips, D.R., (eds). Plenum Press. pp 51-85.

Collar, B.S., Folts, J.D., Smith, S.R., Scudder, L.E. and Jordan, R. 1989 Abolition of In Vivo Platelet Thrombus Formation in Primates With Monoclonal Antibodies to the Platelet GPIIb/IIIa Receptor. Circ.80(6):1766-1774.

Du, X., Plow, E.F., Frelinger, A.L., O'Toole, T.E., Loftus, J.C. and Ginsberg, M.H. 1991 Ligands "Activate" Integrin $\alpha_{IIb}\beta_3$ (Platelet GPIIb-IIIa). Cell 65:409-416.

Escolar, G., Leistikow, E. and White, J.G. 1989 The Fate of the Open Canalicular System in Surface and Suspension-Activated Platelets. Blood 74(6):1983-1988.

Frens, G. 1973 Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Solutions. Nat. Phys. Sci. 241:20-22.

Frojmovic, M., Longmire, K. and van de Ven, T.G.M. 1990 Long-range Interactions in Mammalian Platelet Aggregation. Biophys. J. 58:309-318.

George, J.N. 1989 The Origin and Significance of Platelet IgG. in Platelet Immunobiology, Molecular and Clinical Aspects. Kunicki, T. and George, J. (eds). J.B. Lippincott Co. Penn. pgs 305-336.

George, J.N., Caen, J.P. and Nurden, A.T. 1990 Glanzmann's Thrombasthenia: Spectrum of the Clinical Disease. Blood 75(7):1383-1395.

Isenberg, W.M., McEver, R.P., Phillips, D.R., Shuman, M.A. and Bainton, D.F. 1987 The Platelet Fibrinogen Receptor: An Immunogold-Surface Replica Study of Agonist-induced Ligand Binding and Receptor Clustering. J. Cell Biol. 104:1655-1663.

Kieffer, N. and Phillips, D.R. 1990 Platelet Membrane Glycoproteins: Functions in Cellular Interactions. Annu. Rev. Cell Biol. 6:329-357.

Klomparens, K.L., Flegler, S.L. and Hooper, G.R. 1986 Procedures for Transmission and Scanning Electron Microscopy for Biological and Medical Science. A Laboratory Manual, 2nd ed. Ladd Research Ind., Burlington, VT, p. 146.

Legrand, C., Dubernard, V. and Nurden, A.T. 1989 Studies on the Mechanism of Expression of Secreted Fibrinogen on the Surface of Activated Human Platelets. Blood 73(5):1226-1234.

Longmire, L. and Frojmovic, M. 1990 Long-range Interactions in Mammalian Platelet Aggregation. Evidence from Kinetic Studies in Brownian Diffusion. Biophys. J. 58:299-307.

Mustard, J.F., Packham, M.A., Kinlough-Rathbone, R.L., Perry, D.W. and Regoeczi, E. 1978 Fibrinogen and ADP-induced Platelet Aggregation. Blood 52:453-465.

Nurden, A.T., George, J.N. and Phillips, D.R. 1986 Platelet Membrane Glycoproteins: Their Structure, Function, and Modification in Disease. in Biochemistry of Platelets. Phillips, D.R., Shuman, M.A. (eds). Academic Press, N.Y. pgs 159-224.

O'Brien, J.R. 1990 Shear-induced Platelet Aggregation. Lancet 335:711-713.

Pidard, D. 1986 Studies on the Organization and Function of GP IIIa Within the Plasma Membrane of Intact Human Platelets Using the Monoclonal Antibody AP-2. in Monoclonal Antibodies and Human Blood Platelets. INSERM Symposium No.27. McGregor, J.L. (ed). Elsevier Science Publ. B.V. pp 103-117.

Reynolds, E.S. 1963 The Use of Lead Citrate At High pH as an Electron Opaque Stain in Electron Microscopy. J. Cell Biol. 17:208-212.

Ruggeri, Z.M. 1986 The Use of Monoclonal Antibodies to Probe the Receptor Function of the Glycoprotein IIb/IIIa Complex. in Monoclonal Antibodies and Human Blood Platelets. INSERM Symposium No.27. McGregor, J.L. (ed). Elsevier Science Publ. B.V. pp 83-91.

Shattil, S.J. 1988 Expression, Regulation and Detection of Fibrinogen Receptors on Activated Platelets in Platelet Membrane Receptors: Molecular Biology, Immunology, Biochemistry and Pathology. Jamieson, G.A. (ed). Alan R. Liss, Inc. pp 345-380.

Wencel-Drake, J.D., Plow, E.F., Kunicki, T.J., Woods, V.L., Keller, D.M. and Ginsberg, M.H. 1986 Localization of Internal Pools of Membrane Glycoproteins Involved

in Platelet Adhesive Responses. Am. J. Pathol. 124:324-334.

White, J.G., Leistikow, E.L. and Escolar, G. 1990 Platelet Membrane Responses to Surface and Suspension Activation. Blood Cells 16:43-72.

Woods, V.L., Wolff, L.E. and Keller, D.M. 1986 Resting Platelets Contain a Substantial Centrally Located Pool of Glycoprotein IIb-IIIa Complex Which May Be Accessible to Some but Not Other Extracellular Proteins. J. Biol. Chem. 261(32):15242-15251.

CHAPTER 2

EFFECTS OF LOW-DOSE THROMBIN ON PLATELET ACTIVATION AND GLYCOPROTEIN IIb-IIIa REDISTRIBUTION

INTRODUCTION

Thrombin is the only blood coagulation factor that can directly activate platelets. It is found circulating in the blood stream as prothrombin, an inactive precursor to the enzyme (Shuman and Greenberg, 1986). The enzyme can be activated on the surface of the platelet via a prothrombinase complex, or in the circulatory system, with non-physiologic activators such as snake venom enzymes (Owen and Jackson, 1973; Morita, et al., 1976) or culture filtrates of *Staphylococcus aureus* (Bas, et al., 1974). Thrombin can, in high enough concentrations, activate the platelet to release stored compounds that enhance the aggregation-adhesion response by providing fibrinogen, calcium and ADP (Davey and Lüscher, 1967). This ability of thrombin to bypass contact activation or other receptor-mediated activation pathways, gives it an important role in platelet function.

The morphologic examination of thrombin-induced mobility and fate of platelet surface receptors has been limited to high concentrations of the agonist. These concentrations of thrombin have ranged from 0.2U/ml to as high as 5U/ml (White, J.G. and Escolar, G., 1991; White, et al., 1990; Escolar, et al., 1989). The effect of these thrombin concentrations on F-Au probe distribution are consistent with results described in Chapter

1 for 0.1U/ml of thrombin. It has been shown that platelet activation by thrombin is potentially controlled by more than one membrane receptor. These receptors, associated with two separate coupling mechanisms (McGowen, E.B. and Detwiler, T.C., 1986), are affected by both the concentration (Seiler, et al., 1991; Jamieson, G.A., 1988) and duration of thrombin exposure (Holmsen, et al., 1981). If the thrombin concentration is too low or the duration of exposure to short, it is not clearly understood, whether measurable activation of the platelet still occurs.

Thrombin stimulation is capable of inducing morphologic alterations in Glycoprotein IIb-IIIa (GPIIb-IIIa) receptor that result in the binding of specific adhesive proteins. This receptor, along with one of its ligands, fibrinogen, is instrumental in the aggregation of platelets (Peerschke, E.I.B., 1985). The GPIIb-IIIa receptor can be induced to bind fibrinogen by every platelet agonist tested (Shattil, S.J., 1988), though the amount of binding and resulting degree of platelet activation vary greatly. This implies that the mode of receptor activation is not necessarily through the same mechanism of platelet activation. It has also been demonstrated that with high doses of thrombin, internal stores of the GPIIb-IIIa receptor are exposed to the surface, and are competent to bind ligand (Wencel-Drake, et al., 1986). It was postulated, therefore, that as opposed to high dose thrombin platelet activation, using lower doses may provide more information about GPIIb-IIIa activation and redistribution, particularly, if internal and external populations of the GPIIb-IIIa receptor could be differentiated.

A number of electron dense probes have been used for ultrastructural localization of the GPIIb-IIIa receptor after platelet activation. One of the most popular probes has been colloidal gold. This probe has been used in two general labelling schemes. The first links colloidal gold probe directly to the receptor ligand or a monoclonal antibody that recognizes the receptor. The second binds the probe to a secondary label, such as an antibody for the ligand used to locate the receptor. Non-ligand based probes (monoclonal antibodies) have been used extensively and have been able to distinguish between the activated and non-activated forms of the GPIIb-IIIa receptor (Collar, B.S., 1986; Pidard, et al., 1983). This technology was instrumental in the characterization of the two forms of this receptor. One potential drawback to the use of monoclonal antibodies in the examination of receptor activation is that the antibody is not a native ligand. Therefore, ligand-receptor mediated interactions may not reflect normal platelet response. Sinigaglia and coworkers (1989) demonstrated that occupancy of GPIIb-IIIa with fibrinogen, modulated thrombin activation of human platelets, therefore, the use of a native ligand in my studies was instrumental. The use of a monoclonal antibody specific for the GPIIb-IIIa receptor complex (AP-2) (Pidard, D., 1986), provided a means to control for the contribution of external GPIIb-IIIa receptors in platelet activation and subsequent receptor reorganization.

This study examines the effects of low dose thrombin activation on the GPIIb-IIIa receptor, as well as the effects on the platelet morphology. By surveying the binding of gold-labelled fibrinogen, and localizing its subsequent redistribution, this research study

provides specific data on the threshold level of activation for this receptor in intact platelets, as well as the contribution of external GPIIb-IIIa receptors to platelet activation. Comparison of the relative activation level of the platelet with receptor activation also provides information on these two events.

MATERIALS AND METHODS

Platelet Isolation: Donor blood was drawn into a 30ml syringe charged with 3.8% sodium citrate to give a final anticoagulant concentration of 0.38%. The sample was then spun at 1000 rpm for 10 minutes to obtain platelet rich plasma (PRP). The PRP was carefully removed, and transferred to a new plastic test tube where 1μM Prostaglandin E_1 (PGE_1; final reaction concentration) was added. This suspension was allowed to rest, in a covered tube, 10 minutes before it was spun to concentrate the platelets (2000 rpm, 10 min.). The resulting pellet was resuspended in 1ml Tyrode's Buffer without calcium (TB; 0.2% bovine serum albumin, 130mM NaCl, 2.6mM KCl, 10mM HEPES, 5.5mM glucose, 2mM $MgCl_2$, pH 7.4), and additional PGE_1 (to 1 μM final concentration) was added. The concentrated platelet suspension was covered, and allowed to rest 10 minutes before gel filtration.

Gel Filtration: A 10ml column was prepared using Sepharose 4B-200, equilibrated with TB. The 1ml platelet suspension was layered onto the surface and gel filtered to remove any residual plasma proteins. The first 25 cloudy drops (approx. 1.25mls) were pooled,

the platelets counted, and the concentrate diluted with Tyrode's buffer with calcium (TB+Ca^{++}; 1mM $CaCl_2$) to give a final platelet count of $2x10^8$ cells/ml. This suspension was allowed to rest (30 min) before experimental procedures.

Colloidal Gold Probes: Fifteen to 18nm colloidal gold was prepared according to the methods of Bendayan (1984) and Frens (1973), and was conjugated with fibrinogen and Polyethylene Glycol (PEG) as follows:

Fibrinogen - One hundred and thirty milligrams of lyophilized human fibrinogen (KABI) was dissolved in 3mls 5mM NaCl and fractionated over a 20ml Sephadex G-25 column (to remove contaminating citrate and reduce the NaCl concentration) equilibrated with 5mM NaCl. The 280nm absorbance of the fractions were taken, and the 3 tubes with the highest readings were pooled and filtered to remove macro-aggregates. A Markwell protein assay was performed to determine the protein concentration. The fibrinogen was conjugated to the colloid at pH 6.4 after a minimum protecting protein concentration was determined.

PEG - The minimum protecting concentration of 1% PEG in 5mM NaCl was established, and the PEG was conjugated to the colloidal gold at pH 6.0 to provide a non-specific binding control for the gold labelling.

Agonist: Thrombin from human plasma (Sigma, T-8885) was diluted such that the addition of 20μl provided a final reaction concentration of 0.1U/ml, 0.05U/ml, 0.025U/ml, 0.0125U/ml and 0.006U/ml.

Unstirred Platelet Treatments: Five hundred microliters of the platelet suspension was aliquoted into 1500μl microfuge tubes. To each of these tubes, 100μl of gold probe or TB+Ca^{++} was added, the tube inverted to mix, then allowed to incubate at 37°C for 3 minutes. At the end of the initial incubation 20μl of agonist or TB+Ca^{++} was added, inverted to mix and incubated an additional 5 minutes at 37°C. See Figure 2.1 for treatment listing. The reaction was terminated with the addition of 600μl of 0.1% glutaraldehyde in 0.1M cacodylate buffer (CB), pH 7.4. After 30 minutes, the platelet suspensions were pelleted at 12,800 rpm for 10 seconds.

	PROBE	AGONIST
	(100μl, 3 min, 37°C)	(20μl, 5 min, 37°C)
1	PEG-Au	0.1U/ml Thrombin
2	Fibrinogen-Au	0.1U/ml Thrombin
3	Fibrinogen-Au	0.05U/ml Thrombin
4	Fibrinogen-Au	0.025U/ml Thrombin
5	Fibrinogen-Au	0.0125U/ml Thrombin
6	Fibrinogen-Au	0.006U/ml Thrombin

Figure 2.1. Dose Response Treatment Combinations

Stirred Platelet Treatments: Five hundred microliters of platelet suspension was placed in an aggregation cuvette (with stirbar) along with 100μl probe or TB+Ca^{++}, covered, and incubated 2 minutes at 37°C. After incubation, the cuvette was placed in a Bio/Data Platelet Aggregometer and a baseline trace was taken for 1 minute. Twenty microliters of agonist or TB+Ca^{++} was added, and the trace was monitored until aggregation was

complete or had stabilized (approx. 5 minutes) (Figure 2.1). The reaction was stopped by the addition of 600μl of 0.1% glutaraldehyde in CB, and the mixtures carefully transferred to microfuge tubes. Fixation was allowed to continue for 30 minutes before centrifugation at 12,800 rpm for 10 seconds.

AP-2 Pre-Treatment: To determine the effect of blocking initial fibrinogen binding, the monoclonal antibody AP-2 was used (generous gift of T.J. Kunicki, Blood Center of Southeastern Wisconsin). The saturating concentration of AP-2 was based on the ability to inhibit a 10μM ADP-induced aggregation of PRP. Platelet suspensions were pre-incubated with a saturating concentration of 0.05mgs of the monoclonal antibody / $2x10^8$ cells/ml for 20 minutes at 37°C. then reacted as before using the thrombin dosages in both stirred and unstrirred systems (Figure 2.1).

Transmission Electron Microscopy: After centrifugation, the supernatant was removed and fresh, 2.5% glutaraldehyde in 0.1M CB was added. The pellet was broken up into smaller pieces, and allowed to fix for an additional hour. The samples were centrifuged at 12,000 rpm for 1 minute, then the pellets were removed and suspended in 1% agarose in water. The solidified agarose was cut into pieces, fixed in 2.5% glutaraldehyde in CB (1 hour), post-fixed in 1% aqueous osmium tetroxide, water washed, then dehydrated through a graded series of ethanol. Infiltration in Spurrs-Mollenhauer resin (Klomparens, et al. 1986) was through 3:1, 1:1 and 1:3 (ethanol:resin) for 2 hours each, and in 100% resin overnight. Blocks were polymerized at 65°C. for 48 hours, ultrathin sectioned,

stained with 2% aqueous uranyl acetate and Reynold's lead citrate (1963).

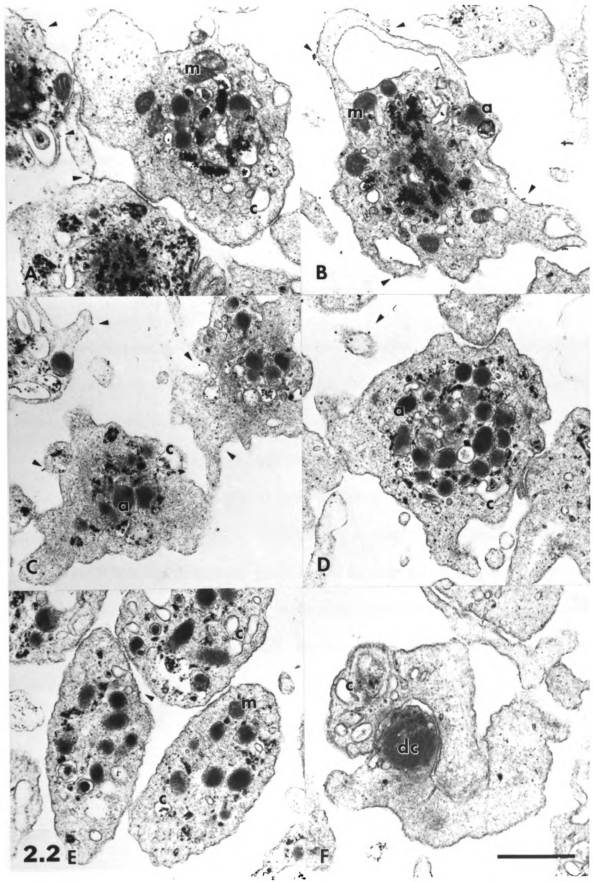
All data based on at least two experimental repetitions, and the observation of at least 20 platelets per experimental treatment.

RESULTS

Unstirred Dose Response: At thrombin concentrations of 0.1U/ml and 0.05U/ml, long cytoplasmic projections were common on most platelets, and a central dense core was apparent. The majority of platelets were monodispersed, with few, if any, aggregates. Labelling with F-Au resulted in internalization of probe that was primarily centralized around the dense core and appeared to be contained in membrane bound vesicles or channels. External labelling was minimal, and when present, consisted of single and clustered gold particles (Figures 2.2a and 2.2b).

A concentration of 0.025U/ml of thrombin did not result in granule centralization as in the higher concentrations of thrombin. The majority of internal labelling was still centralized around the granulomere region, but some level of label could be found throughout the platelet cytoplasm. The amount and distribution of external labelling did not differ significantly from higher concentrations (Figure 2.2c). At a concentration of 0.0125U/ml, the cells appeared activated, through loss of discoid shape, and the presence

Figure 2.2 Unstirred Thrombin Dose Response. Bar = 1μM. OCS = open canalicular system. A) 0.1U/ml thrombin dose. External label visible (arrowheads). OCS packed with label. Mitochondria visible (m) near OCS. B) 0.05U/ml thrombin. External labelling of platelet found (arrowheads). Mitochondria (m) and alpha granules (a) near F-Au packed OCS. C) 0.0250U/ml thrombin. Primarily external labelling (arrowheads). Open canalicular system (OCS) visible (c), with minimal internalization. Alpha granules (a) visible D) 0.0125U/ml thrombin. External labelling (arrowheads). No apparent internal labelling. Alpha granules (a), and OCS (c) present. E) 0.006U/ml thrombin. Minimal external labelling (arrowheads). Mitochondria (m) and OCS (c) present. F) Thrombin-stimulated, PEG-Au incubated control. No apparent labelling. Dense core (dc) and OCS (C) visible.



2.2

of a ruffled plasma membrane. External label was irregularly dispersed, and found as singlets. There was no obvious F-Au internalization (Figure 2.2d).

When treated with 0.006U/ml thrombin, the platelets were discoid in shape, had minimal projections, and maintained intact granules. There was minimal F-Au external labelling, and no apparent internal labelling (Figure 2.2e). The control for non-specific binding showed no PEG-Au internalization after thrombin stimulation (Figure 2.2f).

Stirred Dose Response: Thrombin concentrations of 0.1U/ml and 0.05U/ml resulted in two types of aggregates, large aggregates consisting of an undetermined number of platelets, and those that had 4 or less cells in the aggregate. The large aggregates consisted of a core of platelets with fused granules, that were surrounded by internalized F-Au. Platelets surrounding this platelet core were totally degranulated, and appeared as empty plasma membranes, devoid of any inclusions, showing irregular external labelling, and no internal labelling. Fibrinogen-gold labelling between the aggregated platelets was often extensive (Figures 2.3a and 2.3b). In the small aggregates, a mixture of granulated and degranulated platelets could be found. Internalized label was centralized around the dense cores or granules, was contained in membrane bound vesicles, and often filled what appeared to be the open canalicular system (OCS). External labelling was irregular, and appeared as both singlets and clusters on the external membrane of the platelet. Intra-platelet labelling was also evident (Figures 2.3c and 2.3d). In single platelets, when found, there was an apparent increase in overall external binding (Figures 2.3e and 2.3f).

Figure 2.3 High Thrombin Dose Response of Platelets, Stirred Treatments. Bar = 1μM. OCS = open canalicular system. A) 0.1U/ml thrombin dose. Large aggregate of platelets. Internal label visible (double arrowheads). Outside edges of aggregate cleared of cellular contents and label. B) 0.05U/ml thrombin. Large aggregate of platelets. Internal label visible (double arrowheads). Outside edges of aggregate cleared of cellular contents and label. C) 0.1U/ml thrombin. Medium aggregate of platelets. Some external labelling (arrowheads) present, with the majority found between platelets. Internal label (double arrowheads) in OCS surrounding dense core. D) 0.05U/ml thrombin. Medium aggregate of platelets. Some external labelling (arrowheads) seen. Internal labelling (double arrowheads), centralized in platelet. E) 0.1U/ml thrombin. Small aggregate of platelets. Increased external label (arrowheads). Internal labelling (double arrowheads) centralized around dense core. F) 0.05U/ml thrombin. Small aggregate of platelets. External labelling (arrowheads) present, increased at points of cell contact. Internal label (double arrowheads), found surrounding dense platelet core.

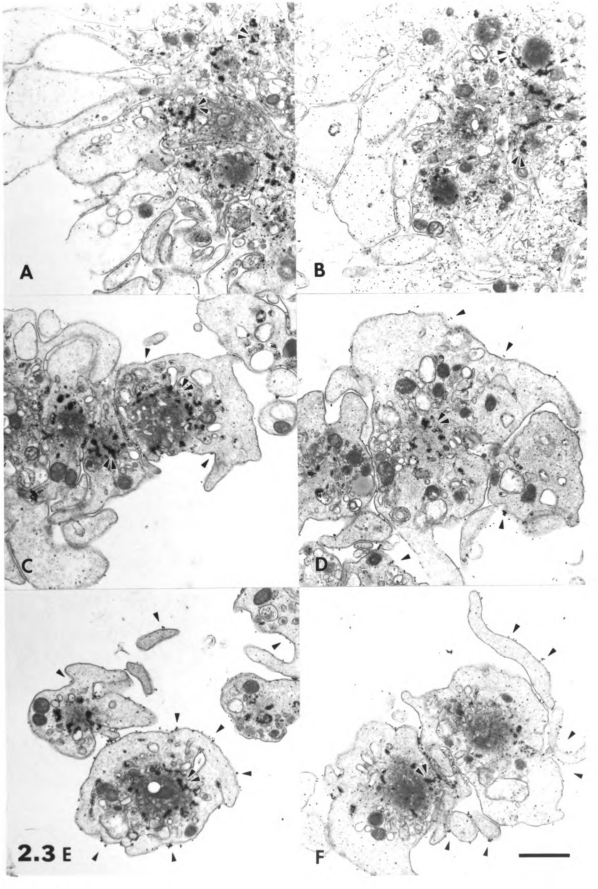

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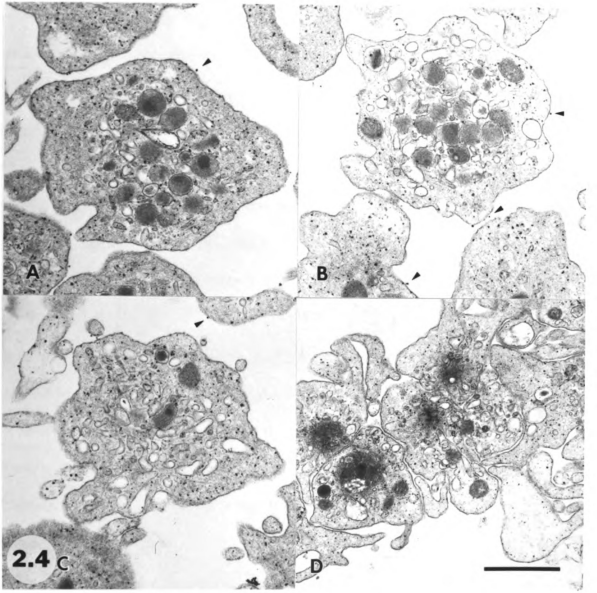
Figure 2.4 Low Thrombin Dose Response of Platelets, Stirred Treatments. Bar = 1μM. A) 0.025U/ml thrombin. External label (arrowhead) sparse and in singlet. No internal labelling. B) 0.0125U/ml thrombin. External label (arrowhead) sparse and in singlet. No internal labelling. C) 0.006U/ml thrombin. External label (arrowhead) sparse and in singlet. No internal labelling. D) 0.1U/ml thrombin-stimulated, PEG-Au control. Aggregate shows no specific labelling, internally or externally.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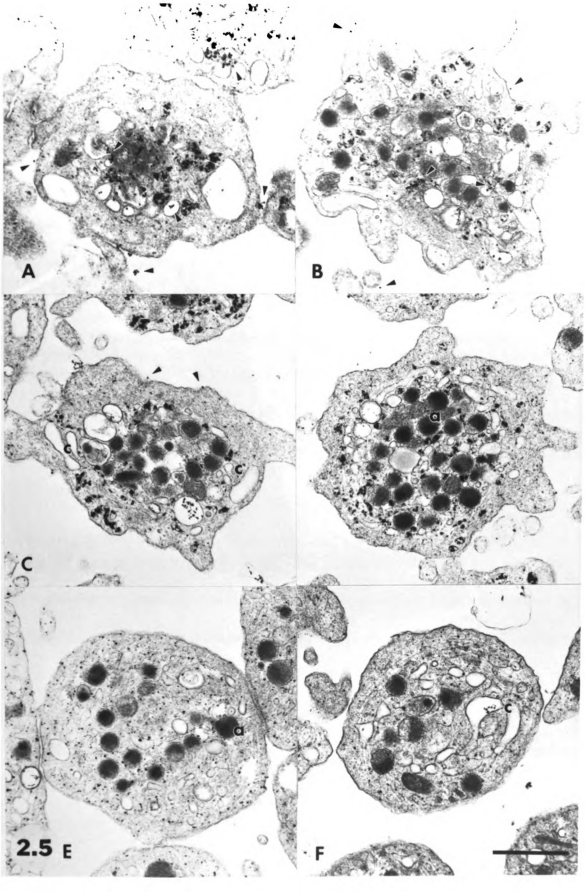
Platelets treated with 0.025U/ml thrombin had long cytoplasmic projections, and centralized granules that often showed some level of fusion. Rare internal labelling could be found. External labelling was in singlets, though clustered probe could be found, all of which were unevenly dispersed around the plasma membrane (Figure 2.4a). A thrombin dose of 0.0125U/ml resulted in blunt cytoplasmic projections, with a general centralization of the platelet granules, and only minimal granule fusion. Minimal internal labelling could be found on occasion. External labelling could be found and it was in singlets and unevenly dispersed (Figure 2.4b). A concentration of 0.006U/ml thrombin resulted in rounded platelets with blunt pseudopodia and intact granules. There was infrequent single label externally, and no label internalization (Figure 2.4c). No non-specific binding was seen in thrombin-stimulated PEG-Au controls (Figure 2.4d).

Unstirred, AP-2 Pre-treatment, Dose Response: A concentration of 0.1U/ml thrombin resulted in cytoplasmic ruffling of the platelet plasma membrane, and very low numbers of intact granules, often replaced with dense platelet cores. The OCS appeared to be swollen and devoid of inclusions except around the dense core, where it contained internalized F-Au. External label was irregularly spaced and consisted of single and doublet label (Figure 2.5a).

With 0.05U/ml thrombin, platelets with dense cores appeared to equal those with intact granules, and pseudopodia formation was no different than in the 0.1U/ml thrombin dose. Internal labelling could be found in increasing concentrations from the plasma membrane

Figure 2.5 Unstirred AP-2 Pre-treated, Thrombin Dose Response. Bar = 1μM. OCS = open canalicular system. A) 0.1U/ml thrombin dose. Internal label (arrowheads) found in membrane bound vesicles, and external label visible (arrowheads). B) 0.05U/ml thrombin. Minimal internal F-Au labelling (arrowheads) contained in membrane bound vesicles, and external labelling of platelet found (arrowheads). C) 0.0250U/ml thrombin. External labelling only (arrowheads). OCS visible (c). D) 0.0125U/ml thrombin. No apparent labelling. Alpha granules present (a). E) 0.006U/ml thrombin. No apparent labelling. Alpha granule (a) visible. F) ADP-stimulated, F-Au incubated control. No apparent labelling. OCS visible (c).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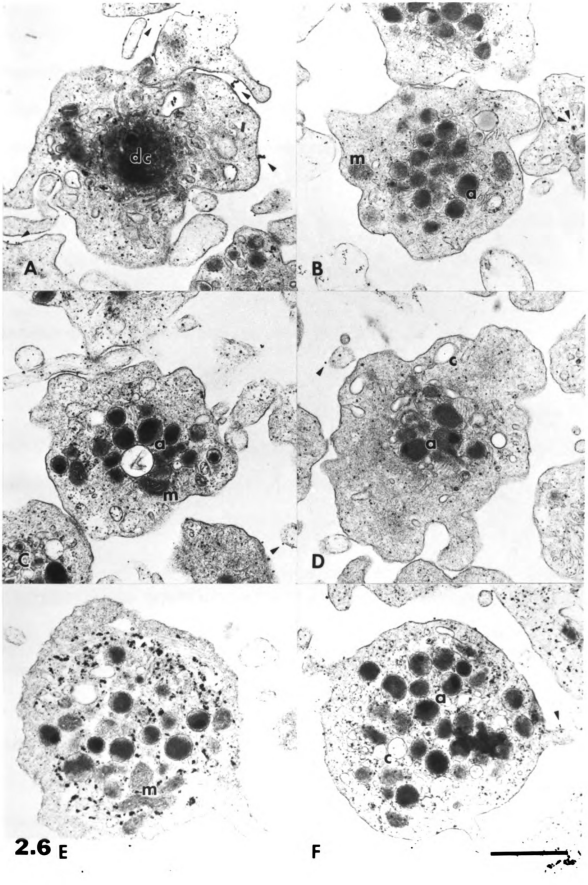
to the area adjacent to the granulomere region. There was minimal external labelling present (Figure 2.5b).

In the 0.025U/ml thrombin dose, the platelet had the same ruffled appearance as did the two highest thrombin concentrations. The number of platelets with intact granules appeared to equal that of the degranulated ones. No apparent internalization of probe was observed, and external labelling was irregular and singlet (Figure 2.5c). Platelets treated with 0.0125U/ml thrombin had intact secretory granules, and blunted cytoplasmic projections. External labelling was absent. There was no internalization (Figure 2.5d). A concentration of 0.006U/ml caused a slight production of blunt pseudopodia. No apparent labelling could be seen internally or externally (Figure 2.5e). In platelets pre-treated identically with AP-2 and stimulated with 10μM ADP, there was no F-Au labelling (Figure 2.5f).

Stirred, AP-2 Pretreatment, Dose Response: The highest thrombin dose resulted in dense core formation in about half the platelets, with no apparent aggregate formation. All platelets had long cytoplasmic projections. Internal label could be found throughout the platelet in membrane bound vesicles, whose numbers increased in frequency going from plasma membrane to the central region. There was an additional increase in labelling around dense cores, not seen with platelets containing intact granules. External labelling was infrequent, and when present was singlet or clustered and irregularly dispersed (Figure 2.6a).

Figure 2.6 Stirred AP-2 Pre-Treated, Thrombin Dose Response. Bar = 1μM. OCS = open canalicular system. A) 0.1U/ml thrombin dose. External label visible (arrowheads). Dense core (dc) visible, with minimal labelling in OCS. B) 0.05U/ml thrombin. Minimal internal label (double arrowheads). External labelling not apparent. Mitochondria (m) and alpha granules (a) found. C) 0.0250U/ml thrombin. Occasional external labelling (arrowheads). No internalization. Alpha granules (a) and mitochondria (m) visible D) 0.0125U/ml thrombin. Minimal external labelling (arrowheads). No apparent internal labelling. Alpha granules (a), and OCS (c) present. E) 0.006U/ml thrombin. No internal or external labelling. Mitochondria (m) present. F) ADP-stimulated, F-Au incubated control. Minimal external labelling (arrowheads). Alpha granules (a) and OCS (c) present.



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In the 0.05U/ml thrombin dose, there were more platelets with intact granulomeres than those with dense cores. Minimal internalization was seen, and then only in platelets with dense cores, or those with swollen OCS near the cell periphery. External labelling was very infrequent, found in singlets, and irregularly dispersed (Figure 2.6b).

A 0.025U/ml thrombin concentration caused blunt cytoplasmic projections from the platelet, and no internalization of F-Au. External labelling was in singlets or clumped, irregularly spaced and infrequent (Figure 2.6c). Platelets activated with 0.0125U/ml of thrombin were rounded with blunt projections, and had intact secretory granules. There was no internalization, and only minimal labelling externally (Figure 2.6d). With 0.006U/ml thrombin, the platelets were morphologically similar to those treated with 0.0125U/ml thrombin, but did not label either internally or externally (Figure 2.6e). In platelets pre-treated with AP-2, stirred, then stimulated with 10μM ADP, there was minimal external F-Au labelling (Figure 2.6f), and no internalization.

Aggregation Curves: In platelets treated with 0.1U/ml or 0.05U/ml thrombin, aggregation traces showed an increase in light transmission consistent with the observation of extensive aggregate formation. Aggregation curves of the lowest three thrombin concentrations (0.025, 0.0125, 0.006U/ml) resulted in shape change alone, that eventually (<5 minutes) returned to baseline level. As the concentration of thrombin decreased there was a decrease in the rate at which shape change occurred, as well as an increase in the time required to return to baseline. In platelets pre-treated with AP-2 prior to thrombin

Figure 2.7 Dose Response Aggregation Curves. Graphs are based on the change in light absorbance (Y-axis) over time (X-axis).

No Pre-incubation: 0.1U/ml thrombin - Trace showing shape change and secondary aggregation. 0.05U/ml thrombin - Shape change present, with delayed secondary aggregation. 0.025, 0.0125 and 0.006U/ml thrombin - Shape change only, with increased delay of onset, respectively.

AP-2 Pre-incubation: 0.1U/ml thrombin - Shape change only. 0.05, 0.025, 0.0125 and 0.006U/ml thrombin - Shape change only, with increased delay of onset, respectively.

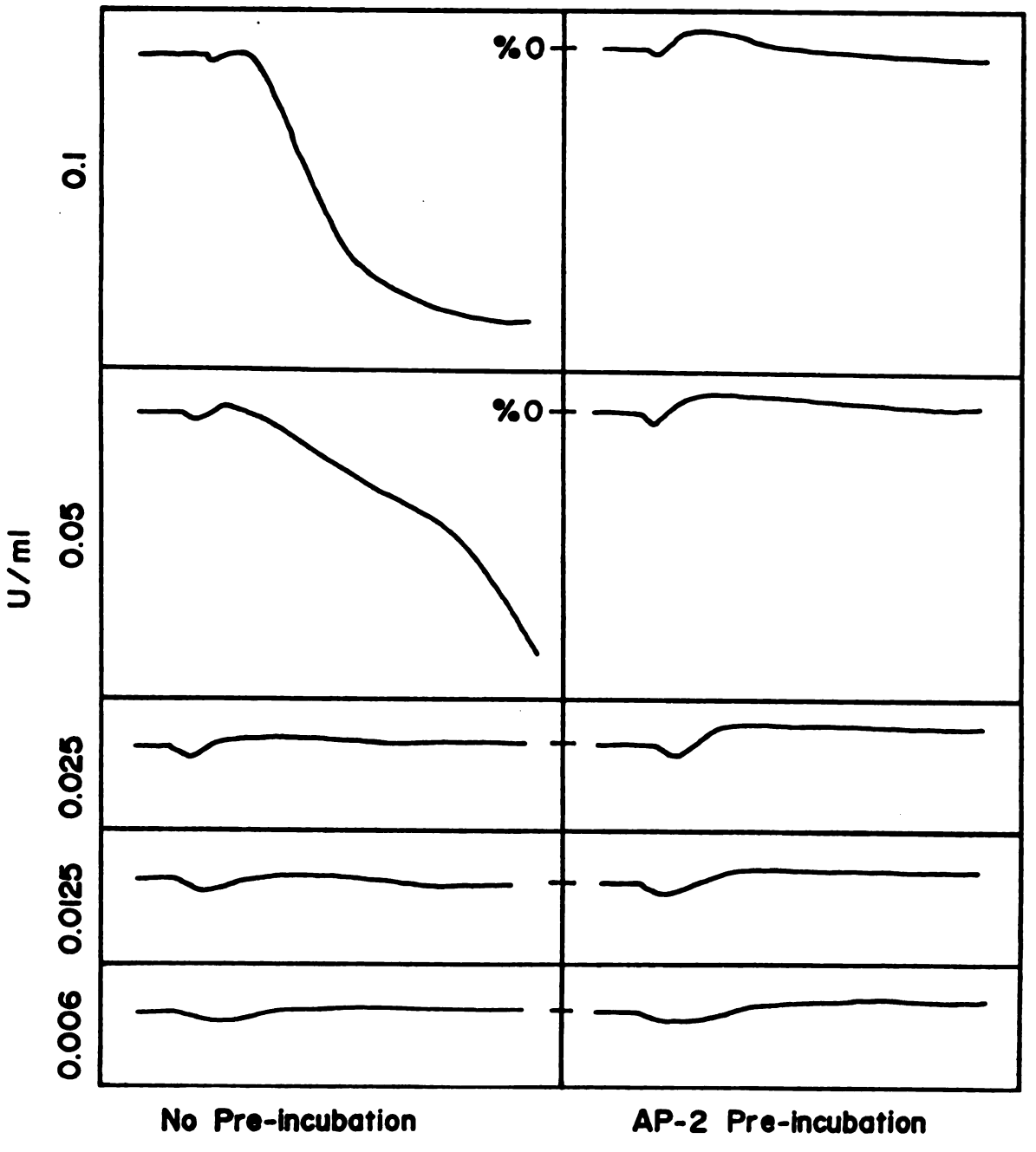


Figure 2.7 Dose Response Aggregation Curves

activation, 0.1U/ml resulted in shape change with a slight increase (approximately 5%) in light transmission over 5 minutes. Thrombin at 0.05U/ml resulted in a shape change that remained maximal and did not fall below its baseline light transmission. In both of these concentrations shape change occurred swiftly without a lag period. The lowest three concentrations of thrombin only induced shape change. The time before onset of shape change increased as the concentration of thrombin decreased (Figure 2.7).

DISCUSSION

Results of this thrombin dose response study on fibrinogen gold labelling, revealed four major findings, 1) there is an effect of stirred versus unstirred platelet micro-environments on cell activation, 2) platelet activation and GPIIb-IIIa receptor activation are most likely regulated separately, but are interdependent, 3) receptor occupation by a ligand has distinctly different effects on platelet response, depending on the specific ligand that is bound, and 4) there exists a threshold thrombin concentration that induces the release of internal stores of GPIIb-IIIa receptors to the platelet surface.

The effect of micro-environment on platelet activation has been reported in platelets stimulated with ADP. It was shown that in an unstirred system, 8 times the concentration of ADP was required to activate platelets to the same degree as those in a stirred system (Longmire and Frojmovic, 1990). In the thrombin activated platelets, the same kind of enhancement of activation in the stirred versus unstirred system was observed in this study. There was a lack of platelet aggregates in the unstirred suspensions, as compared

to the same thrombin concentrations (0.1 and 0.05U/ml) in the stirred/aggregated samples. This phenomenon was not present at the three lower thrombin concentrations, where the platelets showed very little difference between stirred and unstirred preparations. This observation reaffirms that thrombin-induced platelet aggregation requires some threshold concentration of thrombin, in addition to platelet-platelet collision for aggregate formation.

Platelet aggregates, if examined on the basis of size, also demonstrate some interesting differences. Examination of single platlets, show internalized F-Au and heavy external labelling. As the platelet interacts with other platelets, there is an accummulation of label at these interfaces, until the aggregate reaches a point where there is a platelet core (one platelet surrounded by others). At this point, external labelling begins to disappear, and the platelet-platelet interface is cleared of label. The redistribution of the label is apparent, but the subsequent destination of the probe is not known.

Examination of platelet morphological changes as an indicator of the level of thrombin activation, provided a means to correlate GPIIb-IIIa receptor activation in the same cells. Mild activation, associated with shape change from discoid to spherical shape, and the development of pseudopodia, are reversible changes in the platelet, and are considered within the first or primary wave of aggregation (Huang and Detwiler, 1986). With stronger activation, there is platelet-platelet cohesion, fusion of platelet secretory granules, and consequent release of their contents to the immediate micro-environment, enhancing the aggregation response. This level of activation is not reversible, and is referred to as

secondary wave aggregation (Huang and Detwiler, 1986). The aggregation curves generated by each of the thrombin doses demonstrate that only the 0.1 and 0.05U/ml thrombin was enough to cause secondary wave aggregation of the platelets. The three lower doses produced shape change only, that eventually returned to baseline levels, illustrating the reversible activation of the platelet. When the level of F-Au probe binding to the GPIIb-IIIa receptor was compared in these lower dose treatments, they were equivalent to samples that were unactivated (no exogenous agonist addition). This demonstrates a separate, but related association with platelet activation (shape change), GPIIb-IIIa receptor activation (F-Au binding), and finally, GPIIb-IIIa internalization.

Using AP-2, a monoclonal antibody that specifically recognizes the complexed GPIIb-IIIa receptor, I was able to evaluate F-Au binding and receptor redistribution in the absence of external GPIIb-IIIa receptors. Overall, the level of platelet activation was reduced in both the stirred and unstirred systems, where the lowest three concentrations of thrombin stimulation showed no overwhelming differences, either morphologically or in regards to F-Au binding, from a control platelet. The only level of activation that markedly overrode the AP-2 effect was the degranulating concentration of 0.1U/ml thrombin (and to a lesser extent, 0.05U/ml thrombin). In these platelets, there was an abundance of internalized probe, that surrounded a fused granular core. This finding would suggest an additional store of receptors, not accessible to AP-2, that would be available to bind F-Au, and then be internalized. This is in agreement with other researchers that have demonstrated an internal store of GPIIb-IIIa receptors that are liberated to the platelet

surface after degranulating concentrations of agonist (Gogstad, et al., 1981; Woods, et al., 1986; Niiya, et al., 1987).

The major difference between the stirred and unstirred samples pre-incubated with AP-2, was in the 0.05U/ml thrombin treatments, and to a lesser extent, the lower three concentrations. At these concentrations, there was a marked difference between the level of F-Au labelling of the pre-incubated platelets. The stirred platelets showed essentially no probe binding, while the unstirred platelets had both internal and external binding at 0.05U/ml, and external labelling at the other concentrations. The significance of this labelling disparity is unclear, though it is in direct opposition to labelling characteristics reported earlier (Chapter 1) between unstirred and stirred preparations. One possible explaination may due to partial blocking of GPIIb-IIIa receptors by AP2. In the unstirred system, weakened ligand-receptor interactions may not be disrupted, but in the stirred system, higher shear rates may strip the F-Au from the platelet surface.

If the aggregation curves for AP-2 treated platelets are examined, no concentration of thrombin tested, caused a secondary wave of aggregation, and in fact, only minimal primary wave was observed. I have demonstrated previously that 0.1U/ml thrombin is sufficient stimulus to cause platelet aggregation. It was possible, however, that the presence of the monoclonal antibody modified the platelet-platelet interaction by binding exposed GPIIb-IIIa receptors to the extent of inhibiting aggregation, even under degranulating conditions. Isenberg, et al. (1990), reported similar results using

monoclonal antibodies specific for GP IIb and GP IIIa. They found that the antibodies were internalized and interfered with pseudopodia formation and aggregation. Another possibility is that the type of ligand bound to a receptor, either native or introduced, is involved in the activation response of the platelet. In this regard, Torti and coworkers (1991) demonstrated that the GPIIb-IIIa receptor is linked to signal transduction, and is dependent on the ligand bound for a specific response. They demonstrated that there was an inhibitory effect **after** mild thrombin activation by most ligands. Whether this effect is the same with receptor binding prior to activation, has not been previously addressed.

I have shown that the platelet micro-environment plays an important role in thrombin-induced activation, and that the platelet morphological response and GPIIb-IIIa activation are coupled, but not linearly related. The use of a GPIIb-IIIa-specific monoclonal antibody, provided useful information on the role of bound ligand on the activation and function of low dose thrombin-stimulated platelets. And finally, the release of internal platelet GPIIb-IIIa receptor stores are instrumental in platelet aggregation. These findings only reveal the complexity of the effect of thrombin on the platelet. Additional examination of the coupling effects between agonist, receptor, ligand and platelet activation, is essential for the understanding of platelet function and it's role in disease.

These findings are based solely on platlets activated in suspension. To better understand the mechanism of GPIIb-IIIa receptor redistribution, it is also necessary to examine the receptor after contact-activation of the platelet. The comparison of these two activation

mechanisms are explored in the following chapter.

BIBLIOGRAPHY

Bas, B.M., Muller, A.D. and Hemker, H.C. 1974 Purification and Properties of Staphylocoagulase. Biochim. Biophys. Acta 379:164-171.

Bendeyan, M. 1984 Protein-A Gold Electron Microscopic Immunocytochemistry: Methods, Applications, and Limitations. J. Electron Microsc. Tech. 1:243-270.

Collar, B.S. 1986 Mechanism(s) of Exposure of the GPIIb/IIIa Complex Receptor for Adhesive Glycoproteins. in Monoclonal Antibodies and Human Blood Platelets. INSERM Symposium No. 27. McGregor, J.L. (ed). Elsevier Science Publ. pp 93-102.

Davey, M.G. and Lüscher, E.F. 1967 Actions of Thrombin and Other Coagulant and Proteolytic Enzymes on Blood Platelets. Nature 216:857-858.

Escolar, G., Leistikow, E. and White, J.G. 1989 The Fate of the Open Canalicular System in Surface and Suspension-Activated Platelets. Blood 74(6):1983-1988.

Frens, G. 1973 Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Solutions. Nat. Phys. Sci. 241:20-22.

Gogstad, G.A., Hagen, I., Korsmo, R. and Solum, N.O. 1981 Characterization of the Proteins of Isolated Platelet Alpha-granules. Evidence for a Separate Alpha-granule Pool of the Glycoproteins IIb-IIIa. Biochem. Biophys. Acta 670:150-162.

Holmsen, H., Dangelmaier, C.A. and Holmsen, A.K. 1981 Thrombin-induced Platelet Responses Differ in Requirement for Receptor Occupancy. J. Biol. Chem. 256:9393-9399.

Huang, E.M. and Detwiler, T.C. 1986 Stimulus-Response \ Coupling Mechanisms in Biochemistry of Platelets. Phillips, D.R. and Shuman, M.A. (eds). Academic Press, Inc. pp 1-68.

Isenberg, W.M., Bainton, D.F. and Newman, P.J. 1990 Monoclonal Antibodies Bound to Subunits of the Integrin GPIIb-IIIa Are Internalized and Interfere With Filopodia Formation and Platelet Aggregation. Blood 76(8):1564-1571.

Jamieson, G.A. 1988 The Activation of Platelets by Thrombin: A Model for Activation by High and Moderate Affinity Receptor Pathways. in Platelet Membrane Receptors: Molecular Biology, Immunology, Biochemistry, and Pathology. G.A. Jamieson (ed). Alan R. Liss. pp 137-158.

Klomparens, K.L., Flegler, S.L. and Hooper, G.R. 1986 Procedures for Transmission and Scanning Electron Microscopy for Biological and Medical Science. A Laboratory Manual, 2nd ed. Ladd Research Ind., Burlington, VT. p. 146.

Longmire, L. and Frojmovic, M. 1990 Long Range Interactions in Mammalian Platelet Aggregation. I. Evidence from Kinetic Studies in Brownian Diffusion. Biophys. J. 58:299-307.

McGrowan, E.B. and Detwiler, T.C. 1986 Modified Platelet Response to Thrombin. Evidence for Two Types of Receptors or Coupling Mechanisms. J. Biol. Chem. 261:739-746.

Morita, T., Iwanaga, S. and Suzuki, T. 1976 The Mechanism of Activation of Bovine Prothrombin By a Procoagulant Factor from the Venom of *Echis carinatus* Venom and Characterization of the New Active Intermediates. J. Biochem. 79:1089-1108.

Niiya, K., Hodson, E., Bader, R., Byers-Ward, V., Koziol, J.A., Plow, E.F. and Ruggeri, Z.M. 1987 Increased Surface Expression of the Membrane Glycoprotein IIb/IIIa Complex Induced By Platelet Activation. Relationship to the Binding of Fibrinogen and Platelet Aggregation. Blood 70:475-83.

Owen, W.G. and Jackson, C.M. 1973 Activation of Prothrombin with *Oxyuranus scutellatus scutellatus* (Taipan Snake) Venom. Thrombos. Res. 3:705-714.

Peerschke, E.I.B. 1985 The Platelet Fibrinogen Receptor. Semin. Hematol. 22:241-246.

Pidard, D. 1986 Studies on the Organization and Function of GP IIb and GP IIIa Within the Plasma Membrane of Intact Human Platelets Using the Monoclonal Antibody AP-2. in Monoclonal Antibodies and Human Blood Platelets. INSERM Symposium No. 27. McGregor, J.L. (ed). Elsevier Science Publ. pp 103-117.

Pidard, D., Montgomery, R.R., Bennet, J.S. and Kunicki, T.J. 1983 Interaction off AP-2, a Monoclonal Antibody Specific for the Human Platelet Glycoprotein IIb-IIIa Complex, With Intact Platelets. J. Biol. Chem. 258:12582-12584.

Reynolds, E.S. 1963 The Use of Lead Citrate at High pH as an Electron Opaque Stain in Electron Microscopy. J. Cell Biol. 17:208-212.

Seiler, S.M., Goldeberg, H.J., Michel, I.M., Hunt, J.T. and Zavoico, G.B. 1991 Multiple

Pathways of Thrombin-induced Platelet Activation Differentiated by Desensitization and a Thrombin Exosite Inhibitor. Biochem. Biophys. Res. Comm. 181(2):636-643.

Shattil, S.J. 1988 Expression, Regulation and Detection of Fibrinogen Receptors on Activated Human Platelets. in Platelet Membrane Receptors: Molecular Biology, Immunology, Biochemistry, and Pathology. G.A. Jamieson (ed). Alan R. Liss. pp 345-380.

Shuman, M.A. and Greenberg, C.S. 1986 Platelet Regulation of Thrombus Formation in Biochemistry of Platelets. Phillips, D.R. and Shuman, M.A. (eds). Academic Press, Inc. pp 319-346.

Sinigaglia, F., Torti, M., Ramaschi, G. and Balduini, C. 1989 The Occupancy of Glycoprotein IIb-IIIa Complex Modulates Thrombin Activation of Human Platelets. Biochim. Biophys. Acta 00:225-230.

Torti, M., Sinigaglia, F., Ramaschi, G. and Balduini, C. 1991 Platelet Glycoprotein IIb-IIIa is Associated with 21-kDa GTP-binding Protein. Biochim. Biophys. Acta 1070:20-26.

Wencel-Drake, J.D., Plow, E.F., Kunicki, T.J., Woods, V.L., Keller, D.M. and Ginsberg, M.H. 1986 Localization of Internal Pools of Membrane Glycoproteins Involved in Platelet Adhesive Responses. Am. J. Pathol. 124:324-334.

White, J.G. and Escolar, G. 1991 The Blood Platelet Open Canalicular System: A Two-way Street. Eur. J. Cell Biol. 56:233-242.

White, J.G., Leistikow, E.L. and Escolar, G. 1990 Platelet Membrane Responses to Surface and Suspension Activation. Blood Cells 16:43-72.

Woods, V.L., Woof, L.E. and Keller, D.M. 1986 Resting Platelets Contain a Substantial Centrally Located Pool of Glycoprotein IIb-IIIa Complex Which May be Accessible to Some but Not Other Extracellular Proteins. J. Biol. Chem. 261(32):15242-15251.

CHAPTER 3.

EXAMINATION OF GPIIb-IIIa REDISTRIBUTION IN ADHERENT PLATELETS USING CORRELATIVE ULTRA-THIN SECTIONING AND WHOLE MOUNTS

INTRODUCTION

The platelet circulates in the blood stream as a small, anuclear cell fragment, until called to participate in hemostatic plug formation. As a consequence of vascular injury and exposure of the subendothelium, the platelet adheres and spreads to cover defects in the vascular cell wall, and subsequently recruits passing platelets to aid in the formation of a platelet plug. Of the glycoproteins present on the platelet membrane, by far, the most abundant, is Glycoprotein IIb-IIIa (GPIIb-IIIa) (Phillips, et al., 1991). This glycoprotein is responsible for platelet-platelet cohesion in the presence of fibrinogen, a circulating blood protein (Peerschke, E.I.B., 1985), and for platelet spreading after attachment to a surface (Zucker and Vroman, 1969; Jen and Lin, 1991).

The Glycoprotein IIb-IIIa receptor resides as an inactive, calcium-dependent heterodiamer, consisting of one molecule of Glycoprotein IIb and one of Glycoprotein IIIa (Newman, P.J., 1991; Nurden, et al., 1986). The receptor requires activation (Phillips, et al., 1991; Niiya, et al., 1987) before it is competent to bind ligand, of which fibrinogen, von Willibrand factor and fibronectin are included. Of these ligands, fibrinogen is the most significant. The number of receptors per platelet has been estimated at 50,000 (Jennings

and Phillips, 1982), but this number may be greater with the finding that the platelet contains an internal source of the receptor that is released upon platelet activation (Wencel-Drake, et al., 1986; Woods, et al., 1986).

The primary role of GPIIb-IIIa is the binding of fibrinogen, and the subsequent formation of platelet aggregates. In the absence of fibrinogen, it has been shown that platelets stimulated with ADP will form unstable, micro-aggregates without fibrinogen being detectable on the platelet surface (Suzuki, et al., 1988). The mechanism of this platelet cohesion is still not understood. In Glanzmann's thrombasthenia, where the GPIIb-IIIa receptor is lacking, non-functional or reduced in number, aggregation is reduced. This is believed to be due to the inability of the receptor to bind fibrinogen at normal levels (Lee, et al., 1981; Bennett, J.S., 1979). This modification of the GPIIb-IIIa receptor manifests, clinically, with various levels of hemorrhagic diathesis. It is therefore apparent that fibrinogen is necessary for normal platelet function.

Glycoprotein IIb-IIIa is not essential for platelet adhesion, though, under high shear rates, it is thought to assist Glycoprotein Ib, the primary adhesion receptor (Sakariassen, et al., 1986). The GPIIb-IIIa receptor is thought to be involved in the spreading of platelets after initial attachment to a surface. *In vivo*, GPIIb-IIIa's ligand is most likely von Willebrand factor (Weiss, et al., 1986), though, in Glanzmann's thrombastenia, washed platelets, in a static system, show reduced adherence and spreading (Caen and Michel, 1972). Glycoprotein IIb-IIIa is the primary receptor for adhesion only in the case of

fibrinogen and fibrin (Jen and Lin, 1991; Hantgan, et al., 1990). This is based on the high affinity the platelet has for fibrin(ogen) after activation, which results in recruitment of platelets to a growing thrombus (de Groot and Sixma, 1990).

In an attempt to determine what influence the GPIIb-IIIa receptor has on adherence and spreading, many investigators have examined the receptor activation and fate using labelled fibrinogen. The technique most often employed to view the receptor redistribution has been the use of whole mounts, which is faster and easier than ultrathin sectioning of adherent platelets (Kieffer, et al., 1992; White and Escolar, 1990; White, et al., 1990; Albrecht, et al., 1989: Escolar, et al., 1989). Determination of the location of the probe has been estimated through the use of stereo imaging micrographs of the whole mounts (Lewis, et al., 1990; Olorundare, et al., 1992). Though this method will approximate the location of the particle, only thin sectioning of the adherent platelets can give the actual location.

Platelets in suspension bind gold-labelled fibrinogen (F-Au) in a monodispersed fashion (Isenberg et al., 1989). In contrast, using a GPIIb-IIIa specific gold conjugated monoclonal antibody (MoAb-Au) it has been shown that patching, capping and eventual surface membrane clearing of the label occurs (Santoso et al., 1986). If the same probe is used in a calcium free system, receptor reorganization does not occur (Isenberg et al., 1987) indicating a calcium dependent step, possibly associated with microfilament reorganization. Stimulation of platelets in suspension with ADP did not alter the

monodisperse pattern of the MoAb-Au binding (Isenberg et al., 1987 and 1989). However, platelets activated with thrombin, which induces degranulation and release of internal stores of fibrinogen, resulted in receptor clustering followed by surface membrane clearing and aggregation (Escolar et al., 1989; White et al., 1990).

Like platelets in suspension, adherent platelets have similar labelling properties that may depend on the level of activation. Unlike platelets in suspension the role of the adherent platelet is distinctly different. Its primary function is to cover large areas requiring significant increases in surface area. Adherent platelets labelled with F-Au show migration of the label from the margins of the cells toward the granulomere (Albrecht et al., 1989; Lewis et al., 1990), a phenomenon similar to receptor capping. If calcium is not present (EDTA in media), no binding and therefore no migration is seen (White et al., 1990). This is possibly more related in this instance to the calcium requirement for fibrinogen binding than to calcium dependent alterations in the platelet cytoskeleton. Platelets labelled prior to adhesion demonstrate centralized migration of the fibrinogen-gold, but much of the label is internalized (Albrecht et al., 1989). This again suggests a separate sequence of events in suspension versus what is occurring following adhesion and spreading.

This study compared the fate of the GPIIb-IIIa complex redistribution in suspension after activation, with that of platelets attached to a surface. The use of whole mounts to obtain a general distribution of the probe was correlated with information from thin sections of

both the suspension and adherent platelets to better determine the localization of the fibrinogen probe, and, therefore, the GPIIb-IIIa receptor.

MATERIALS AND METHODS

Platelet Isolation: Donor blood was drawn into a 30ml syringe charged with 3.8% sodium citrate to give a final anticoagulant concentration of 0.38%. The sample was then spun at 1000 rpm for 10 minutes to obtain platelet rich plasma (PRP). The PRP was carefully removed, and transferred to a new plastic test tube where 1μM Prostaglandin E_1 (PGE_1; final concentration) was added. This suspension was allowed to rest, covered, 10 minutes before it was spun to concentrate the platelets (2000 rpm, 10 min.). The resulting pellet was resuspended in 1ml Tyrode's Buffer without calcium (TB; 0.2% bovine serum albumin, 130mM NaCl, 2.6mM KCl, 10mM HEPES, 5.5mM glucose, 2mM $MgCl_2$, pH 7.4), and additional PGE_1 (to 1μM) was added. The concentrated platelet suspension was covered, and allowed to rest 10 minutes before gel filtration.

Gel Filtration: A 10ml column was prepared using Sepharose 4B-200, equilibrated with TB. The 1ml platelet suspension was layered onto the surface and gel filtered to remove any residual plasma proteins. The first 25 cloudy drops were pooled, the platelets counted, and the concentrate diluted with Tyrode's buffer with calcium (TB+Ca^{++}; 1mM $CaCl_2$) to give a final platelet count of $2x10^8$ cells/ml. This suspension was allowed to rest (30 min) before experimental procedures.

Colloidal Gold Probes: Fifteen to 18nm colloidal gold was prepared according to the methods of Bendayan (1984), and was conjugated with fibrinogen and Polyethylene Glycol (PEG) as follows;

Fibrinogen - One hundred and thirty milligrams of lyophilized human fibrinogen (KABI) was dissolved in 3mls 5mM NaCl and fractionated over a 20ml Sephadex G-25 collum equilibrated with 5mM NaCl. The 280nm absorbance of the non-precipitated fractions were taken, and the 3 tubes with the highest readings were pooled. A Markwell protein assay was performed to determine the protein concentration. The fibrinogen was conjugated to the colloid at pH 6.4 after a minimum protecting protein concentration was determined.

PEG - The minimum protecting concentration of 1% PEG in 5mM NaCl was established, and the PEG was conjugated to the colloidal gold at pH 6.0 to provide a non-specific binding control for the gold labelling.

Agonists: Thrombin from human plasma (Sigma, T-8885) was diluted to provide a final reaction concentration of 0.1U/ml. Adenosine diphosphate (ADP) was made as a 2.4 x 10^{-4} stock, and 200μl aliquots were stored at -60°C until use. The ADP was diluted to a final reaction concentration of 10μM.

Adherent Surfaces: For transmission electron microscopic (TEM) cross-sections, tissue-culture plastic coverslips (Thermonox, Miles Scientific) were quartered and notched (to maintain orientation), then carbon-coated. For whole mount preparations, 300 mesh

nickel grids were coated with 0.5% Formvar plastic then carbon-coated. Platelets were allowed to attach and spread directly on the coated surfaces.

Pre-adhesion Ligand Binding: Five hundred microliters of the platelet suspension was incubated with 100μl of gold probe or TB+Ca^{++} (37°C, 3 min.). Twenty microliters of agonist or TB+Ca^{++} was added to the suspension, was mixed by inversion and applied to the appropriate surface (Fig. 3.1). Platelets were allowed to attach and spread at 37°C, 10 minutes, in a humidified chamber. After incubation, the unattached platelets were

	PROBE	AGONIST
	(50μl, 3 min, 37°C)	(add 10μl, then apply to surface, 10 min, 37°C)
1	PEG-Au	TB+Ca^{++}
2	PEG-Au	10μM ADP
3	PEG-Au	0.1U/ml Thrombin
4	Fibrinogen-Au	TB+Ca^{++}
5	Fibrinogen-Au	10μM ADP
6	Fibrinogen-Au	0.1U/ml Thrombin

Figure 3.1. Pre-Adhesion Treatment Combinations

removed by washing with TB+Ca^{++}, and the samples fixed for electron microscopy.

Post-adhesion Ligand Binding: Platelets were allowed to attach to the surfaces, 10 minutes at 37°C in a humid chamber. At the end of this time, the unattached platelets were removed by washing with TB+Ca^{++}. The remaining cells were overlaid with a

reaction mixture cocktail made from 250μl of TB+Ca^{++}, 50μl of probe or buffer, and 20μl of agonist or buffer (Fig. 3.2). This was allowed to incubate on the cells at 37°C for 5 minutes, then was washed off with buffer to remove unreacted solutions. These surfaces were then prepared for electron microscopy.

Unstirred Platelet Treatments: Five hundred microliters of the platelet suspension was aliquoted into 1500μl microfuge tubes. To each of these tubes, 100μl of gold probe or TB+Ca^{++} was added, the tube inverted to mix, then allowed to incubate at 37°C for 3 minutes. At the end of the initial incubation 20μl of agonist or TB+Ca^{++} was added,

	PROBE	AGONIST
	(50μl diluted in TB+Ca^{++})	(add 10μl to labelling cocktail, then apply to cells, 5 min, 37°C)
1	TB+Ca^{++}	TB+Ca^{++}
2	TB+Ca^{++}	10μM ADP
3	TB+Ca^{++}	0.1U/ml Thrombin
4	PEG-Au	TB+Ca^{++}
5	PEG-Au	10μM ADP
6	PEG-Au	0.1U/ml Thrombin
7	Fibrinogen-Au	TB+Ca^{++}
8	Fibrinogen-Au	10μM ADP
9	Fibrinogen-Au	0.1U/ml Thrombin

Figure 3.2. Post-Adhesion Treatment Combinations

inverted to mix and incubated an additional 5 minutes at 37°C. See Figure 3.3 for

particular experimental variables. The reaction was terminated with the addition of 600μl of 0.1% glutaraldehyde in 0.1M cacodylate buffer (CB), pH 7.4. After 30 minutes, the platelet suspensions were pelleted at 12,800 rpm for 10 seconds.

Fixation and Embedding: Platelets on their support structures were fixed in 2.5% glutaraldehyde in 0.1M Cacodylate buffer (CB) for 1 hour at 27°C.

Transmission electron microscopy - Whole mounts - Grids were washed after primary fixation with dH_2O and stained with 0.5% aqueous uranyl acetate for 15 minutes at 27°C. Samples were washed again in dH_2O and transferred to porous baskets for the duration

	PROBE	AGONIST
	(100μl, 3 min, 37°C)	(20μl, 5 min, 37°C)
1	TB+Ca^{++}	TB+Ca^{++}
2	TB+Ca^{++}	10μM ADP
3	TB+Ca^{++}	0.1U/ml Thrombin
4	PEG-Au	TB+Ca^{++}
5	PEG-Au	10μM ADP
6	PEG-Au	0.1U/ml Thrombin
7	Fibrinogen-Au	TB+Ca^{++}
8	Fibrinogen-Au	10μM ADP
9	Fibrinogen-Au	0.1U/ml Thrombin

Figure 3.3. Suspension Treatment Combinations

of the dehydration in graded ethanols and critical point drying. Samples were examined

directly at 80kV in a JEOL 100CXII TEM.

Thin sections - Samples were post-fixed in aqueous 1% osmium tetroxide for 30 minutes at RT, washed with dH_2O, en-bloc stained in an aqueous 0.5% uranyl acetate solution for 30 minutes at RT, then dehydrated in a graded ethanol series to 100%. Resin infiltration was carried out in ratios of 3:1, 1:1 and 1:3 (100% ethanol to resin) for 3 hours each on a rotator. The final infiltration into 100% Spurrs-Mollenhauer's (Klomparens, et al. 1986) resin was overnight on a rotator. The coverslips were removed from the vials, the backs wiped free of resin with ethanol, and placed on microscope slides. Fresh resin was placed on the coverslips to form a slightly convex layer over the cells, and was polymerized at 65°C for 48 hours. The samples were allowed to cool, then were peeled away from the coverslips, cut into smaller pieces and re-embedded in molds, convex side down. Ultrathin sections were stained in a saturated aqueous solution of uranyl acetate followed by Reynolds lead (Reynolds, 1963), then examined in a JEOL 100CXII TEM at 80kV.

All data based on at least two experimental repetitions, and the observation of at least 20 platelets per experimental treatment.

RESULTS

Examination of the control treatments, where PEG-Au was the labelling probe, revealed a lack of specific binding to the platelet, in all treatments (Figures 3.4 and 3.5). In

platelets pre-incubated with the PEG-Au label and allowed to adhere, there was a background of non-specific labelling over both the plastic film and the attached platelets. Cross-sections of the platelets showed no internalization and minor trapping of the probe beneath the platelet (Figure 3.4). In platelets that were attached first to the surface then incubated with the PEG-Au reaction mixtures, minimal gold labelling was seen in thin sections, though small clusters of the probe could occasionally be found over the granulomere region in the whole mounts (Figure 3.5). Platelets incubated in suspension showed little to no specific PEG-Au probe binding (Figure 3.6), and only then in thrombin-stimulated platelets.

Pre-incubated Aherent Platelets: Examination of platelets pre-incubated with F-Au cocktails, then allowed to spread on a surface are shown in Figure 3.7. Whole mounts demonstrated labelling that was centralized in patches of clumped probe (Figure 3.7c). Cross-sections demonstrate that the labelling is both surface oriented and internalized, with little or no gold between the platelet and the adhesion surface (Figure 3.7a). Some non-specific attachment of probe on the plastic support film also occurred (Figure 3.7b). Adenosine diphosphate-activation prior to attachment caused an apparent increase in the amount of internalizated F-Au probe, and a reduction in surface labelling (Figure 3.7d). The whole mounts showed a general clearing of the peripheral web, with centralization of the probe over areas where intact secretory granules could be found. Probe labelling near the periphery was sparse with distinct clusters as well as singlet probe (Figure 3.7f). Thrombin activation of F-Au pre-incubated platelets, resulted in platelets devoid of

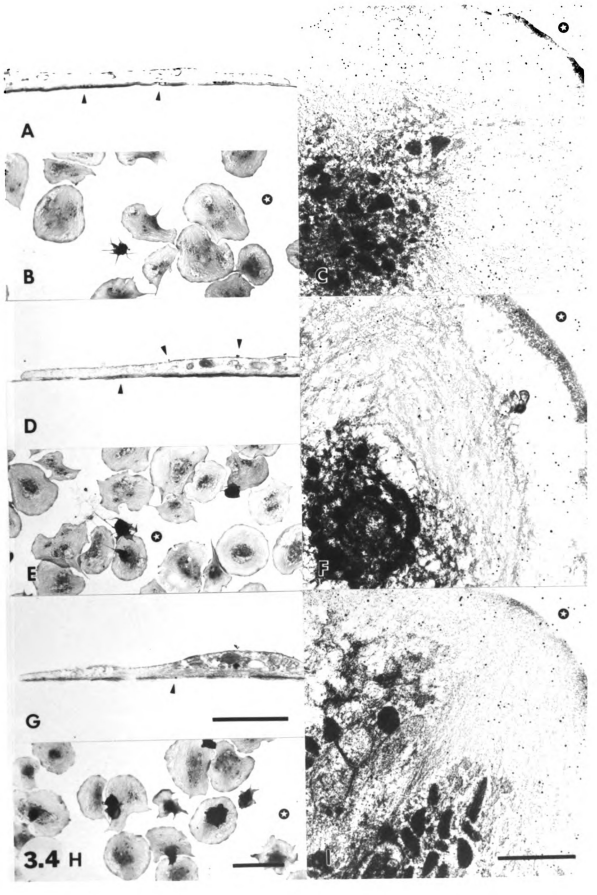
Figure 3.4 Pre-adhesion Controls. PEG-Au Incubation.

PEG-Au incubated, no agonist: A) Ultra-thin section. Minor labelling at platelet adhesion interface (arrowheads). B) Whole mount. Some non-specific labelling present on plastic film (star). C) Whole mount. Non-specific binding of label on platelet and plastic surface (star).

PEG-Au incubated, ADP-stimulated: D) Ultra-thin section. Minor labelling at platelet adhesion interface and on exposed surface (arrowheads). E) Whole mount. Some non-specific labelling present on plastic film (star). F) Whole mount. Non-specific binding of label on platelet and plastic surface (star).

PEG-Au incubated, thrombin-stimulated: G) Ultra-thin section. Minor labelling at platelet adhesion interface and on exposed surface (arrowheads). H) Whole mount. Some non-specific labelling present on plastic film (star). I) Whole mount. Non-specific binding of label on platelet and plastic surface (star).

Bar = 1μM in A, C, D, F, G, I. Bar = 5μM in B, E, H.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B
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3.4 H
I

Figure 3.5 Post-adhesion Controls. PEG-Au Incubation.

PEG-Au incubated, no agonist: A) Ultra-thin section. No labelling internally or externally. B) Whole mount. No non-specific labelling present. C) Whole mount. No non-specific binding of label on platelet or plastic surface.

PEG-Au incubated, ADP-stimulated: D) Ultra-thin section. No labelling at platelet adhesion interface or on exposed surface. E) Whole mount. No non-specific labelling present. F) Whole mount. Minimal non-specific labelling present.

PEG-Au incubated, thrombin-stimulated: G) Ultra-thin section. No labelling present at platelet adhesion interface or on exposed surface. H) Whole mount. No non-specific labelling present. I) Whole mount. Minor non-specific binding of label on platelet and plastic surface.

Bar = 1 micrometer in A, D, G. Bar = 1 micrometer in C, F, I. Bar = 5 micrometers in B, E, H.

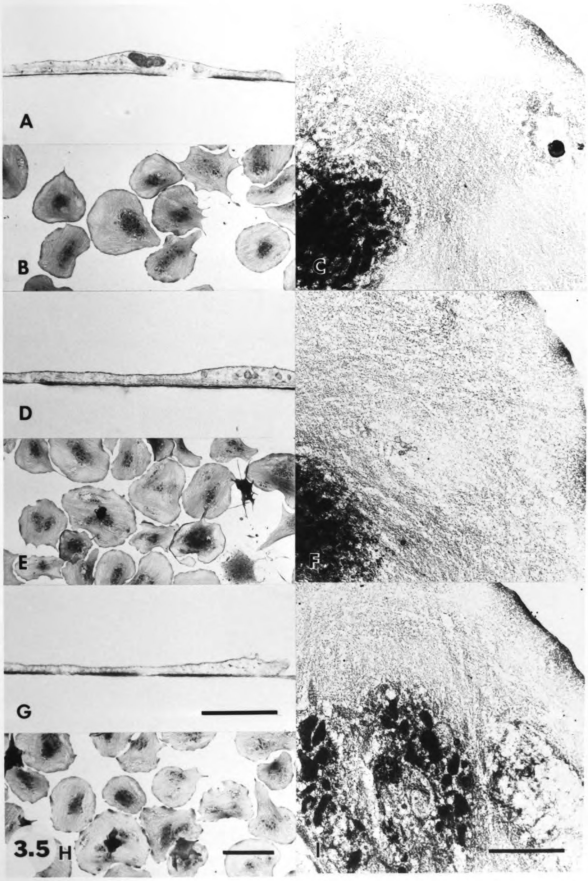
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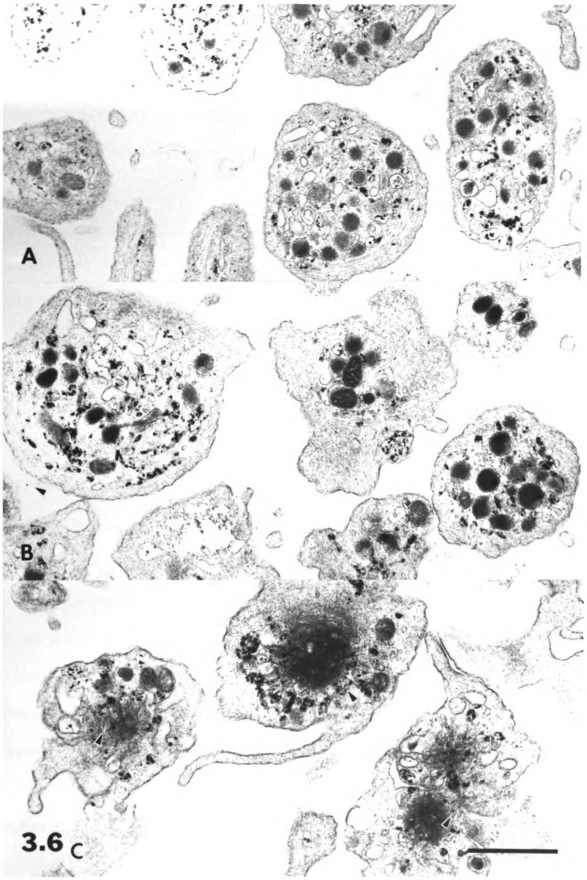
Figure 3.6 Unstirred Suspension Controls. Bar = 1μm. Dense granular areas in platelet represent glycogen deposits. A) Platelets treated with PEG-Au without agonist addition. No apparent labelling of platelets B) ADP-stimulated, PEG-Au incubated platelets. Occassional probe found (arrowheads), though non-specific in attachment. C) Thrombin-stimulated, PEG-Au incubated treatment. No external labelling, with occasional internalization (arrowheads).



3.6

surface labelling (Figure 3.7g), and increased internalization into the OCS. Whole mounts show a lack of probe except over the granulomere region of the platelet (Figure 3.7i). The whole mounts showed some platelet aggregation, typified by rounded un-spread cells layered on the adherent platelets (Figure 3.7h).

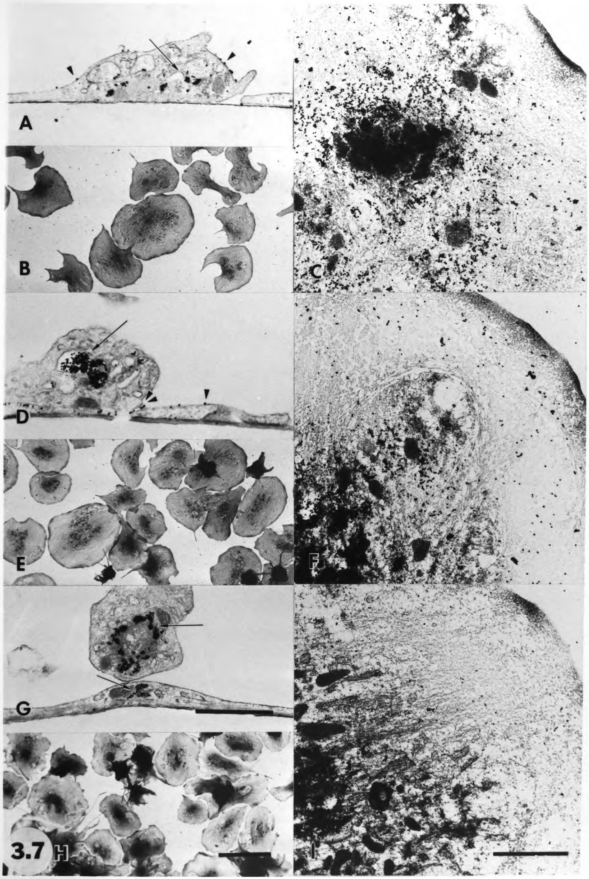
Post-incubated Adherent Platelets: Platelets, allowed to adhere then treated with F-Au, showed heavy labelling of the whole mounts that increased in density from the membrane edge towards the granulomere region (Figure 3.8c). Cross-sections of these platelets, revealed that the majority of the label was external and could be found both on the surface of the platelet as well as beneath. Very little F-Au was located internally and, if found, was in membrane bound vesicles (Figure 3.8a). The probe appeared clumped in both whole mounts and the sectioned samples. In ADP activated, F-Au samples, labelling could be found both externally on the surface and beneath the platelets, and internally in the OCS (Figure 3.8d). Whole mounts demonstrated a more sparse dispersement of the label along the peripheral web of the platelet, that was clumped and irregular (Figure 3.8f), as compared to F-Au only (Figure 3.8c). The highest density of label was found in the granulomere region as was evidenced in both whole mounts and cross-section. Thrombin-activated specimens did not differ greatly from ADP-activated platelets. Distribution in whole mounts had the same characteristics (Figure 3.8i), though thin sections appeared to have increased external labelling over the granulomere region of the platelet (Figure 3.8g). The general morphology of the platelets in the Thrombin/F-Au treatment, changed from the rounded appearance of F-Au and ADP/F-Au (Figures 3.8b

Figure 3.7 Pre-adhesion Treatments. F-Au Incubation. OCS = open canalicular system. F-Au incubated, no agonist: A) Ultra-thin section. External label (arrowheads) single and clustered. Minimal labelling at platelet adhesion interface. Internal labelling (arrow) in OCS. B) Whole mount. Minimal non-specific labelling present on plastic film. C) Whole mount. Clumped labelling most dense around granulomere, with a cleared area near platelet peripheral membrane.

F-Au incubated, ADP-stimulated: D) Ultra-thin section. Concentrated internal labelling in OCS (arrow). Single probe external labelling (arrowhead) on exposed platelet surface. Increased external labelling at platelet-platelet contact (double arrowheads). E) Whole mount. Some non-specific labelling present on plastic film. F) Whole mount. Labelling concentrated in granulomere region. Clustered probe found from platelet periphery to central region. Singlet probe scattered throughout platelet.

F-Au incubated, thrombin-stimulated: G) Ultra-thin section. No to rare labelling present at platelet surface. Internal labelling in OCS (arrows) of both adherent and unattached platelets. H) Whole mount. No non-specific labelling present on plastic film. I) Whole mount. Platelet periphery almost cleared of probe binding. Majority of probe centralized in granulomere region.

Bar = 1μM in A, C, D, F, G, I. Bar = 5 micrometers in B, E, H.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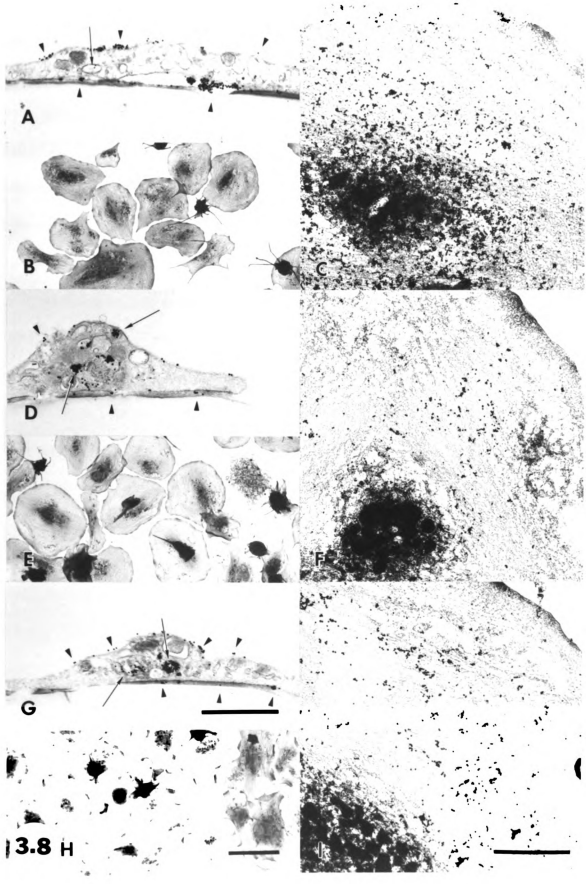
3.7

Figure 3.8 Post-adhesion Treatments. F-Au Incubation. OCS = open canalicular system. F-Au incubated, no agonist: A) Ultra-thin section. External label (arrowheads) some single but mostly clustered. Labelling at platelet adhesion interface. Minimal internal labelling (arrow) in OCS. B) Whole mount. No non-specific labelling present on plastic film. C) Whole mount. Clustered labelling most dense around granuomere region, that decreases nearer platelet peripheral membrane.

F-Au incubated, ADP-stimulated: D) Ultra-thin section. Concentrated internal labelling in OCS (arrow). Single probe and clustered external labelling (arrowhead) on exposed platelet surface, and at platelet adhesion interface. E) Whole mount. No non-specific labelling present on plastic film. F) Whole mount. Labelling concentrated in granulomere region. Clustered probe found from platelet periphery to central region in patches.

F-Au incubated, thrombin-stimulated: G) Ultra-thin section. Single and clustered labelling present at platelet surface, and adhesion interface (arrowheads). Internal labelling in OCS (arrows). H) Whole mount. No non-specific labelling present on plastic film. I) Whole mount. Probe clustered and in patches across platelet. Majority of probe centralized in granulomere region.

Bar = 1μM in A, C, D, F, G, I. Bar = 5μM in B, E, H.

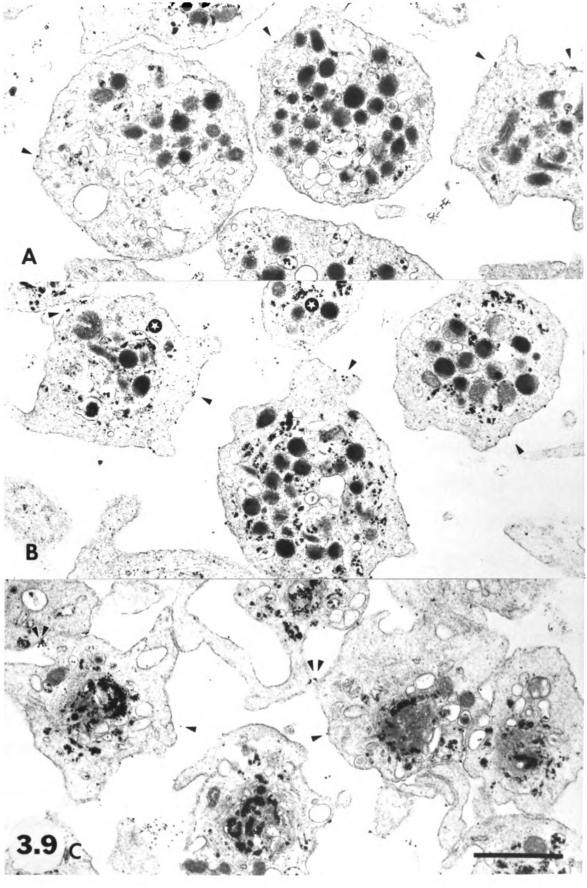


3.8

and 3.8e), to cells irregular in shape (Figure 3.8h).

Suspension Platelets: Platelets incubated with the reaction cocktails in suspension then fixed, showed limited F-Au binding to unactivated platelets. This minimal binding was consistant with previous observations, and only found externally (Figure 3.9a). Samples activated with ADP/F-Au showed an increase in labelling that could be found both internally as well as externally (Figure 3.9b). The label was primarily mono-dispersed on the cell surface, and when internalized, could be found in membrane bound vesicles. The ADP/F-Au samples showing internalization was estimated to be approximately 7% of the total platelet population. Thrombin stimulation of the platelets resulted in marked internalization into the OCS. Areas of contact between platelets contained probe, and there was sparse, mono-dispersed F-Au on the membrane surface (Figure 3.9c).

Figure 3.9 Unstirred Suspension Treatments. Bar = 1μM. A) Platelets treated with F-Au without agonist addition. Sparse, singlet probe labelling of external platelet membrane (arrowheads). B) ADP-stimulated, F-Au incubated platelets. Clustered probe on external membrane (arrowheads). Internal labelling (star) in membrane bound vesicles. C) Thrombin-stimulated, F-Au incubated treatment. Sparse external labelling (arrowheads), with clustering (double arrowheads) at points of cell contact.


A
B
3.9 C

DISCUSSION

Whole mount preparations to determine probe localization on the platelet has been extensively used. The procedure eliminates the need for thin sections for transmission electron microscopy (TEM), which is both difficult and time-consuming. The cells are spread on plastic-coated TEM grids, and due to the thinness of the platelet, are viewed directly without embedding. Examination of the whole mounts provides general information on the distribution of the probe, though no information on whether the probe is internally or externally bound can be accurately determined. Thin sectioning of adherent platelets is the only accurate method to determine probe localization.

The control samples demonstrated that the PEG-Au conjugate was not specifically bound to the cells in any of the treatments, either in suspension or as adherent platelets. There was an increase in background labelling in the pre-incubation samples, where platelets in the labelling cocktail were allowed to spread on the plastic film. This high background was most likely due to a net negative charge on the fresh plastic film, that had not been dampened by protein in the reaction buffer. In contrast, on the grids where the platelets were allowed to attach and spread prior to labelling, there was no visible background.

Comparison of the suspension and pre-incubated adherent samples show some expected similarities. In suspension samples treated only with F-Au, the labelling was limited to the outside surface of the platelet. With the enhancement of platelet contact-activation,

F-Au incubated adherent platelets demonstrated external labelling, as well as internal labelling. No labelling could be found at the adhesion interface. The ADP/F-Au treatments showed an increase in external and minimal internal (7%) labelling in suspension. In contrast, adherent platelets showed an increase in internalization with a decrease in external labelling. These observations suggest a transfer of label internally after contact activation of the platelet.

Thrombin stimulation and pre-incubation with F-Au produced a platelet in suspension with moderate external labelling, and extensive internalization of probe into the OCS. This result was similar to the pre-incubated adherent platelet in the amount of internalization of marker, but differed greatly in the amount of probe bound to the surface. In the adherent platelet, there was no surface labelling. Again, this suggested an internalization of surface probe after contact activation. One possible explanation for this phenomenon is based on the micro-environment of the treatments. In an unstirred suspension, the platelet was in an unnatural, static, environment. Researchers have demonstrated that platelets in an unstirred system, required more stimulus for activation than in a stirred system (Longmire and Frojmovic, 1990). In addition, there was also created a micro-environment of extended agonist exposure, because the agonist was not removed by flow as it normally would be in the circulation. This factor is pertinent because it has been shown that strength and duration of agonist exposure has profound effects on the level of activation of the platelet (Seiler, et al., 1991; Jamieson, G.A., 1988; Holmsen, et al., 1981). These two factors may be eliciting a protective mode in the

platelets, where it removes available receptors from surface exposure to prevent embolism formation (Torti, et al., 1991). It has been demonstrated that given equal exposure to thrombin in stirred and unstirred systems, the unstirred treatment does not result in aggregate formation, while aggregates were formed in the stirred treatments (Chapter 1). When these pre-incubated platelets were allowed to attach to a surface, there was an increase in internalization of probe, with a simultaneous decrease in external labelling, as the agonist potency increases. This again, may be carry-over from the protective response found in suspension. In the case of F-Au labelling with no agonist, the platelet responded as it would be expected in a contact activated situation. The probe remains on the surface, with little internalization, available for platelet recruitment. It was therefore apparent, that the micro-environment, in conjunction with agonist stimulation of the platelet, had a distinct effect on the platelet, and that the platelet may have limited means to control aggregate formation.

Membrane movement in suspension appears to be considerably different than when following contact activation. In suspension, the platelet develops a spherical shape and forms pseudopodia upon activation, with no real change in the size of the cell. In contrast, following contact activation, the platelet goes from a 2-4μm disc, to a cell that is approximatley 15μm in diameter. Some researchers believe that this phenomonmen involves the recruitment of membrane from platelet stores, the most likely source being, the OCS (Escolar, et al., 1989). If F-Au has been internalized into the OCS, and the OCS evaginates to the adhesion surface to allow spreading, then the ligand probe would be

expected to be deposited at this site. In my studies, the surface-activated platelets maintain the majority of the F-Au probe internally, in obvious, OCS channels, though the platelets are fully spread. Therefore, platelet spreading may be a more closely related phenomenon to the hypothesis of Behnke and Bray (1988), who patterns the spreading mechanism of platelets to that of fibroblasts and endothelial cells, both of which do not contain an OCS.

Platelets that have been allowed to attach to a surface have gone through contact activation, though, the level of activation of the platelet has not been completely elucidated (Lüscher, E.F., 1985). In this work, receptor redistribution was also observed following the responses of the platelet to contact activation. When spread platelets were incubated with F-Au alone, there was extensive probe labelling on the platelet surface. This observation has been previously reported by a number of investigators (Albrecht, et al., 1989; Lewis, et al, 1990; White, et al., 1990). Of interest, was the finding of F-Au at the adhesion interface, and minimal apparent internalization of probe into the platelet interior. The migration of probe to the adhesion interface has not previously been reported. This may be due to resolution difficulties when using stereo-paired whole mounts to determine probe localization. The second observation, of no F-Au internalization into spread platelets, is in contrast with White, et al. (1990), who reported marked internalization into the platelet OCS. The experimental conditions surrounding the ultra-thin section shown in their paper were not clear, and therefore, the variation seen between our data may be due to experimental differences. My data strongly suggests that

the difference is related to the point at which the receptor is occupied (ie. pre- verus post-incubation). The surface labelling would be available for platelet cohesion, and the migration of probe to the adhesion interface may act as an adhesion/spreading mechanism to more completely anchor the cell.

In contrast, agonist addition to the F-Au pre-incubation, resulted in internalization of probe that was not observed in the F-Au-only treatment. The internalization increased with the potency of the agonist, and may be due to receptor recycling or newly exposed receptors (Wencel-Drake,et al., 1986; Woods, et al., 1986). The apparent clearing of the platelet membrane surface may be the result of the newly exposed receptors binding released, endogenous stores of fibrinogen (Gralnick, et al., 1991). This hypothesis must be examined before further conjecture.

The difference between the pre-incubation adhesion samples and those allowed to attach prior to labelling, may be related to the hypothesis of Watson (1991) which postulates "function follows form". The GPIIb-IIIa receptor redistribution may be linked to an alternate series of intercellular signals based on whether the ligand is bound in suspension or after the platelet has attached to a surface. As stated before, the clearing of ligand/receptor complexes into the platelet OCS may be protective in pre-incubation situations, while surface exposure of the same complexes in adherent platelets may aid in platelet recruitment. This potentially demonstrates a much more intricate redistribution system for the GPIIb-IIIa receptor.

As shown in this study, the micro-environment, the potency of agonist, and the method of platelet activation have profound effects on the redistribution of the GPIIb-IIIa receptor. This redistribution may be involved with platelet activation protection from unnatural stimuli, and in more natural settings, as a means to enhance platelet recruitment. In addition, the point at which the receptor is occupied by ligand (pre- or post-adhesion) appears to be instrumental to where the receptor/ligand complex is relocated. This information would not be available using conventional means of data collection. Whole mounts, used in tandem with thin sections, were required to accurately determine the redistribution of the receptor, that whole mounts alone, could not provide.

The importance of the ligand/receptor interaction has been shown to be important in its susequent redistribution after platelet activation. To further examine this interaction, the effects of soluble verus particulate ligand probe will be examined and compared in the following chapter.

BIBLIOGRAPHY

Albrecht, R.M., Goodman, S.L. and Simmons, S.R. 1989 Distribution and Movement of Membrane-Associated Platelet Glycoproteins: Use of Colloidal Gold with Correlative Video-Enhanced Light Microscopy, Low-Voltage High-Resolution Scanning Electron Microscopy, and High-Voltage Transmission Electron Microscopy. Am. J. Anat. 185:149-164.

Behnke, O. and Bray, D. 1988 Surface Movements During the Spreading of Blood Platlets. Eur. J. Cell Biol. 46:207-216.

Bendeyan, M. 1984 Protein-A Gold Electron Microscopic Immunocytochemistry: Methods, Applications, and Limitations. J. Electron Microsc. Tech. 1:243-270.

Bennett, J.S. 1976 Exposure of Platelet Fibrinogen Receptors by ADP and Epinephrine. J. Clin. Invest. 64:1393-1399.

Caen, J.P. and Michel, H. 1972 Platelet Shape Change and Aggregation. Nature 240:148-149.

de Groot, P.G. and Sixma, J.J. 1990 Platelet Adhesion . Brit. J. Haematol. 75:308-312.

Escolar, G., Leistikow, E. and White, J.G. 1989 The Fate of the Open Canalicular System in Surface and Suspension-Activated Platelets. Blood 74(6):1983-1988.

Gralnick, H.R., Williams, S., McKeown,L., Connaghan, G., Shafer, B., Hansmann, K., Vail, M. and Fenton, J. 1991 Endogenous Platelet Fibrinogen Surface Expression on Activated Platelets. J. Lab. Clin. Med. 118(6):604-613.

Hantgan, R.R., Nichols, W.L. and Ruggeri, Z.M. 1990 Von Willebrand Factor Competes With Fibrin for Occupancy of GPIIb:IIIa on Thrombin-Stimulated Platlets. Blood 75(4):889-894.

Holmsen, H., Dangelmaier, C.A. and Holmsen, A.K. 1981 Thrombin-induced Platelet Responses Differ in Requirement for Receptor Occupancy. J. Biol. Chem. 256:9393-9399.

Isenberg, W.M., McEver, R.P., Phillips, D.R., Shuman, M.A. and Bainton, D.F. 1987 The Platelet Fibrinogen Receptor: An Immunogold-Surface Replica Study of Agonist-Induced Ligand Binding and Receptor Clustering. J. Cell Biol. 104:1655-1663.

Isenberg, W.M., McEver, R.P., Phillips, D.R., Shuman, M.A. and Bainton, D.F. 1989 Immunogold-Surface Replica Study of ADP-Induced Ligand Binding and Fibrinogen Receptor Clustering in Human Platelets. Am. J. Anat. 185:142-148.

Jamieson, G.A. 1988 The Activation of Platelets by Thrombin: A Model for Activation by High and Moderate Affinity Receptor Pathways. in Platelet Membrane Receptors: Molecular Biology, Immunology, Biochemistry, and Pathology. G.A. Jamieson (ed). Alan R. Liss. pp 137-158.

Jen, C.J. and Lin, J.S. 1991 Direct Observation of Platelet Adhesion to Fibrinogen- and Fibrin-coated Surfaces. Am. J. Physiol. 261:H1457-H1463.

Jennings, K.L. and Phillips, D.R. 1982 Purification of Glycoproteins IIb and III From Human Platelet Plasma Membranes and Characterization of a Calcium-dependent

Glycoprotein IIbIII Complex. J. Biol. Chem. 257:10458-10466.

Kieffer, N., Guichard, J. and Brenton-Gorius, J. 1992 Dynamic Redistribution of Major Platelet Surface Receptors After Contact-induced Platelet Activation and Spreading. Am. J. Pathol. 140(1):57-73.

Klomparens, K.L., Flegler, S.L. and Hooper, G.R. 1986 Procedures for Transmission and Scanning Electron Microscopy for Biological and Medical Science. A Laboratory Manual, 2nd ed. Ladd Research Ind., Burlington, VT, p. 146.

Lee, H., Nurden, A.T., Thomaidis, A. and Caen, J.P. 1981 Relationship Between Fibrinogen Binding and the Platelet Glycoprotein Deficiences in Glanzmann's Thrombasthenia Type I and Type II. Br. J. Haematol. 48:47-52.

Lewis, J.C., Hantgan, R.R., Stevenson, S.C. Thornburg, T., Keiffer, N., Guichard, J. and Breton-Gorius, J. 1990 Fibrinogen and Glycoprotein IIb/IIIa Localization During Platelet Adhesion. Am. J. Pathol. 136(1):239-252.

Longmire, L. and Frojmovic, M. 1990 Long-range Interactions in Mammalian Platelet Aggregation. Evidence from Kinetic Studies in Brownian Diffusion. Biophys. J. 58:299-307.

Lüscher, E.F. 1985 Plasma Membrane Receptors and Platelet Response in Platelet Membrane Glycoproteins. George, J.N., Nurden, A.T., Phillips, D.R. (eds). Plenum Press. New York. pp 3-9.

Newman, P.J. 1991 Platelet GPIIb-IIIa: Molecular Variations and Alloantigens. Thrombos. Haemost. 66(1):111-118.

Niiya, K., Hodson, E., Bader, R., Byers-Ward, V., Koziol, J.A., Plow, E.F. and Rugger, Z.M. 1987 Increased Surface Expression of the Membrane Glycoprotein IIb/IIIa Complex Induced by Platelet Activation: Relationship to the Binding of Fibrinogen and Platelet Aggregation. Blood 70:475-483.

Nurden, A.T., George, J.N. and Phillips, D.R. 1986 Platelet Membrane Glycoproteins: Their Structure, Function, and Modification in Disease in Biochemistry of Platelets. Phillips and Shuman (eds). Academic Press. pp 159-224.

Olorundare, O.E., Simmons, S.R. and Albrecht, R.M. 1992 Cytochalasin D and E: Effects on Fibrinogen Receptor Movement and Cytoskeletal Reorganization in Fully Spread, Surface-Activated Platelets: A Correlative Light and Electron Microscopic Investigation. Blood 79(1): 99-109.

Peerschke, E.I.B. 1985 The Platelet Fibrinogen Receptor. Semin. Hematol. 22:241-59.

Phillips, D.R., Charo, I.F. and Scarborough, R.M. 1991 GPIIb-IIIa: The Responsive Intergrin. Cell 65:359-362.

Reynolds, E.S. 1963 The Use of Lead Citrate At High pH as an Electron Opaque Stain in Electron Microscopy. J. Cell Biol. 17:208-212.

Sakariassen, K.S., Nievelstein, P.F.E.M., Coller, B.S. and Sixma, J.J. 1986 Role of Platelet Membrane Glycoproteins Ib and IIbIIIa in Platelet Adhesion to Human Artery Subendothelium. Brit. J. Haematol. 63:681-691.

Santoso, S., Zimmermann, U., Neppert, J. and Mueller-Eckhardt, C. 1986 Receptor Patching and Capping of Platelet Membranes Induced by Monoclonal Antibodies. Blood 67(2):343-349.

Seiler, S.M., Goldeberg, H.J., Michel, I.M., Hunt, J.T. and Zavoico, G.B. 1991 Multiple Pathways of Thrombin-induced Platelet Activation Differentiated by Desensitization and a Thrombin Exosite Inhibitor. Biochem. Biophys. Res. Comm. 181(2):636-643.

Suzuki, H., Kinlough-Rathbone, R.L., Packham, M.A., Tanoue, K., Yamazaki, H. and Mustard, J.F. 1988 Immunocytochemical Localization of Fibrinogen on Washed Human Platelets. Lack of Requirement for Fibrinogen During Adenosine Diphosphate Induced Responses and Enhanced Fibrinogen Binding In a Medium With Low Calcium Levels. Blood 71:850-860.

Torti, M., Sinigaglia, F., Ramaschi, G. and Balduini, C. 1991 Platelet Glycoprotein IIb-IIIa is Associated with 21-kDa GTP-Binding Protein. Biochim. Biophys. Acta 1070:20-26.

Watson, P.A. 1991 Function Follows Form: Generation of Intracellular Signals by Cell Deformation. FASEB J. 5:2013-2019.

Weiss, H.J., Turitto, V.T. and Baumgartner, H.R. 1986 Platelet Adhesion and Thrombus Formation on Subendothelium in Platelets Deficient in Glycoproteins IIb-IIIa and Storage Organelles. Blood 67:322-331.

Wencel-Drake, J.D., Plow, E.F., Kunicki, T.J., Woods, V.L., Keller, D.M. and Ginsberg, M.H. 1986 Localization of Internal Pools of Membrane Glycoproteins Involved in Platelet Adhesive Responses. Am. J. Pathol. 124:324-334.

White, J.G. and Escolar, G. 1990 Induction of Receptor Clustering, Pathching, and Capping on Surface-Activated Platelets. Lab. Invest. 63(3):332-340.

White, J.G., Leistikow, E.L. and Escolar, G. 1990 Platelet Membrane Responses to

Surface and Suspension Activation. Blood Cells 16:43-72.

Woods, V.L., Wolff, L.E. and Keller, D.M. 1986 Resting Platelets Contain a Substantial Centrallly Located Pool of Glycoprotein IIb-IIIa Complex Which May Be Accessible to Some but Not Other Extracellular Proteins. J. Biol. Chem. 261(32):15242-15251.

Zucker, M.B. and Vroman, L. 1965 Platelet Adhesion Induced by Fibrinogen Adsorbed onto Glass. Proc. Soc. Exp. Biol. Med. 131:318-320.

CHAPTER 4

THE EXAMINATION OF GLYCOPROTEIN IIb-IIIa USING BIOTINYLATED FIBRINOGEN AS A SOLUBLE-PHASE LIGAND PROBE, TO EXAMINE RECEPTOR REDISTRIBUTION IN ACTIVATED HUMAN PLATELETS

INTRODUCTION

To date, most studies designed to evaluate Glycoprotein IIb-IIIa (GPIIb-IIIa) receptor mobility have centered around the use of particulate probes such as cationized ferritin (Behnke, O., 1987) or specific antibody (Olufunke, et al., 1992; Escolar, et al., 1989) or adhesive protein colloidal gold probes (Horsewood, et al., 1991; Lewis, et al., 1990; Albrecht, et al., 1989). Evidence suggests the platelet responds differently to particulate probes than it does to soluble phase ligands (Estry and Mattson, 1989). In order to eliminate the need for an electron dense particulate probe, the ligand, fibrinogen, was biotinylated in order to trace the path of receptor reorganization, in both platelets activated in suspension, as well as platelets adherent and spread on a foreign surface.

Examination of the receptor using soluble-labelled fibrinogen has not been widely attempted. Most studies have employed monoclonal antibodies to the GPIIb-IIIa receptor, or anti-fibrinogen antibodies. These methods will localize the receptor, but are limited in the information that can be interpreted from the labelling. In the case of monoclonal antibodies against the receptor, if the ligand interaction with the receptor is necessary for

triggering physiological responses, the antibody may not provide the appropriate stimulus. With anti-fibrinogen labelling, the ligand/receptor problem is solved, but the determination of the particular population of fibrinogen being labelled now becomes unclear. Differentiation between exogenous and endogenous stores of fibrinogen cannot be separated using a ligand-specific antibody. With these barriers, it is necessary to find a method to label fibrinogen that maintains physiological activity, and provides an accurate method for localization.

Biotinylation is a widely used method for the labelling of proteins, and has been investigated in great detail (Bayer and Wicheck, 1990; Berti, et al., 1983; Hsu, et al., 1981). The technique is based on the binding of biotin, a 244 dalton vitamin found in blood and tissue, to the molecule of interest. This procedure normally uses avidin, a 66K dalton protein found in egg whites, or streptavidin, a highly purified product of *Streptococcus avidinii* to subsequently localize the biotin. The avidin-biotin interaction is the strongest known, non-covalent linkage between protein and ligand ($K_a=10^{15}$ M^{-1}), which makes it an invaluable tool in research (Green, N.M., 1975). In this work, biotin was used because of its small size, not because of its interaction with avidin. It was necessary to find a form of protein labelling, without having to rely on radioisotopic labelling, that would have the least effect on the biological activity of the fibrinogen. Biotinylation of proteins can be controlled by using various molar ratios of the biotin to ligand, then assaying the fibrinogen for maintenance of various biological activities. Localization of the biotinylated molecules can then be accomplished using avidin, or an

anti-biotin antibody complexed with an electron dense marker, such as colloidal gold (Diamandis, E.P. and Christopoulos, T.K., 1991). In this instance, avidin was avoided due to its more rapid dissociation from colloidal gold probes, leaching from the surface of the beads in a matter of weeks (personal observation).

In these experiments, post-embedding labelling with anti-biotin-gold was chosen to prevent the possible effects of the particulate probe on the receptor redistribution.

By maintaining near normal biological activity of the fibrinogen with this type of labelling, it was then necessary to modify the protocol used in previous studies, to prevent fibrin polymer formation when thrombin is added to the probe. Under physiological conditions, thrombin was instrumental in the conversion of the monomer, fibrinogen to fibrin (Blombäck, B., 1985). One of the most effective thrombin inactivators is hirudin. The mechanism of inhibition by hirudin is based on the binding of the inhibitor to the protein binding sites of the enzyme, thus rendering it incapable of enzymatic actions (Stocker, K., 1991). By the use of hirudin, the platelet can still bind thrombin and be activated, and still have available fibrinogen to bind with the GPIIb-IIIa receptor.

This study was designed to assess the effect that soluble phase fibrinogen has on receptor mediated internalization of bound ligand, and to determine what changes, if any, are seen in the redistribution of the GPIIb-IIIa receptor.

MATERIALS AND METHODS

Platelet Isolation: Donor blood was drawn into a 30ml syringe charged with 3.8% sodium citrate to give a final anticoagulant concentration of 0.38%. The sample was then spun at 1000 rpm for 10 minutes to obtain platelet rich plasma (PRP). The PRP was carefully removed, and transferred to a new plastic test tube where 1μM Prostaglandin E_1 (PGE_1; final reaction concentration) was added. This suspension was allowed to rest, in a covered tube, 10 minutes before it was spun to concentrate the platelets (2000 rpm, 10 min.). The resulting pellet was resuspended in 1ml Tyrode's Buffer without calcium (TB; 0.2% bovine serum albumin, 130mM NaCl, 2.6mM KCl, 10mM HEPES, 5.5mM glucose, 2mM $MgCl_2$, pH 7.4), and additional PGE_1 (to 1μM final concentration) was added. The concentrated platelet suspension was covered, and allowed to rest 10 minutes before gel filtration.

Gel Filtration: A 10ml column was prepared using Sepharose 4B-200, equilibrated with TB. The 1ml platelet suspension was layered onto the surface and gel filtered to remove any residual plasma proteins. The first 25 cloudy drops (approx. 1.25mls) were pooled, the platelets counted, and the concentrate diluted with Tyrode's buffer with calcium ($TB+Ca^{++}$; 1mM $CaCl_2$) to give a final platelet count of $2.2x10^8$ cells/ml. This suspension was allowed to rest (30 min.) before experimental procedures.

Colloidal Gold Probe: Fifteen to 18nm colloidal gold was prepared according to the

methods of Goodman, et al. (1981) and Frens (1973), and was conjugated with rabbit anti-biotin, IgG fraction (Rockland Ind.).

Biotinylation of Fibrinogen: I. Biotinylation Protocol: One hundred and twenty milligrams of human fibrinogen (Kabi) was dissolved in 25mls 0.1M sodium bicarbonate, 0.15M NaCl at pH 8.5. This solution provided a fibrinogen concentration of approximately 3.0mgs/ml. The solution was then dialysed 4 hrs at room temperature (RT) with two changes of the bicarbonate buffer. A protein assay was performed, and the fibrinogen solution was diluted to 2.5mg/ml, and a total of 10mgs fibrinogen was used per biotin reaction. Four molar ratios of biotin to fibrinogen were chosen along with a control (6:1, 12:1, 24:1, 48:1 and 0:1) and the appropriate amount of biotin was weighed out and placed in glass test tubes. Four milliliters of the fibrinogen solution was added to each of the biotin ratios and allowed to react in the dark for 1 hr at RT. Each of the solutions were dialysed in 0.15M NaCl for 2 hrs at RT with 2 changes of buffer, then dialysed overnight in 0.1M Phosphate Buffered Saline (PBS), pH 7.4 at RT.

II. Determination of Biotin:Fibrinogen Molar Ratios: The amount of biotin per fibrinogen molecule was determined by the modified procedure of Green, N.M., 1975. In this procedure HABA (4'-hydroxyazobenzene-2-carboxylic acid) is displaced from avidin, by biotin (indicated by a decrease in absorbance), and measured photometrically at a wavelength of 500nm. Briefly, 3mls of 0.5 mgs/ml avidin in 0.05M PBS, pH 6.0 was combined with 75μl of 10mM HABA in 10mM NaOH. The absorbance of this

solution was taken, and reread after each addition of 2μl of a 0.5mM biotin in 0.05M PBS solution, pH 6.0. From these readings, a standard curve was generated, where the change in absorbance was plotted against the biotin concentration. The biotinylated fibrinogen samples were in turn added in 15μl aliquots to fresh avidin/HABA, read at 500nm, and the amount of biotin determined from the standard curve.

III. Determination of Biotinylated Fibrinogen Activity: Relative biological reactivity of the biotinylation preparations were determined by thrombin clotting time, and aggregation studies using 10μM ADP as agonist. These results were compared to control (non-labelled) fibrinogen values. All incubations of fibrinogen with platelets provided 0.5mgs fibrinogen per $2.2x10^8$ cells/ml.

Agonists: Thrombin from human plasma (Sigma, T-8885) was diluted to provide a final reaction concentration of 0.1U/ml. Adenosine diphosphate (ADP) was made as a 2.4 x 10^{-4} stock, and 200μl aliquots were stored at -60°C until use. The ADP was diluted to a final reaction concentration of 10μM.

Hirudin Inactivation: Hirudin (Sigma, H-5126) inactivation of thrombin was used to prevent the polymerization of the biotinylated fibrinogen. Platelets were first activated with thrombin, then a hirudin concentration equal to thrombin (0.1U/ml) was added prior to the addition of fibrinogen.

I. Suspension Experiments

Unstirred Platelet Treatments: Four hundred and fifty microliters of the platelet suspension was aliquoted into 1500μl microfuge tubes. To each of these tubes, 150μl of biotinylated fibrinogen (F-Biotin) or unlabelled fibrinogen was added, the tube inverted to mix, then allowed to incubate at 37°C for 3 minutes. At the end of the initial incubation 20μl of agonist or TB+Ca^{++} was added, inverted to mix and incubated an additional 5 minutes at 37°C. In hirudin-treated samples, the platelet suspensions were

	PROBE	AGONIST
	(150μl, 3 min, 37°C)	(20μl, 5 min, 37°C)
1	Control Fibrinogen	0.1U/ml Thrombin
2	F-Biotin	TB+Ca^{++}
3	F-Biotin	10μM ADP
4	F-Biotin	0.1U/ml Thrombin
5	F-Biotin	Thrombin/Hirudin

Figure 4.1. Biotinylated Suspension Combinations

first incubated with thrombin, 3 minutes at 37°C, hirudin was added and allowed to incubate an additional 2 minutes, then the fibrinogen added as with the other treatments. See Figure 4.1 for particular experimental variables. The reaction was terminated with the addition of 600μl of 0.1% glutaraldehyde in 0.1M cacodylate buffer (CB), pH 7.4. After 30 minutes, the platelet suspensions were pelleted at 12,800 rpm for 10 seconds.

Stirred Platelet Treatments: Four hundred and fifty microliters of platelet suspension

was placed in an aggregation cuvette (with stirbar) along with 150μl F-Biotin, covered, and incubated 2 minutes at 37°C. After incubation, the cuvette was placed in a Bio/Data Platelet Aggregometer and a baseline trace was taken for 1 minute. Agonist or TB+Ca^{++} was added, and the trace was collected until aggregation was complete or had stabilized (approx. 5 minutes) (Figure 4.1). In hirudin inactivated samples, thrombin was added as above, then inactivated just after the onset of shape change. The reaction was stopped by the addition of 600μl of 0.1% glutaraldehyde in CB, and the mixtures carefully transferred to microfuge tubes. Fixation was allowed to go for 30 minutes before centrifugation at 12,800 rpm for 10 seconds.

Transmission Electron Microscopy: After centrifugation, the supernatant was removed and fresh, 2.5% glutaraldehyde in 0.1M CB was added. The pellet was broken up into smaller pieces, and allowed to fix for an additional hour. The samples were centrifuged at 12,000 rpm for 1 minute, then the pellets were removed to be suspended in 1% agarose in water. The solidified agarose was cut into pieces, fixed in 2.5% glutaraldehyde in CB (1 hour), post-fixed in 1% aqueous osmium tetroxide, water washed, then dehydrated through a series of graded ethanols. Infiltration in Spurrs-Mollenhauer resin (Klomparens, et al. 1986) was through 3:1, 1:1 and 1:3 (ethanol:resin) for 2 hours each, and in 100% resin overnight. Blocks were polymerized at 65°C. for 48 hours.

II. Adhesion Experiments

Adherent Surfaces: For transmission electron microscopic (TEM) cross-sections, tissue-

culture plastic coverslips (Thermonox, Miles Scientific) were quartered and notched (to maintain orientation), then carbon-coated. Platelets were allowed to attach and spread directly on the coated surfaces.

Pre-adhesion Ligand Binding: Five hundred microliters of the platelet suspension was incubated with 100μl F-Biotin (37°C, 3 min.). Twenty microliters of agonist or TB+Ca^{++} was added to the suspension, was mixed by inversion and applied to the coverslip (Figure 4.2). Platelets were allowed to attach and spread at 37°C, 10 minutes, in a humidified chamber. After incubation, the unattached platelets were removed by washing with TB+Ca^{++}, and the samples fixed for electron microscopy.

	PROBE	AGONIST
	(75μl, 3 min. 37°C)	(add 10μl, then apply to surface, 10 min, 37°C)
1	Control Fibrinogen	0.1U/ml Thrombin
2	F-Biotin	10μM ADP
3	F-Biotin	0.1U/ml Thrombin
4	F-Biotin	Thrombin/Hirudin

Figure 4.2. Biotinylated Pre-adhesion Combinations

Post-adhesion Ligand Binding: Platelets were allowed to attach to the plastic surface, 10 minutes at 37°C in a humid chamber. At the end of this time, the unattached platelets were removed by washing with TB+Ca^{++}. The remaining cells were overlaid with a reaction mixture made from 250μl of TB+Ca^{++}, 50μl of probe, and 10μl of agonist or

buffer (Figure 4.3). This was allowed to incubate on the cells at 37°C for 5 minutes, then was washed off with buffer to remove unreacted solutions. These surfaces were then prepared for electron microscopy.

	PROBE	AGONIST
	(75μl diluted in TB+Ca^{++})	(add 10μl to labelling cocktail, then apply to cells)
1	F-Biotin	TB+Ca^{++}
2	F-Biotin	10μM ADP
3	F-Biotin	0.1U/ml Thrombin/Hirudin

Figure 4.3. Biotinylated Post-adhesion Combinations

Thin Sections: Platelets on their support structures were transfered to 1500μl microfuge tubes, fixed in 2.5% glutaraldehyde in 0.1M Cacodylate buffer (CB) for 1 hour at 27°C, post-fixed in 1% aqueous osmium tetroxide, water washed, then dehydrated through a series of graded ethanols. Infiltration in Spurrs-Mollenhauer resin (Klomparens, et al. 1986) was through 3:1, 1:1 and 1:3 (ethanol:resin) for 2 hours each. The coverslips were removed from the microfuge tubes draining as much excess resin as possible, the backside of the coverslip wiped clean of resin with 100% ethanol, and transfered to a clean microscope slide. Fresh resin was added to the coverslip until a miniscus formed and the samples were polymerized at 65°C. for 48 hours. Samples were allowed to cool, were transferred to the freezer for 5 minutes and the resin/monolayer popped off the plastic coverslip. These samples were dried, then re-embedded in blocks, sample side toward mid-block, and polymerized as before.

Post-Embedding Labelling with Anti-Biotin Gold: Samples were ultrathin sectioned and collected on 300 mesh nickel grids. Plastic sections were etched using an aqueous 0.4% sodium meta-periodate solution for 30 minutes at RT, were washed in dH_2O, then blocked with 3.0% ovalbumin, 0.1% Tween-20 in PBS for 30 minutes at RT. Incubation in anti-biotin gold followed the blocking directly and lasted 30 minutes at RT. Grids were washed in dH_2O, and allowed to dry throughly before staining the unetched side with Reynold's lead citrate (Reynold, 1963). Specimens were examined using a JEOL 100CXII TEM.

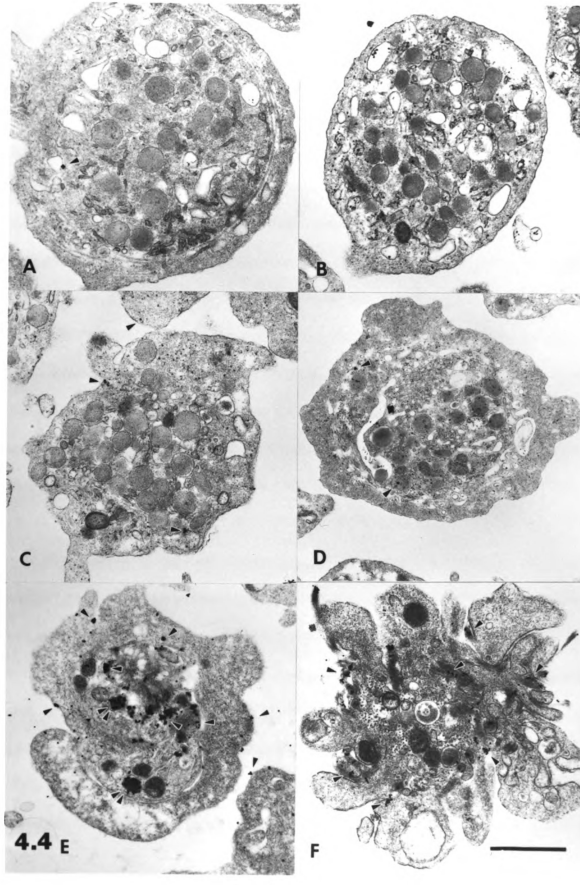
All data based on at least two experimental repetitions, and the observation of at least 20 platelets per experimental treatment.

RESULTS

Biotinylation of Fibrinogen: Using the criteria of aggregation curves and thrombin clotting times, an average assayed, molar ratio of 22.6:1 (biotin:fibrinogen) was used. The 22.6:1 value represents the assayed molar ratio that was estimated to be 24:1 at the onset of the biotinylation.

Suspension Treatments: Post-embedding labelling of the suspension treatments resulted in very little anti-biotin gold binding to the samples incubated with F-Biotin alone (Figure 4.4a) or those activated with ADP/F-Biotin (Figure 4.4c) in the unstirred preparations.

Figure 4.4 Unstirred versus Stirred Platelet Suspensions. Bar = 1μM. A) Unstirred, F-Biotin incubated, no agonist. Minimal internal labelling (arrowhead), possibly associated with membrane. B) Stirred, F-Biotin incubated, no agonist. Minimal to no internal labelling. C) Unstirred, ADP-stimulated, F-Biotin incubated. Minimal internal labelling (arrowheads) not apparently associated with any cellular structure. D) Stirred, ADP-stimulated, F-Biotin incubated. Internal labelling (arrowheads) near OCS. E) Unstirred, thrombin-stimulated, F-Biotin incubated. Concentrated internal labelling (double arrowheads) associated with granules, and in cytoplasm (arrowheads) both single and clustered. Clustered external label (arrowheads) also present. F) Stirred, thrombin-stimulated, F-Biotin incubated. Platelet showed internal labelling (arrowheads) primarily towards periphery of platelet that is clumped. External labelling (arrowheads) was mostly single and infrequent.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F

The stirred preparations showed very little binding as well, though in the ADP/F-Biotin sample (Figure 4.4d) there was probe localization over membrane bound granules. The only substantial labelling was found in the thrombin-stimulated platelets. Figure 4.4e demonstrates the labelling in an unstirred sample treated with thrombin/F-Biotin. External labelling was clumped, and irregularly distributed along the membrane. Internal labelling was found singularly and in clumps distributed throughout the platelet, with an increase in probe density towards the granulomere region. Except for the label localized over the membrane- bound granules (double arrowheads), most of the labelling was without distinct association with any cellular structure, including the OCS. In the stirred suspension, gold probe was also internalized within the platelet aggregate (Figure 4.4f), and again showed no distinct association with any cellular component. There was an overall loss of external labelling, and when found, was associated with what appeared to be fibrin strands.

Adherent Platelets: Interestingly, platelets pre-incubated with the F-Biotin and no agonist, labelled almost exclusively at the platelet adhesion interface (Figure 4.5a). This labelling was mostly singlet gold, and evenly distributed below the platelet. The addition of ADP to the reaction mixture (Figure 4.5c), resulted in internalization of probe into the platelet, some of it granule associated (arrow). External labelling was again, exclusively at the adhesion interface, and resembled that of F-Biotin alone. Thrombin/F-Biotin stimulation of the platelets in suspension, then allowing them to adhere, resulted in a marked increase in overall labelling (Figure 4.5e). The exposed platelet surface showed slight labelling

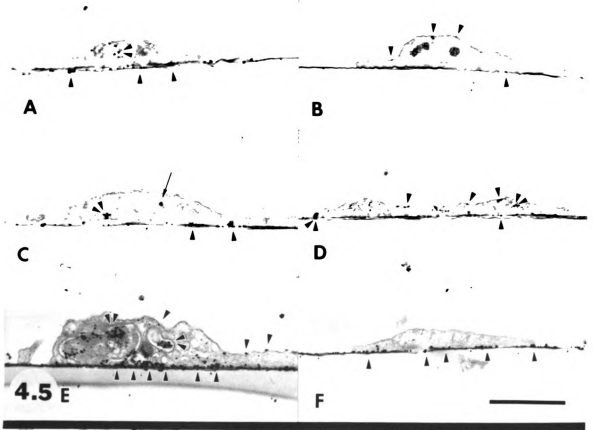
with no apparent distribution pattern. Internal probe appeared to be associated with internal membranes and was often in large clusters, though it did not appear to be membrane enclosed (double arrows). The majority of the label was, again, found at the adhesion interface.

Adherent platelets incubated with F-Biotin alone (Figure 4.5b), labelled sparsely on the exposed platelet surface, as well as at the adhesion interface. There was no apparent internalization. Platelets treated with ADP/F-Biotin (Figure 4.5d) showed no difference in probe localization than those treated with F-Biotin alone, though the relative amount of probe increased with ADP stimulation. Thrombin/F-Biotin treated platelets (Figure 4.5f), did not label on the exposed platelet surface, and only minimal label was noted internally. All anti-biotin gold was found at the platelet adhesion interface. This level of binding exceeded that of the other two treatments, F-Biotin alone and ADP/F-Biotin (Figures 4.5b and 4.5d, respectively).

Labelling Controls: Biotinylated fibrinogen allowed to polymerize with thrombin treatment, was labelled with anti-biotin gold (Figure 4.6a) to serve as a positive control. Thrombin/Control Fibrinogen-stimulated platelets show no significant labelling by anti-biotin gold probe (Figure 4.6b).

Figure 4.5 Pre- and Post-adhesion Incubation With F-Biotin. Bar = 1μM. A) Pre-adhesion, F-Biotin incubated, no agonist. No external labelling. Minimal internal labelling (double arrowheads) found possibly associated with open canalicular system. Probe at platelet adhesion interface (arrowheads). B) Post-adhesion, F-Biotin incubated, no agonist. No internal labelling. Minimal external labelling (arrowheads), and rare interface labelling. C) Pre-adhesion, ADP-stimulated, F-Biotin incubated. No exposed surface external labelling. Minimal internal labelling (double arrowheads) clustered and not apparently associated with any cellular structure, and internal labelling associated with granules (arrow). Labelling at platelet adhesion interface (arrowheads). D) Post-adhesion, ADP-stimulated, F-Biotin incubated. Internal labelling (double arrowheads) infrequent. External labelling (arrowhead) clustered. Some label at platelet adhesion interface. E) Post-adhesion, thrombin-stimulated, F-Biotin incubated. Concentrated internal labelling (double arrowheads) associated with internal membranes, and in cytoplasm both single and clustered. Clustered external label (arrowheads) also present. Platelet adhesion interface contained majority of probe. F) Post-adhesion, thrombin-stimulated, F-Biotin incubated. Platelet showed majority of labelling (arrowheads) at platelet adhesion interface. Minimal internal label present. No external labelling on exposed platelet surface.

Figure 4.6 F-Biotin Labelling Controls. Bar = 1 micrometer. A) Polymerized F-Biotin labelled with anti-biotin-gold. B) Thrombin-stimulated platelet incubated with control F-Biotin (0:1, biotin:fibrinogen). No labelling present.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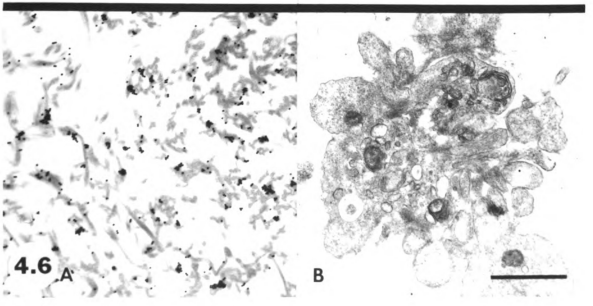

4.6 A
B

DISCUSSION

There were four major differences found between the soluble-phase fibrinogen (biotinylated, F-Biotin) and that reported with solid-phase fibrinogen (colloidal gold, F-Au). One, there is an overall reduction in the amount of label found on the biotinylated fibrinogen samples. Two, there is an obvious migration of F-Biotin to the adhesion surface interface, not seen in comparable samples treated with F-Au (Chapter 3). Three, there was a lack of obvious internal structure association with the internalized probe. And fourth, most striking, was the incorporation of F-Biotin into secretory granules of both suspension and adherent platelets.

The reduction of post-embedding labelling using antibodies bound to colloidal gold has been noted previously by many researchers. This reduction has been primarily attributed to loss of antigenicity due to fixation and embedding media (Bendayan, M., 1984). This effect can be reduced by etching the ultrathin sections with strong oxidizing agents to expose antigenic sites. This procedure allows the maintenance of ultrastructural detail, otherwise lost by using lower concentrations of fixative. The advantages of using the soluble phase probe to localize the GPIIb-IIIa receptor, outweighs the disadvantages of the possibility of reduced antibody-antigen interaction, and therefore, labelling. It was necessary then, to approach this form of labelling as a strict localization technique, and not one for quantitation.

The migration/deposition of F-Biotin exclusively to the adhesion surface interface in these samples, whether pre- or post-incubated with F-Biotin, is in contrast with the literature using a particulate fibrinogen probe. The work of White, et al. (1990) reports the internalization of probe into the OCS of the platelet. This may be due to the particulate nature of the fibrinogen probe. Behnke and Bray (1988) demonstrated the internalization of particulates (ferritin and lentil-conjugated gold particles) into the platelet interior, specifically, the OCS, upon spreading. Soluble-phase ligand appeared to by-pass the OCS and migrate to the attachment surface. This migration may be the actual physiological receptor redistribution mechanism, and may be explained by the postulated role of GPIIb-IIIa in platelet spreading after adhesion. In an *in vivo* situation, the role of platelet adhesion and spreading is to cover large areas. This phenomenon, whether the surface is platelet or non-platelet, still must occur for the platelet to fulfill its part in the repair of vascular damage. Deposition of fibrinogen by the platelet onto the spreading surface would provide additional attachment points, and therefore, enhance platelet adhesion during its hemostatic role. Fibrinogen bound to colloidal gold, may be too large a complex to be transported efficiently to the adhesion interface, and may remain trapped in the open canalicular system where the majority of investigators report its localization.

The internalized, biotinylated ligand did not appear to be associated with the OCS as it has been demonstrated previously with ligand conjugated to colloidal gold probes (Chapter 1; Chapter 2; White, et al., 1990). This differential redistribution may indicate two possible mechanisms of handling occupied receptors, dependent on the physical

properties of the bound ligand. The platelet may be responding to the ligand-gold probe as a forgien body, and may be actually clearing it from the platelet surface. In contrast, the soluble ligand was found cytoplasmicly and in the secretory granules, and not associated with the OCS. These results indicate the possible sequestering of fibrinogen and/or GPIIb-IIIa receptors into the platelet for future use.

The labelling of platelet granules with the antibody-gold complex in the F-Biotin system may correlate with the work of Morgenstern and co-workers (1992) who reported the transport of anti-GPIIb-IIIa antibodies into the alpha granules of unstimulated platelets. Positive identification of the alpha granules in the F-Biotin ultrathin sections, was hindered by the etching required for labelling. This procedure reduces the duel density characteristic of the granule, and makes verification of the granule type difficult without additional labelling procedures. Also, the amount of labelling on some of the granules was heavy enough to obscure the internal structure by itself. Circumstantial evidence supports these structures to be alpha granules. The mechanism of transport to the granules, is still unclear.

This research demonstrated that soluble-phase fibrinogen and solid-phase fibrinogen differ significantly in the localization of GPIIb-IIIa after platelet activation both in suspension and on a non-platelet surface. The major differences in the localization was in the deposition of soluble-phase fibrinogen to the adhesion surface interface and in alpha granules. Both of these differences may be due to the non-soluble nature of gold colloid

conjugates and their inability to be transported freely through the platelet. In addition, the soluble-phase ligand was not associated with the OCS, this finding is in direct contrast to particulate-phase ligand studies demonstrating F-Au internalization into the OCS. These results suggest alternate receptor internalization mechanisms dependent on the ligand physical properties.

The demonstration of another active, receptor internalization mechanism, other than the OCS, suggests the role of endocytic vesicle formation in receptor redistribution. To examine this mechanism, bovine platelets, which lack an OCS, were compared to human platelets, following agonist-induced platelet activation in the ensuing chapter.

BIBLIOGRAPHY

Albrecht, R.M., Goodman, S.L. and Simmons, S.R. 1989 Distribution and Movement of Membrane-Associated Platelet Glycoproteins: Use of Colloidal Gold with Correlative Video-Enhanced Light Microscopy, Low-Voltage High-Resolution Scanning Electron Microscopy, and High-Voltage Transmission Electron Microscopy. Am. J. Anat. 185:149-164.

Bayer, E.A. and Wilchek, M. 1990 Protein Biotinylation in Methods in Enzymology. Vol. 184. Avidin-Biotin Technology. Wilchek, M. and Bayer, E.A. (eds). Academic Press. pp 138-160.

Behnke, O. 1987 Surface Membrane Clearing of Receptor-Ligand Complexes in Human Blood Platelets. J. Cell Sci. 87:465-472.

Behnke, O. and Bray, D. 1988 Surface Movements During the Spreading of Blood Platelets. Eur. J. Cell Biol. 46:207-216.

Bendeyan, M. 1984 Protein-A Gold Electron Microscopic Immunocytochemistry: Methods, Applications, and Limitations. J. Electron Microsc. Tech. 1:243-270.

Berti, E., Monti, M., Cavicchini, S. and Caputo, R. 1983 The Avidin-Biotin Peroxidase Complex (ABC-Px) in Skin Immunoelectron Microscopy. Arch. Dermatol. Res. 275:134-138.

Blombäck, B. 1985 Fibrinogen to Fibrin - An Overview in Fibrinogen-Structural Variants and Interactions. Henschen, A., Hessel, B., McDonagh, J., Saldeen, T. (eds). Walker de Gruyter and Co. New York.

Diamandis, E.P. and Christopoulos, T.K. 1991 The Biotin-(Strept)Avidin System: Principles and Applications in Biotechnology. Clin. Chem. 37(5):625-636.

Escolar, G., Leistikow, E. and White, J.G. 1989 The Fate of the Open Canalicular System in Surface and Suspension-activated Platelets. Blood 74(6):1983-1988.

Estry, D.W. and Mattson, J.C. 1989 Comparison of Fibrinogen Receptor Distribution on Adherent Platelets Using Both Soluble Fibrinogen and Fibrinogen Immobilized on Gold Beads. FASEB J. 3:A310.

Goodman, S.L., Hodges, G.M., Trejdosiewicz, L.K. and Livingston, D.C. 1981 Colloidal Gold Markers and Probes for Routine Application in Microscopy. J. Microscopy 123(2):201-213.

Green, N.M. 1975 Avidin in Advances in Protein Chemistry. Academic Press. Vol. 29. pp 85-133.

Horsewood, P., Hayward, C.P.M., Warkentin, T.E. and Kelton, J.G. 1991 Investigation of the Mechanisms of Monoclonal Antibody-induced Platelet Activation. Blood 74(4):1019-1026.

Hsu, S-M., Raine, L. and Fanger, H. 1981 Use of Avidin-Biotin-Peroxidase Complex (ABC) in Immunoperoxidase Techniques. A Comparison Between ABC and Unlabelled Antibody (PAP) Procedures. J. Histochem. Cytochem. 29:1349-1353.

Klomparens, K.L., Flegler, S.L. and Hooper, G.R. 1986 Procedures for Transmission and Scanning Electron Microscopy for Biological and Medical Science. A Laboratory Manual, 2nd ed. Ladd Research Ind., Burlington, VT, p. 146.

Lewis, J.C., Hantgan, R.R., Stevenson, S.C., Thornburg, T., Kieffer, N., Guichard, J. and Breton-Gorius, J. 1990 Fibrinogen and Glycoprotein IIb/IIIa Localization During Platlet Adhesion. Am. J. Pathol. 136:239-252.

Olufunke, E.O., Simmons, S.R. and Albrecht, R.M. 1992 Cytochalasin D and E: Effets on Fibrinogen Receptor Movement and Cytoskeletal Reorganization in Fully Spread, Surface-Activated Platelets: A Correlative Light and Electron Microscopic

Investigation. Blood 79(1):99-109.

Reynolds, E.S. 1963 The Use of Lead Citrate At High pH as an Electron Opaque Stain in Electron Microscopy. J. Cell Biol. 17:208-212.

Stocker, K. 1991 Laboratory Use of Hirudin. Sem. Thrombos. Hemost. 17(2):113-121.

White, J.G., Leistikow, E.L. and Escolar, G. 1990 Platelet Responses to Surface and Suspension Activation. Blood Cells 16:43-72.

CHAPTER 5

COMPARISION OF BOVINE PLATELETS WITH HUMAN PLATELETS IN THE REDISTRIBUTION OF THE GLYCOPROTEIN IIb-IIIa RECEPTOR, USING A COLLOIDAL GOLD PROBE BOUND TO ITS LIGAND, FIBRINOGEN

INTRODUCTION

The fate of the Glycoprotein IIb-IIIa receptor after activation and ligand binding, has been addressed by many investigators. Research has postulated that the human platelet may have two possible systems involved in the clearing of the surface membranes, endocytosis and the open canalicular system (OCS) (Wencel-Drake et al., 1990). Both of these systems are based on the rearrangement of the platelet cytoskeleton after activation. It is unclear whether one, or both, of these mechanisms are responsible for the receptor/ligand redistribution seen after platelet activation. To better understand this process, bovine platelets, reportedly lacking an OCS (Zucker-Franklin, et al., 1985), were examined and compared to human platelets.

Platelets have been shown to contain an extensive membrane cytoskeleton composed of actin filaments, actin-binding protein and two major membrane glycoproteins, Ib and Ia (Fox and Phillips, 1983). GP Ib has been shown to be part of the platelet cytoskeleton that is dissociated from the actin network as a consequence of the calcium dependent protease (Fox et al., 1985, 1988). These findings, coupled with the observation that the

GP Ib receptor remains evenly distributed throughout the platelet membrane, provide support for the role of membrane-cytoskeletal linkages in platelet receptor mobility. The findings with GP Ib are distinctly different than those made following attachment of a monoclonal antibody to GPIIb-IIIa (Wencel-Drake, 1990). GPIIb-IIIa, has been shown to redistribute and be cleared following ligand-receptor activation, and has been demonstrated to contain a subpopulation of receptors that are bound to the cytoskeleton via actin filaments (Painter et al., 1985). This actin linkage may be important in the receptor redistribution and removal after ligand binding the pattern of which has been shown to be similar to cytoskeletal organization (Debus et al., 1981).

Endocytosis in the platelet has been observed using particulate tracers bound to fibrinogen and albumin. In these studies, both tracers were endocytosed over time. These observations were based on the presence of coated pits on the surface membrane and in conjunction with the OCS, and vesicle transfer of probe to internal granules (Behnke, 1989). In another approach, freeze fracture of platelets was performed after incubation with latex particles (0.3μm - 1.0μm). This work showed two possible routes of internalization: 1) where the larger particles were internalized via the surface membrane, and 2) the smaller particles entered the OCS and were endocytosed from there (Zucker-Franklin, 1981).

Migration of particulate-labelled ligands into the OCS of the platelet has been demonstrated as well. In one study, suspensions of platelets demonstrated clearing of

cationized ferritin to the OCS directly, while an immunoglobulin-gold probe exhibited patching, capping and eventual internalization into the OCS, though the two probes were contained in separate areas (Behnke, 1987). In related studies, F-Au was used to follow internalization in adherent platelets (Escolar et al., 1989; White et al., 1990). They found that as platelets spread on a surface the amount of surface probe diminished. Upon examination of the interior of the cells they found the probe concentrated within the OCS.

This study was designed to examine the labelling properties of both bovine and human platelets in suspension in order to elucidate a possible mechanism for the clearance of the GPIIb-IIIa receptor/ligand complex from the cell surface.

MATERIALS AND METHODS

Platelet Isolation: Donor blood was drawn into a 30ml syringe charged with 3.8% sodium citrate to give a final anticoagulant concentration of 0.38%. The sample was then spun at 1000 rpm for 10 minutes to obtain platelet rich plasma (PRP). The PRP was carefully removed, and transferred to a new plastic test tube where 1μM Prostaglandin E_1 (PGE_1; final reaction concentration) was added. This suspension was allowed to rest, in a covered tube, 10 minutes before it was spun to concentrate the platelets (2000 rpm, 10 min.). The resulting pellet was resuspended in 1ml Tyrode's Buffer without calcium (TB; 0.2% bovine serum albumin, 130mM NaCl, 2.6mM KCl, 10mM HEPES, 5.5mM glucose, 2mM $MgCl_2$, pH 7.4), and additional PGE_1 (to 1μM final concentration) was added. The

concentrated platelet suspension was covered, and allowed to rest 10 minutes before gel filtration.

Gel Filtration: A 10ml column was prepared using Sepharose 4B-200, equilibrated with TB. The 1ml platelet suspension was layered onto the surface and gel filtered to remove any residual plasma proteins. The first 25 cloudy drops (approx. 1.25mls) were pooled, the platelets counted, and the concentrate diluted with Tyrode's buffer with calcium ($TB+Ca^{++}$; 1mM $CaCl_2$) to give a final platelet count of $2x10^8$ cells/ml. This suspension was allowed to rest (30 min.) before experimental procedures.

Colloidal Gold Probes: Fifteen to 18nm colloidal gold was prepared according to the methods of Bendayan (1984) and Frens (1973), and was conjugated with fibrinogen and Polyethylene Glycol (PEG) as follows:

Fibrinogen - One hundred and thirty milligrams of lyophilized human fibrinogen (KABI) was dissolved in 3mls 5mM NaCl and fractionated over a 20ml Sephadex G-25 column (to remove contaminating citrate and reduce the NaCl concentration) equilibrated with 5mM NaCl. The 280nm absorbance of the fractions were taken, and the 3 tubes with the highest readings were pooled and filtered to remove macro-aggregates. A Markwell protein assay was performed to determine the protein concentration. The fibrinogen was conjugated to the colloid at pH 6.4 after a minimum protecting protein concentration was determined.

PEG - To determine if the platelets would internalize the gold probe in the course

of activation, a control gold probe was made. The control was produced in tandem with F-Au, where the colloidal gold was conjugated instead with PEG, and was used as a control in all treatments to parallel fibrinogen gold labelling. The minimum protecting concentration of 1% PEG in 5mM NaCl was established, and the PEG was conjugated to the colloidal gold at pH 6.0 to provide a non-specific binding control for the gold labelling.

Agonists: Thrombin from human plasma (Sigma, T-8885) was diluted to provide a final reaction concentration of 0.1U/ml. Adenosine diphosphate (ADP) was made as a 2.4 x 10^{-4} stock, and 200μl aliquots were stored at -60°C until use. The ADP was diluted to a final reaction concentration of 10μM.

Unstirred Platelet Treatments: Five hundred microliters of the platelet suspension was aliquoted into 1500μl microfuge tubes. To each of these tubes, 100μl of gold probe or TB+Ca^{++} was added, the tube inverted to mix, then allowed to incubate at 37°C for 3 minutes. At the end of the initial incubation 20μl of agonist or TB+Ca^{++} was added, inverted to mix and incubated an additional 5 minutes at 37°C. See Figure 5.1 for particular experimental variables. The reaction was terminated with the addition of 600μl of 0.1% glutaraldehyde in 0.1M cacodylate buffer (CB), pH 7.4. After 30 minutes, the platelet suspensions were pelleted at 12,800 rpm for 10 seconds.

Stirred Platelet Treatments: Five hundred microliters of platelet suspension was placed

in an aggregation cuvette (with stirbar) along with 100μl probe or TB+Ca^{++}, was covered, and incubated 2 minutes at 37°C. After incubation, the cuvette was placed in a Bio/Data Platelet Aggregometer and a baseline trace was taken for 1 minute. Agonist or TB+Ca^{++} was added, and the trace was collected until aggregation was complete or stabilized (approx. 5 minutes) (Figure 5.1). The reaction was stopped by the addition of 600μl of 0.1% glutaraldehyde in CB, and the mixtures carefully transferred to microfuge tubes. Fixation was allowed to go for 30 minutes before centrifugation at 12,800 rpm for 10 seconds.

	PROBE	AGONIST
	(100μl, 3 min, 37°C)	(20μl, 5 min, 37°C)
1	TB+Ca^{++}	TB+Ca^{++}
2	TB+Ca^{++}	10μM ADP
3	TB+Ca^{++}	0.1U/ml Thrombin
4	PEG-Au	TB+Ca^{++}
5	PEG-Au	10μM ADP
6	PEG-Au	0.1U/ml Thrombin
7	Fibrinogen-Au	TB+Ca^{++}
8	Fibrinogen-Au	10μM ADP
9	Fibrinogen-Au	0.1U/ml Thrombin

Figure 5.1. Bovine and Human Platelet Treatment Combinations

Transmission Electron Microscopy: After centrifugation, the supernatant was removed and fresh, 2.5% glutaraldehyde in 0.1M CB was added. The pellet was broken up into smaller pieces, and allowed to fix for an additional hour. The samples were centrifuged

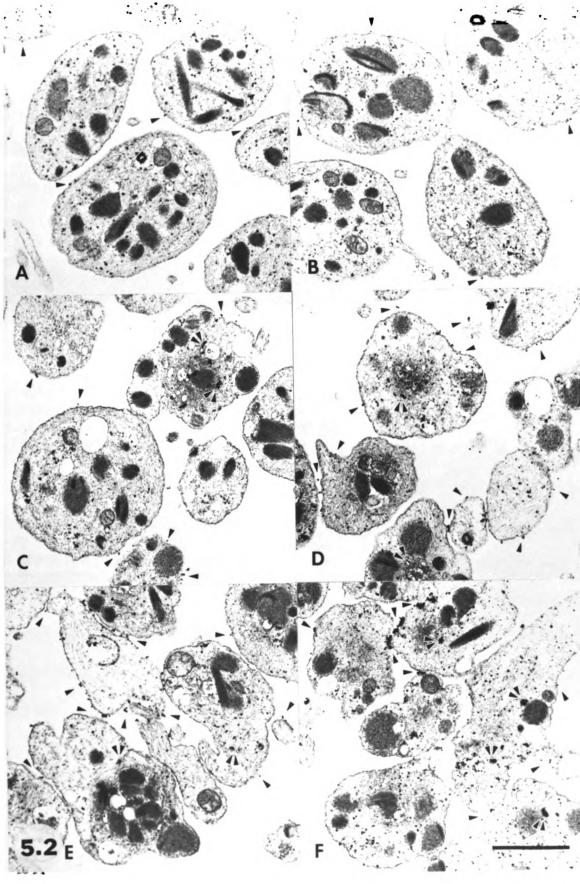
at 12,000 rpm for 1 minute, then the pellets were removed to be suspended in 1% agarose in water. The solidified agarose was cut into pieces, fixed in 2.5% glutaraldehyde in CB (1 hour), post-fixed in 1% aqueous osmium tetroxide, water washed, then dehydrated through a graded ethanol series. Infiltration in Spurrs-Mollenhauer resin (Klomparens, et al. 1986) was through 3:1, 1:1 and 1:3 (ethanol:resin) for 2 hours each, and 100% resin overnight. Blocks were polymerized at 65°C. for 48 hours, ultrathin sectioned, and stained with 2% aqueous uranyl acetate and Reynold's lead citrate (1963) before examination in a JEOL 100CXII TEM.

All data based on at least two experimental repetitions, and the observation of at least 20 platelets per experimental treatment.

RESULTS

Bovine platelets bound small amounts of F-Au without additional agonist in both the unstirred (Figure 5.2a) and stirred treatments (Figure 5.2b). There was no apparent internalization. In ADP-stimulated, F-Au incubated samples, there was internal labelling and an increase in external label, in both suspension treatments. Internalized probe was contained in membrane bound vesicles (double arrowheads), and tended to be more centralized in the platelets. External label appeared to be more frequent in the stirred sample (Figure 5.2d) compared to the unstirred treatment (Figure 5.2c). Probe was also seen in areas of cell to cell contact in the stirred treatment (Figure 5.2d).

Figure 5.2 Unstirred versus Stirred Bovine Platelet Treatments. Bar = 1μM. A) Unstirred, F-Au incubated, no agonist. No internal labelling. External labelling (arrowheads) found that was single and infrequent. B) Stirred, F-Au incubated, no agonist. No internal labelling. External labelling (arrowhead) infrequent. C) Unstirred, ADP-stimulated, F-Au incubated. Internal labelling (double arrowheads) in membrane bound vesicles. External label (arrowhead) both single and clustered. D) Stirred, ADP-stimulated, F-Au incubated. Internal labelling (double arrowheads) present. External labelling (arrowheads) both single and clustered, increased at platelet-platelet contact. E) Unstirred, thrombin-stimulated, F-Au incubated. Some internal labelling (double arrowheads) near centralized granules. External labelling (arrowheads) both single and clustered more pronounced. F) Stirred, thrombin-stimulated, F-Au incubated. Marked external labelling (arrowheads) at platelet-platelet contact points. Internal labelling (double arrowheads) present.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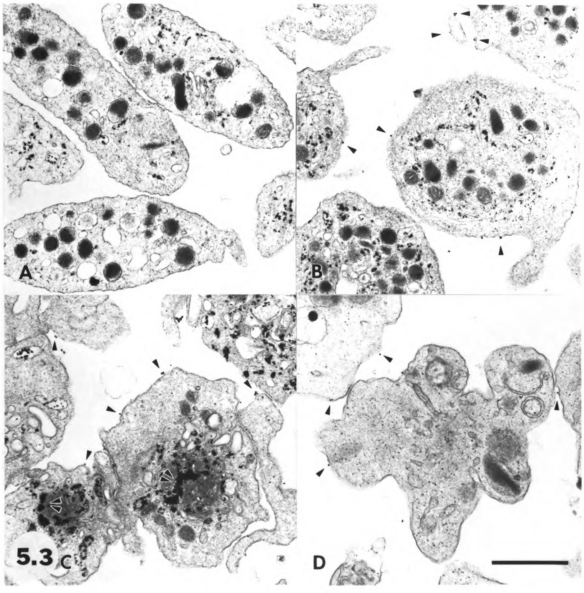
The thrombin stimulation and incubation with F-Au, produced a variation in effect between the unstirred and stirred samples. In the unstirred preparation (Figure 5.2e), external labelling was increased compared to that of the unstirred, ADP-stimulated sample. External label was randomly distributed along the plasma membrane, with slight increases at platelet-platelet interactions. Internal labelling could still be found (double arrowheads, Figure 5.2e). In stirred, thrombin/F-Au treated platelets (Figure 5.2f), there was a general clearing of the probe from the plasma membrane, with an increase in concentration at points of cell contact with other platelets. The level of probe internalization appeared to be similar to the corresponding unstirred treatment.

Unstirred human platelets, incubated with F-Au showed no to minimal probe attachment to the platelet surface, and no activation based on morphological changes (discoid shape maintained) (Figure 5.3a). Platelets stimulated with ADP and incubated with F-Au, demonstrated increased external labelling (Figure 5.3b), that was random and rarely clumped. Internalization of probe appears in approximately 7% of the platelets, and is contained in membrane bound vesicles. Thrombin-stimulation of the platelets caused extensive internalization of the F-Au probe, and clustering of the externally-bound label (Figure 5.3c). Bovine platelets stimulated with thrombin and incubated with PEG-Au, showed some external labelling, and no internalization (Figure 5.3d).

Figure 5.4 illustrates the differences between aggregation traces of bovine and human platelets treated identically. An identical increase in light transmission due to dilution

Figure 5.3 Unstirred Human Platelet Treatments. Bar = 1μM. A) F-Au incubated, no agonist. No internal labelling. External labelling rare to absent. B) ADP-stimulated, F-Au incubated. Internal labelling not present in sample. External label (arrowhead) both single and clustered. C) Thrombin-stimulated, F-Au incubated. Marked internal labelling (double arrowheads) near centralized granules, and packed into the open canalicular system. External labelling (arrowheads) both single and clustered. D) Bovine platelet, thrombin-stimulated, PEG-Au incubated. External labelling (arrowheads) as primarily single particles. Internal labelling not present.



5.3

Figure 5.4 Platelet Aggregation Curves. Graphs are based on the change in light absorbance (Y-axis) over time (X-axis).

Bovine Platelets: A) F-Au, PEG-Au or buffer incubation. No agonist addition. Increased light transmission due to dilutional effect. B) ADP-stimulated: PEG-Au or buffer incubation resulted in shape change alone that returned to baseline levels. F-Au incubation resulted in reduced shape change in comparison to PEG-Au or buffer incubation. C) Thrombin-stimulated: Buffer incubation gave secondary aggreagtion and granule release. PEG-Au incubation showed a truncated shape change, and delayed secondary aggregation onset. F-Au incubation resulted in delayed shape change, with a gradual secondary aggregation.

Human Platelets: A) F-Au, PEG-Au or buffer incubation. No agonist addition. Increased light transmission due to dilutional effect. B) ADP-stimulated: PEG-Au or buffer incubation resulted in shape change alone that returned to baseline levels. F-Au incubation resulted in shape change with primary aggregation, but no granule release. C) Thrombin-stimulated: Buffer incubation gave secondary aggreagtion and granule release. PEG-Au incubation had a slightly reduced rate of shape change onset, though secondary aggregation onset was equivalent to the buffer control. F-Au incubation resulted in a control rate shape change, with delayed secondary aggregation.

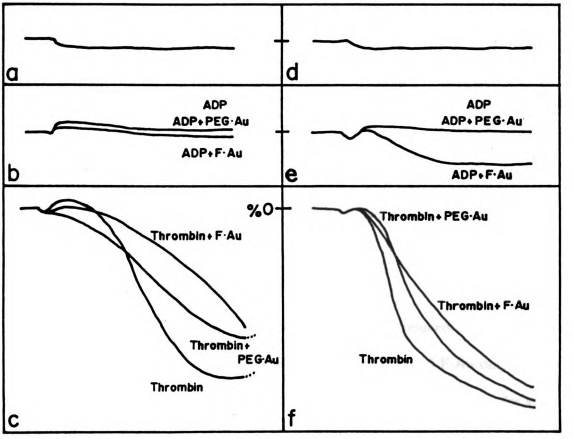


Figure 5.4 Platelet Aggregation Curves

was seen in the bovine platelets incubated without agonist and F-Au, PEG-Au or no probe (Figure 5.4a). Bovine platelets incubated with or without probe, and ADP-stimulated (Figure 5.4b) resulted in shape change only. Thrombin-stimulated bovine platelets resulted in three separate curves dependent on the probe (Figure 5.4c). Thrombin alone, without probe addition, resulted in the most complete aggregation, that over time, began to reverse (dotted line). Thrombin/PEG-Au gave an aggregation curve similar to the thrombin control, but did not attain the same maximal amount of light transmission, nor showed appreciable shape-change at the curve onset. This tracing also showed signs of aggregation reversal after 5 minutes. Thrombin/F-Au treatment of bovine platelets demonstrated an inhibition of aggregation through the delay of onset of shape change and primary aggregation.

Aggregation traces of the various human platelet treatments produced dilutional effects (an overall drop in baseline trace) in the control, PEG-Au only and F-Au only samples (Figure 5.4d). Adenosine diphosphate resulted in platelet shape change only, for all the treated samples, and primary wave aggregation with F-Au incubation (Figure 5.4e). Thrombin stimulation gave a typical aggregation curve, resulting in a secondary wave of aggregation. The extent of aggregation was progressively reduced from the thrombin control, to thrombin/F-Au and thrombin/PEG-Au, respectively (Figure 5.4f). All thrombin treatments maintained their maximal aggregation levels.

DISCUSSION

Theories on the function of the OCS and endocytic internalization in the redistribution of the GPIIb-IIIa receptor after ligand binding, are numerous. Some investigators believe that the OCS is singularly involved with receptor internalization, as well as secretory granule release (White and Escolar, 1991; White, et al., 1990). Other researchers propose an endocytic system, that is intimately involved with OCS (Behnke, O., 1989; Santoso, et al., 1986: Zucker-Franklin, et al., 1985). At this time, the actual mechanism of internalization is still unknown. By employing bovine platelets, that have been reported to lack an OCS, this study provides additional information on the phenomenon of receptor internalization.

One of the most significant differences found between the bovine and human platelets, rested with the variation in response to the agonists employed to cause platelet activation. The bovine platelets showed a much lower level of activation than the human platelets. Agonist stimulation in both the bovine and human platelets enhanced the GPIIb-IIIa receptor binding of labelled fibrinogen to the platelet surface, but internalization levels were vastly different. While the human platelets internalized almost all the ligand, bovine platelets maintained the majority of the labelled fibrinogen on the external surface membrane. Internalization in the bovine platelets was in membrane bound vesicles, as was the case in human platelets. In both species, probe could be found in long canal-like structures, as well as small round structures. There was no apparent difference in the

morphology of these vesicles, the only difference was in the amount internalized.

Aggregation studies showed that the bovine platelets were able to complete aggregation without the massive receptor internalization shown in the human platelets. These platelets maintained the majority of the labelled fibrinogen at sites of platelet-platelet interaction, with very little internalization. These results are quite different than those observed with human platelets. One explaination for these variations may be based on the morphological differences between these two species' platelets. Human platelets have been demonstrated to have internal stores of the GPIIb-IIIa receptor available that are released under strong enough agonist stimulation (Wencel-Drake, et al., 1986; Woods, et al., 1986). This could account for the apparent lack of F-Au at the platelet-platelet junctions seen in human platlets with thrombin stimulation. This suggests that internalization of the GPIIb-IIIa receptor may be linked to the fact that the platelet has additional stores of the receptor, and the receptors responsible for initial fibrinogen gold binding, may not be the same receptors responsible for subsequent platelet aggregation. Fibrinogen conjugated to colloidal gold does not support platelet aggregation to the same extent as native fibrinogen. Full aggregation only occurs when the platelet is induced to release internal stores of unlabelled fibrinogen. The OCS then, in human platelets, may act as a temporary storage facility for receptors that have bound foreign material (non-functional ligand), but not native ligand, ligand bound to another platelet, or ligand bound to other non-internalizable structures, such as a fibrin strand.

Receptor/ligand redistribution studies using both particulate and soluble ligand probes on human platelets, demonstrate a differential redistribution of the complexes. Platelets labelled with fibrinogen coupled to collodial gold resulted, in the internalization of the complex into the OCS (Chapter 1; Chapter 2; White, et al, 1990). In contrast, platelets incubated with biotinylated fibrinogen, a soluble-phase probe, did not demonstrate any complex localization into the OCS, instead, labelling appeared cytoplasmic, with an accumulation of probe in the secretory granules (Chapter 4). These results indicate the human platelet OCS is responsible for the clearing of particulate ligand, and has an alternate mechanism for soluble receptor/ligand complex clearing.

Agonist levels required to activate the bovine platelets also may explain the differences seen. Higher concentrations of agonist was needed to give the same aggregation level, without reversal, in the bovine platelet (Bondy and Gentry, 1989; Marzec, et al., 1975). With an additional store of receptors and a lower activation threshold, human platelets need a mechanism to prevent accidental aggregate formation. This may be the function of the OCS function in human platelets.

This study indicates important differences between platelet systems which contain an open canalicular system, as in humans, and those that do not. If, in fact, the OCS is responsible for the removal of foreign particulate material from the platelet surface, as well as act as a mediator of GPIIb-IIIa receptor expression, it may serve as a protective mechanism in these platelets to prevent premature aggregate formation. The higher

activation theshold, and lack of an additional store of receptors in the bovine platelets may explain the lack of need to have an OCS.

BIBLIOGRAPHY

Behnke, O. 1989 Coated Pits and Vesicles Transfer Plasma Components to Platelet Granules. Thrombos. Haemostas. 62(2).

Behnke, O. 1987 Surface Membrane Clearing of Receptor-Ligand Complexes in Human Blood Platelets. J. Cell Sci. 87:465-472.

Bendayan M. 1984 Protein A-Gold Electron Microscopic Immunocytochemistry: Methods, Applications, and Limitations. J. Electron Microscopy Tech. 1:243-270.

Debus, E., Weber, K. and Osborn, M. 1981 The Cytoskeleton of Blood Platelets Viewed by Immunofluorescence Microscopy. Europ. J. Cell Biol. 24:45-52.

Escolar, G., Leistikow, E. and White, J.G. 1989 The Fate of the Open Canalicular System in Surface and Suspension-Activated Platelets. Blood 74(6):1983-1988.

Fox, J.E.B. 1985 Identification of Actin-binding Protein as the Protein Linking the Membrane Skeleton to Glycoproteins on Platelet Plasma Membranes. J. Biol. Chem. 260(5):11970-11977.

Fox, J.E.B. and Phillips, D.R. 1983 Polymerization and Organization of Actin Filaments Within Platelets. Semin. Hematol. 20(4):243-260.

Fox, J.E.B., Boyles, J.K., Berndt, M.C., Steffen, P.K. and Anderson, L.K. 1988 Identification of a Membrane Skeleton in Platelets. J. Cell Biol. 106:1525-1538.

Frens, G. 1973 Controled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Solutions. Nat. Phys. Sci. 241:20-22.

Klomparens, K.L., Flegler, S.L. and Hooper, G.R. 1986 in Procedure for Transmission and Scanning Electron Microscopy for Biological and Medical Science. A Laboratory Manual, 2nd Edition. Ladd Research Ind., Burlington, VT. p146.

Painter, R.G., Prodouz, K.N. and Gaarde, W. 1985 Isolation of a Subpopulation of

Glycoprotein IIb-III from Platelet Membranes That Is Bound to Membrane Actin. J. Cell Biol. 100:652-657.

Reynolds, E.S. 1963 The Use of Lead Citrate at High pH as an Electron Opaque Stain in Electron MIcroscopy. J. Cell Biol. 17:208-212.

White, J.G., Leistikow, E.l. and Escolar, G. 1990 Platelet Membrane Responses to Surface and Suspension Activation. Blood Cells 16:43-72.

Wencel-Drake, J.D. 1990 Plasma Membrane GPIIB/IIIa. Evidence for a Recycling Receptor Pool. Amer. J. Pathol. 136(1):61-70.

Zucker-Franklin, J. 1981 Endocytosis by Human Platelets: Metabolic and Freeze-Fracture Studies. J. Cell Biol. 91:706-715.

Zucker-Franklin, D. 1985 Absence of a Surface-Connected Canalicular System in Bovine Platelets. Blood 65(1):241-244.

SUMMARY

The goal of this research was to examine platelet receptor Glycoprotein IIb-IIIa redistribution under various conditions. The experimental variables reflected the main questions posed at the onset; 1) is receptor redistribution affected by mode of activation, and 2) does the solubility of the receptor ligand influence receptor mobility?

The first question was approached by using platelets in suspension both stirred and unstirred, and platelets adherent on a plastic support. The findings suggest: 1) suspension platelets undergo a low level of activation in a stirred system, that results in activation-dependent ligand binding without addition of exogenous agonist. In combination with a weak agonist, such as ADP, this stirring results in receptor redistribution similar to that seen with a stronger agonist, such as thrombin. 2) thrombin-stimulation of platelets in suspension can be shown to have a threshold level of activation, that is affected by blocking of the GPIIb-IIIa receptor with monoclonal antibodies. Further, blocking studies suggest a significant contribution of internal receptors to the overall receptor redistribution seen with F-Au. 3) internalization of probe in the human platelet may be a mechanism to remove unanchored ligand from the platelet surface, to prevent unwanted thrombosis formation. Finally, 4) based on the time at which activated receptors are exposed to ligand and whether platelet adhesion is involved, receptors appear to have distinctly

different redistribution characteristics.

The question of soluble-phase ligands verus solid-phase ligands in the redistribution of the receptor, used fibrinogen conjugated to colloidal gold and biotinylated fibrinogen as the soluble label. In these experiments, the receptor redistribution using the particulate probe resulted in localization of the recptor into the OCS whenever internalized. In contrast, biotinylated fibrinogen, migrated to adhesion interfaces, or was internalized into cytoplasmic organelles, believed to be alpha granules. These finding make apparent, the marked effect that particulate markers have on ligand/receptor redistribution.

Related to these findings, the OCS in human platelets may act as a clearing mechaniasm for foreign particulate material in contact with the platelet plasma membrane.

BIBLIOGRAPHY

BIBLIOGRAPHY

Brass, L.F. and Shattil, S.J. 1984 Identification and Function of the High Affinity Binding Sites for Ca^{2+} on the Surface of Platelets. J. Clin. Invest. 73:626-632.

Caen, J.P., Castaldi, P.A., Leclerc, J.C., Inceman, S., Larrieu, M.J., Probst, M. and Bernard, J. 1966 Congenital Bleeding Disorders with Long Bleeding Time and Normal Platelet Count. Am. J. Med. 41:4-26.

Caen, J.P. and Michel, H. 1972 Platelet Shape Change and Aggregation. Nature 240:148-149.

Calvete, J.J., Alvarez, M.V. and González-Rodríguez, J. 1986 Quantitation and Subcellular Distribution of GP IIb and GP IIIa in Human Platelets and in the External Platelet Surface. Characterization of These Glycoproteins Isolated from the Different Subcellular Fractions in Monoclonal Antibodies and Human Blood Platelets. McGregor, J.L. (ed). Elsevier Sci. Publ. pp 179-190.

Calvete, J.J., Rivas, G., Maruri, M., Alvarez, M., McGregor, J.L., Hew, C.L> and Gonzalez-Rodriguez, J. 1988 Tryptic Fragment of Human GP IIIa. Isolation and Biochemical Characterization of the 23kDa N-terminal Gylcopeptide Carrying the Antigenic Determinant for a Monoclonal Antibody (P37) which Inhibits Platelet Aggregation. Biochem. J. 260:697-704.

Carrell, n., Fitzgerald, L.A., Steiner, B., Erickson, H.P. and Phillips, D.R. 1985 Structure of Human Platelet Membrane Glycoproteins IIb and IIIa as Determined by Electron Microscopy. J. Biol. Chem. 260:1743-1749.

Cheresh, D.A., Berliner, S.A., Vicente, V. and Ruggeri, Z.M. 1989 Recognition of Distinct Adhesive Sites on Fibrinogen by Related Integrins on Platelets and Endothelial Cells. Cell 58:945-953.

Cohen, I. 1985 The Mechanism of Clot Retraction in Platelet Membrane Glycoproteins. George, Nurden, Phillips (eds). Plenum Press. pp 299-323.

Davies, T.A., Drotts, D.I., Weil, G.J. and Simons, E.R. 1989 Cytoplasmic Ca^{2+} Is

Necessary for Thrombin-Induced Platelet Activation. J. Biol. Chem. 264(33):19600-19606.

De Christofaro, R., Landolfi, R., Di Cera, E., Castagnola, M., De Candia, E. and Bizzi, B. 1989 Inhibition of Fibrinogen Binding to Human Platelets by Blockage of Na^+/ H^+ Exchange. Biochem. Biophys. Res. Comm. 161(3):1228-1232.

D'Souza, S.E., Ginsberg, M.H., Burke, T.A., Lam, S.C-T., and Plow, E.F. 1988 Localization of a Arg-Gly-Asp Recognition Site Within an Integrin Adhesion Receptor. Science 242:91-93.

Escolar, G., Leistikow, E. and White, J.G. 1986 The Fate of the Open Canalicular System in Surface and Suspension-Activated Platelets. Blood 74(6):1983-1988.

Fitzgerald, L.A. and Phillips, D.R. 1985 Calcium Regulation of the Platelet Membrane Glycoprotein IIb-IIIa Complex. J. Biol. Chem. 260:11366-11374.

Fox, J.E.B. 1986 Platelet Contractile Proteins in Biochemistry of Platelets. Phillips, Shuman (eds). Academic Press. pp 115-157.

Fox, J.E.B. 1985 The Organization of Platelet Contractile Proteins in Platelet Membrane Glycoproteins. George, Nurden, Phillips (eds). Plenum Press. pp 273-298.

Fox, J.E.B., Baughan, A.K. and Phillips, D.R. 1983 Direct Linage of GP Ib to a M_r = 250,000 Polypeptide in Platelet Cytoskeletons. Blood 62:255a.

George, J.N, and Nurden, A.T. 1987 Inherited Disorders of the Platelet Membrane: Glanzmann's Thrombasthenia and Bernard-Soulier Syndrome in Hemostasis and Thrombosis. Basic Principles and Clinical Practice. 2nd Ed. Colman, Hirsh, Marder, Salzman (eds). J.B. Lippincott. pp 726-740.

George, J.M., Nurden, A.T. and Phillips D.R. 1984 Molecular Defects in Interactions of Platelets with the Vessel Wall. N. Engl. J. Med. 311:1084-1098.

George, J.N. and Shattil, S.J. 1991 The Clinical Importance of Acquired Abnormalities of Platelet Function. N. Engl. J. Med. 324:27-39.

Ginsberg, M.H., Lightsey, A., Kunicki, T.J., Kaufmann, A., Marguerie, G. and Plow, E.F. 1986 Divalent Cation Regulation of the Surface Oreintation of Platelet Membrane Glycoprotein IIb. Correlation with Fibrinogen Binding Function and Defination of a Novel Varient of Glanzmann's Thrombasthenia. J. Clin. Invest. 78:1103-1111.

Ginsberg, M.H., Loftus, J. and Plow, E.F. 1988 Platelets and the Adhesion Receptor Superfamily. in Platelet Membrane Receptors: Molecular Biology, Immunology,

Biochemistry and Pathology. Vol. 283. Jamieson, G.A. (ed). Alan Liss. pp 171-195.

Gogstad, G.O., Hagen, I., Korsmo, R. and Solum, N.O. 1981 Characterization of the Proteins of Isolated Human Platelet Alpha-Granules. Evidence for a Separate Alpha-Granule Pool of the Glycoproteins IIb and IIIa. Biochim. Biophys, Acta. 670:150-162.

Gogstad, G.O., Hagen, I., Krutnes, M.B. and Solum, N.O. 1982 Dissociation of the Glycoprotein IIb-IIIa Complex in Isolated Human Platelet Membranes. Dependence of pH and Divalent Cations. Biochim. Biophys. Acta. 689:21-31.

Gralnick, H.R., Williams, S., McKeown, L., Connaghan, G., Shafer, B., Hansmann, K., Vail, M. and Fenton, J. 1991 Endogenous Platelet Fibrinogen Surface Expression on Activated Platelets. J. Lab. Clin. Med. 118(6):604-613.

Isenberg, W.M., McEver, R.P., Phillips, D.R., Shuman, M.A. and Bainton, D.F. 1989 Immunogold-Surface Replica Study of ADP-Induced Ligand Binding and Fibrinogen Receptor Clustering in Human Platelets. Am. J. Anat. 185:142-148.

Jennings, L.K. and Phillips, D.R. 1982 Purification of Glycoproteins IIb and III From Human Platelet Plasma Membranes and Characterization of a Calcium-Dependent Glycoprotein IIb-III Complex. J. Biol. Chem. 257:10458-10466.

Kamiyama, M., Arkel, Y.S., Chen, K. and Shido, K. 1991 Inhibition of Platelet GPIIb/IIIa Binding to Fibrinogen By Serum Factors: Studies of Circulating Immune Complexes and Platelet Antibodies in Patients with Hemophilia, Immune Thrombocytopenic purpura, Human Immunodeficiency Virus-Related Immune Thrombocytopenic Purpura, and Systemic Lupus Erythematosus. J. Lab. Clin. Med. 117:209-217.

Kieffer, N. and Phillips, D.R. 1990 Platelet Membrane Glycoproteins: Functions in Cellular Interactions. Annu. Rev. Cell Biol. 6:329-357.

Kloczewiak, M., Timmons, S., Lukas, T.J. and Hawiger, J. 1984 Platelet Receptor Recognition Site on Human Fibrinogen. Synthesis and Structure-Function Relationship of Peptides Corresponding to the Carboxyterminal Segment of the Gamma Chain. Biochem. 23:1767-1774.

Kroll, M.H. and Schafer, A.I. 1989 Biochemical Mechanisms of Platelet Activation. Blood 74(4):1181-1195.

Kunicki, T.J., Pidard, D., Rosa, J.P. 1981 The Formation of Ca^{++}-Dependent Complexes of Platelet Glycoproteins IIb and IIIa in Solution as Determined by

Crossedimmunoelectroporesis. Blood 58:268-278.

Lam, S.C., Plow, E.F., Smith, M.A., Andrieux, A., Ryckwaert, J.J. 1987 Evidence that Arginyl-Glycyl-Aspartate Peptides and Fibrinogen Gamma Chain Peptides Share a Common Binding Site on Platelets. J. Biol. Chem. 26:947-950.

Lewis, J.C., Hantgan, R.R., Stevenson, S.C., Thornburg, T., Kieffer, N., Guichard, J. and Breton-Gorrius, J. 1990 Fibrinogen and Glycoprotein IIb/IIIa Localization During Platelet Activation. Localization to the Granulomere and Sites of Platelet Interaction. Am. J. Pathol. 136(1):239-252.

Moake, J.L. 1990 Platelet Function, Surface Structure and Antigenicity in Aphersis. Alan Liss. pp 41-43.

Nicholson, N.S., Panzer-Knodle, S.G., Salyers, A.K., Taite, B.B., King, L.W., Miyano, M., Gorczynski, R.J., Williams, M.H., Zupec, F.S., Tjoeng, F.S., Adams, S.P. and Feigen, L.P. 1991 Antiplatelet and Antithrombic Effects of Platelet Glycoprotein IIb/IIIa (GP IIb/IIIa) Inhibition by Arginine-Glycine-Aspartic Acid-Serine (RGDS) and Arginine-Glycine-Aspartic Acid (RGD) (O-me)Y (SC-46749). J. Pharmacol. Exp. Therap. 256(3):876-882.

Nurden, A.T. and Caen, J.P. 1975 An Abnormal Platelet Glycoprotein Pattern in Three Cases of Glanzmann's Thrombasthenia. Br. J. Haematol. 28:253-260.

Painter, R.G. and Ginsberg, M. 1982 Concanavalin A Induces Interactions Between Surface Glycoproteins and the Platelet Cytoskeleton. J. Cell Biol. 92:565-573.

Parise, L.V., Helgerson, S.L., Steiner, B., Nannizzi, L. and Phillips, D.R. 1987 Synthetic Peptides Derived from Fibrinogen and Fibronectin Change the Conformation of Purified Platelet Glycoprotein IIb-IIIa. J. Biol. Chem. 262(26):12597-12602.

Phillips, D.R. and Agin, P.P. 1977 Platelet Membrane Defects in Glanzmann's Thrombasthenia: Evidence for Decreased Amounts of Two Major Glycoproteins. J. Clin. Invest. 60:535-545.

Phillips, D.R., Jennings, L.K. and Edwards, H.H. 1980 Identification of Membrane Proteins Mediating the Interaction of Human Platelets. J. Cell Biol. 86:77-86.

Pierschbacher, M.D. and Ruoslahti, E. 1984 Cell Attachment Activity of Fibronectin Can be Duplicated by Small Synthetic Fragments of the Molecule. Nature 309:30-33.

Pidard, D. 1986 Studies on the Organization and Function of GP IIb and GP IIIa within the Plasma Membrane of Intact Human Platelets Using the Monoclonal Antibody AP-2 in Monoclonal Antibodies and Human Blood Platelets. McGregor, J.L. (ed).

Elsevier Sci. Publ. pp 103-117.

Pidard, D., Frelinger, A.L., Bouillot, C. and Nurden, A.T. 1991 Activation of the Fibrinogen Receptor on Human Platelets Exposed to Alpha Chymotrypsin. Eur. J. Biochem. 200:437-447.

Plow, E.F., Ginsberg, M.H. and Marguerie, G.A. 1986 Expression and Function of Adhesive Proteins on the Platelet Surface in Biochemistry of Platelets. Phillips, Shuman (eds). Academic Press. pp 225-256.

Plow, E.F., Srouji, A.H., Meyer, D., Marquerie, G. and Ginsberg, M.H. 1984 Evidence that Three Adhesive Proteins Interact with a Common Recognition Site on Activated Proteins. J. Biol. Chem. 259:5388-5391.

Polley, M.J., Leung, L.L.K., Clark, F.Y. and Nachman, R.L. 1981 Thrombin-Induced Platelet Membrane Glycoprotein IIb and IIIa Complex Formation. J. Exp. Med. 154:1058-1068.

Rendu, F. and Dupuy, E. 1991 Hereditary Disorders of Platelet Function in Blood Cell Biochemistry. Vol. 2. Harris, J.R. (ed). Plenum Publ. pp 77-112.

Rivas, G.A. and González-Rodríguez, J. 1991 Calcium Binding to Human Platelet Integrin GPIIb/IIIa and to its Constituent Glycoproteins. Biochem. J. 276:35-40.

Ruggeri, Z.M. 1986 The Use of Monoclonal Antibodies to Probe the Receptor Function of the Glycoprotein IIb/IIIa Complex in Monoclonal Antibodies and Human Blood Platelets. McGregor, J.L. (ed). Elsevier Sci. Publ. pp 83-91.

Ruggeri, Z.M. and Hodson, E.M. 1989 Use of Murine Monoclonal Antibodies to Determine Platelet Membrane Glycoprotein Structure and Function in Platelet Immunology. Molecular and Clinical Aspects. Kunicki and George (eds). J.B. Lippincott. pp 235-254.

Ruoslahti, E. and Pierschbacher, M.D. 1987 New Perspectives in Cell Adhesion: RGD and Integrins. Science. 238:491-497.

Santoso, S., Zimmermann, U., Neppert, J. and Mueller-Eckhardt, C. 1986 Receptor Patching and Capping of Platelet Membranes Induced by Monoclonal Antibodies. Blood 67(2):343-349.

Shattil, S.J. 1988 Expression, Regulation and Detection of Fibrinogen Receptors on Activated Platelets in Platelet Membrane Receptors: Molecular Biology, Immunology, Biochemistry and Pathology. Jamieson, G.A. (ed). Alan Liss, Inc. pp 345-380.

Solom, N.O. 1985 Platelet Membrane Proteins. Semin. Hematol. 22(4):289-302.

Solom, N.O., Olsen, T. and Gogstad, G. 1983 GP Ib in the Triton-Soluble (cytoskeletal) Fraction of Platelets (abstract). Thrombos. Haemost. 50:372a.

Steiner, B., Cousot, D., Trzeciak, A., Gillessen, D. and Hadvery, P. 1989 Ca^{2+}-Dependent Binding of Synthetic Arg-Gly-Asp (RGD) Peptide to a Single Site on the Purified Platelet Glycoprotein IIb-IIIa Complex. J. Biol. Chem. 264:13102-13108.

Steiner, B., Parise, L.V., Leung, B. and Phillips, D.R. 1991 Ca^{2+}-Dependent Structural Transitions of the Platelet Glycoprotein IIb-IIIa Complex. J. Biol. Chem. 266(23):14986-14991.

Sugiyama, T., Pidard, D., Wautier, M-P. and Wautier, J-L. 1990 Comparative Immunochemical Analysis of Membrane Proteins from Human Platelets and Endothelial Cells. Nouv. Rev. Fr. Hematol. 32:199-205.

Suzuki, H., Tanoue, K. and Yamazaki, H. 1991 Morphological Evidence for the Association of Plasma Membrane Glycoprotein IIb/IIIa with the Membrane Skeleton in Human Platelets. Histochem. 96:31-39.

Taylor, D.G., Mapp, R.J. and Crawford, N. 1975 The Identification of Actin Associated with Pig Platelet Membranes and Granules. Biochem. Soc. Trans. 3:161-164.

Thiagarajan, P., Shapiro, S.S., Levine, E., DeMarco, L. and Yalcin, A. 1985 A Monoclonal Antibody to Human Platelet Glycoprotein IIIa Detects a Related Protein in Cultured Human Endothelial Cells. J.Clin. Invest. 75:896-901.

Wencel-Drake, J.D., Okita, J.R., Annis, D.S. and Kunicki, T.J. 1991 Activation of Calpain I and Hydrolysis of Calpain Substrates (Actin-Binding Protein, Glycoprotein Ib, and Talin) Are Not a Function of Thrombin-Induced Platelet Aggregation. Arteriosc. Thrombos. 11:882-891.

Wencel-Drake, J.D., Plow, E.F., Kunicki, T.J., Woods, V.L., Keller, D.M. and Ginsberg, M.H. 1986 Localization of Internal Pools of Membrane Glycoproteins Involved in Platelet Adhesive Responses. Am. J. Pathol. 124:324-334.

White, J.G. and Escolar, G. 1990 Mobility of GP IIb-IIIa Receptors Within Membranes of Surface- and Suspension-Activated Platelets Does Not Depend on Assembly and Contraction of Cytoplasmic Actin. Eur. J. Cell Biol. 52:341-348.

Woods, V.L., Wolff, L.E. and Keller, D.M. 1986 Resting Platelets Contain a Substantial Centrally Located Pool of Glycoprotein IIb-IIIa Complex Which May be Available to Some but not Other Extracellular Proteins. J. Biol. Chem. 261(32):15242-15251.

Zucker-Franklin, D. and Grutzy, G. 1972 The Actin and Myosin Filaments of Human and Bovine Blood Platelets. J. Clin. Invest. 51:419-430.