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DUAL-AGONIST ACTIVATED PLATELETS: AN EMERGING COMPONENT OF THE PRIMARY HEMOSTATIC RESPONSE

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ABBREVIATIONS

α_2-AP = α-2 antiplasmin

ACD = acid citrate dextrose

BSA = bovine serum albumin

BSGC = buffered saline-glucose-citrate

CP_{15} = casein peptide (SVLSLSQSKVLPVPE)

COAT = collagen and thrombin (activated)

ECM = extracellular matrix

Fbg = fibrinogen

FcRT = Fc receptor and thrombin (activated)

Fn = fibronectin

FV = factor V

FXIII = factor XIII

FITC = fluorescein isothiocyanate

GAMG = goat anti-mouse IgG

GFP = gel-filtered platelet

HEPES = N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

HPLC = high performance liquid chromatography

mAb = monoclonal antibody

MP = microparticule

PAR1/PAR4 = protease activated receptor

PBS = phosphate-buffered saline

PE = phycoerythrin

PRP = platelet rich plasma

PS = phosphatidylserine

RT = room temperature

SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis

TG = transglutaminase

TO = thiazole orange

Tsp = thrombospondin

vWF = von Willebrand factor

KEYWORDS

platelet activation, thrombin, collagen, convulxin, serotonin, transglutaminase

INTRODUCTION

Platelets activated at sites of vascular injury play two key roles in normal hemostasis. First, by adhering to exposed subendothelium, binding adhesive proteins and aggregating, they create a physical barrier that limits blood loss (1). Second, platelets accelerate coagulation by providing a surface which promotes two procoagulant reactions, generation of Xa and thrombin (2). A specific subset of dual-agonist activated platelets described in this thesis may affect both of these roles.

Platelets activated simultaneously with collagen and thrombin reveal two distinc populations of activated cells. One population expresses very high levels of several α-granule born procoagulant proteins including factor V (FV), fibrinogen (Fbg), von Willebrand factor (vWF), fibronectin (Fn), α_2-antiplasmin (α_2-AP), and thrombospondin (Tsp) while other does not. Those cells retaining high levels of procoagulant proteins are referred to as COAT-platelets, an acronym for **co**llagen **a**nd **t**hrombin activated platelets. This thesis discusses the characteristics and possible physiological implications of COAT-platelets.

Alberio *et al.* initially observed that human gel-filtered platelets (GFP) stimulated simultaneously with two agonists, thrombin and collagen, produced two polulations of activated cells regard to factor V expression (3). The population of cells with high levels of surface FV represented 20 to 40% of the total activated platelets. These two populations could also be observed upon platelet activation with thrombin plus convulxin. Convulxin is a snake venom protein activating the glycoprotein VI (GPVI) receptor on platelets (4). Subsequently we have examined additional α-granule proteins on the surface of COAT-platelets and demonstrated that not solely FV but fibrinogen, vWF, fibronectin, α_2-antiplasmin and thrombospondin are also present on thrombin plus collagen (or convulxin) activated platelets. Antisera against IgG and albumin, which are also α-granule born proteins, did not exhibit this „COAT-like" population upon dual stimulation. All of these adhesive and procoagulant proteins as well as FV are substrates for transglutaminases (TG), and inhibitors of transglutaminase prevented the production of COAT-platelets.

To identify the possible binding site for these proteins on the platelet mebrane we have synthetised a transglutaminase substrate (CP_{15}). This 15-residue peptide derived from β-casein included a transglutaminase-active glutamine (**5**) and was prepared either biotinylated (biotin-CP_{15}) or fluorescein labeled (Fl-CP_{15}). When biotin-CP_{15} was included in a COAT-platelet assay, the biotin label was incorporated into COAT-platelets, and this incorporation could be blocked with transglutaminase inhibitors. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of biotin-CP_{15} incorporated COAT-platelets revealed no high molecular weight platelet membrane proteins. Therefore Fl-CP_{15}-labeled COAT-platelets were extracted with ethanol and analyzed on reverse phase HPLC. The protuct than was analyzed by mass spectrometry and found to be the starting material Fl-CP_{15} plus 176 Da. The most common constituent in platelets with a molecular weight of 176 Da is serotonin. Direct evidence of serotonin's role was provided by identifying conjugated serotonin on fibrinogen isolated from COAT-platelets.

Alberio *et al.* also demonstrated that young platelets, identified by thiazole orange (TO) binding, produced a higher fraction of COAT-platelets than did aged, TO-negative cells (3, **6**). COAT-platelets were also found to express maximum levels of phosphatidyl-serine (PS) as demonstrated by annexin V binding. With the exception of ionophore A23187, the gold standard of PS exposure, no single agonist produced platelets with significant PS expression. However, dual-agonist stimulation with convulxin plus thrombin or collagen plus thrombin elicited strong binding of annexin V in the same population of cells retaining FV. Additionally we have confirmed that all of other α-granule proteins but IgG and albumin were present in a higher fraction on the surface of thiazole orange binding young platelets than on aged ones.

Similar subpopulation of platelets can also be generated by the combined stimulation of FcγRIIA and thrombin receptors (**7**). Platelets activated in this manner are referred to as Fc receptor and thrombin-activated (FcRT) platelets, and they share many of the characteristics of the formerly observed COAT-platelets, including aminophospholipid exposure, adhesive and procoagulant protein enrichment, increased frequency among young platelets, and sensitivity to transglutaminase inhibitors. Although FcγRIIA receptor activation can be achieved either with anti-CD9 monoclonal antibodies (ALB-6 and ML-13) or with direct Fc receptor cross-linking, FcRT-platelet generation occurs

only with concurrent or slightly delayed thrombin stimulation. FcRT-platelet formation in platelet poor plasma and whole blood was also investigated, and results were similar to those observed with gel-filtered platelets. Previous experiments with COAT-platelet formation used physiologic agonists (collagen and thrombin) that might be encountered under either physiologic or pathologic conditions; however, these experiments (**7**) with Fc receptor stimulation have offered the first example in which these highly prohemostatic platelets are likely to be strictly pathogenic.

We do not yet know the physiologic significance of COAT-platelets, but there are several observations that suggest these cells are important components of the primary hemostatic system. The most obvious finding is exposure of surface PS and the generation of significant prothrombinase activity by COAT-platelets (3). Clearly, PS exposure is a critical component of the primary hemostatic system, and the selective exposure of PS by COAT-platelets after collagen plus thrombin stimulation suggests an important role for these cells.

This generation of two populations of activated cells is emphasized by the disparate nature of the final products. COAT-platelets with their armor strongly adherent, procoagulant proteins differ dramatically form the non-COAT population where classical bleeding and retention of adhesive and prohemostatic proteins is observed. The full impact of COAT-platelets in physiologic and pathologic aspects of hemostasis remains to be determined.

BACKGROUND AND SIGNIFICANCE

Platelet activation and adhesive protein binding

Vessel wall injury triggers prompt platelet activation and platelet plug formation, followed by coagulant activity and the formation of fibrin-containing thrombi (so called the primary hemostatic plug) that occlude the site of injury (**8**). These events are crucial to limit blood loss at sites of vascular injury but may also block diseased vessels, leading to ischemia and infarction of vital organs. One of the major clinical problems in the developed world is arterial thrombosis caused by rupture or erosion of an atherosclerotic plaque, leading to platelet adhesion and subsequent thrombus formation in coronary and cerebral arteries causing myocardial infarction and stroke, respectively. Therefore, a detailed understanding of the mechanisms underlying the formation of arterial thrombosis is required in order to control ischemic cardiovascular diseases while retaining hemostasis.

The first step in the hemostatic cascade is platelet interaction with the exposed extracellular matrix (ECM) at sites of injury. Among the macromolecular constituents of the ECM, collagen is considered to play a major role in this process, as in vitro it not only supports platelet adhesion through direct and indirect pathways but it also directly activates the cells initiating aggregation and coagulant activity (9). Platelet adhesion and aggregation on collagen is an integrated process that involves several platelet agonists that act through a variety of surface receptors, including integrins, immunoglobulin-like receptors, and G-protein-coupled receptors.

The rate of platelet activation is another important variable that contributes to the development of the primary hemostatic platelet plug (**8**). Collagen is a key modulator of platelet activation through its stimulation of at least three platelet receptors (10). Platelets have two major primary receptors for collagens, the integrin $\alpha 2\beta 1$ and the platelet-specific receptor glycoprotein VI. A third receptor that created prominently at the very onset of adhesion, the GPIb complex, is also a collagen receptor. It is collagen that captures the vWF molecule by binding to most likely its A3 domain, thereby localizing it and somehow altering its conformation to make it bind via its A1 domain with greater avidity to the GPIb complex. This interaction is relatively weak and short in duration, resulting in a slowing of platelet motion and a tethering or rolling of the

platelet across the thrombogenic collagen-rich surface. It is at this point that the two major receptors, α2β1 and GPVI, become involved. GPVI binds to collagens weakly, and plays an important role in transduction of signals leading to platelet activation. The more avid stable platelet attachment afforded by α2β1 leads to a stable monolayer of activated platelets that serves as a nidus for prothrombin conversion and thrombus formation.

Platelet-collagen interactions are believed to have the greatest significance at the medium and high shear rates found in arteries and diseased vessels. At the very high shear rates found in small arteries and arterioles, the rapid onset of interaction between glycoprotein Ib-V-IX (GPIb-V-IX) and von Willebrand factor immobilized on collagen is crucial for the initial tethering (or capture) of flowing platelets (11,12).

Platelet adhesion to the subendothelium is another important initial step in platelet activation. The subendothelium is composed of ECM proteins, such as collagen, fibronectin, von Willebrand factor, thrombospondin, and laminin (13), many of which are ligands for receptors on the platelet surface. These adhesive proteins are exposed when the endothelial layer is disrupted. Because of the large number of ECM proteins and a high density of platelet surface receptors, platelet adhesion to areas of vascular injury is extremely rapid. Von Willebrand factor secreted into the ECM from endothelial cells, and facilitates platelet adhesion by binding to platelet GPIb/IX/V receptors, especially at high shear rates (14-16). Platelets can also adhere to vascular wall–associated fibrin or fibrinogen through interaction with platelet surface glycoprotein IIb/IIIa (16,17). After adhering to the subendothelium, platelets undergo a cytoskeletal activation that leads to a shape change with development of pseudopods (**8**). Intracellular signaling processes lead to increased cytoplasmic calcium and then initiate a secretory release reaction, whereby products from the α-granules (platelet factor 4, β-thromboglobulin, thrombospondin, platelet-derived growth factor, fibrinogen, vWF) and dense granules (ADP, serotonin) are released into the surrounding milieu (18). The granule membranes contain many integral glycoproteins on their inner leaflet, such as P-selectin (CD62p) in the α-granule and GP53 (CD63) in the lysosome, which become expressed on the outer platelet membrane after the release reaction (19). The release of ADP from the dense granules, together with calcium mobilization, leads

to a conformational change of the glycoprotein IIb/IIIa receptor complex (20). This conformational change of the fibrinogen receptor initiates the process of aggregation, whereby a glycoprotein IIb/IIIa receptor on one platelet is bound in a homotypic fashion to the same receptor on adjacent platelets via a central fibrinogen molecular bridge. Beside ADP, other agonists, such as epinephrine, thrombin, collagen, and platelet-activating factor, can initiate platelet aggregation by interaction with membrane receptors. This platelet release reaction and aggregation lead to the recruitment of many other platelets to the vessel wall with the formation of the primary hemostatic platelet plug. Activated platelets also play a vital procoagulant role that serves as a link between platelet function and coagulation activation. Platelet membrane phospholipids undergo a rearrangement during activation with a transfer of phosphatidylserine from the inner table to the outer leaflet of the platelet membrane, providing a binding site for phospholipid-dependent coagulation complexes that activate both factor X (FX) and prothrombin (21). In addition increased thrombin generation is also potentiated by the coagulation system via activated factor VII (FVIIa) resulting in subsequent platelet activation at the site of vascular injury **(22)**.

Platelet activation by single agonists has been exetensively examined (23-26). The first suggestion that platelets were not homogeneous was provided in 1965 by Webber and Firkin (27), who showed morphological differences among hypotonically treated platelets. Behnke and colleagues, however, were the first to describe biochemical differences among platelets that delineated clear subpopulations of cells (28,29). These workers observed that histological stains for a cytoplasmic phosphatase activity identified two populations of platelets, and subsequent experiments suggested that the phosphatase was more specifically a phospho-tyrosine phosphatase. The absence of this phosphatase activity appeared to correlate with more reactive platelets. Additional examples of platelet heterogeneity are present in the literature. For example, Heemskerk et al. (30) observed that approximately 50% of platelets adherent to a collagen matrix expressed phosphatidylserine as monitored by annexin-V binding. Inclusion of thrombin in the adhesion reaction increased the percentage of PS-positive cells to more than 80%. Similarly, Pasquet et al. (31) observed that stimulation of platelets with collagen plus thrombin resulted in two populations of platelets relative to PS exposure, and when PS-positive platelets were isolated by fluorescent-activated cell sorting, they were found to have decreased levels of tyrosine phosphorylation. Also, Feng and Tracy

reported that stimulation of platelets with high doses of thrombin resulted in two distinct populations of cells when analyzed for surface bound factor Va or factor Xa, and platelets expressing both FVa and FXa represented ~38% of the total population (32).

Platelet activation by thrombin occurs through cleavage of PAR-1 and/or PAR-4 (protease activated receptor) present on human platelets (**8**, 33, 34). Both thrombin receptors are seven transmembrane, G-protein-coupled receptors (**8**, 33). Platelets respond to collagen via a number of different receptors (10, 35). While there is still some disagreement within the field, a general scheme has emerged. The integrin $\alpha_2\beta_1$, binds collagen and is considered primarily responsible for collagen-mediated immobilization of platelets (36). GPVI, which has only recently been cloned (37), also interacts with various collagen types and appears to be primarily responsible for activation events associated with collagen (38). Other collagen receptors, including CD34 and p65 are less well understood (35). Signaling events promoted by GP VI have not been fully elucidated although it is clear that a pathway from syk to PLCγ1 to PI-3-kinase is activated by GP VI (39, 40) and that inhibitors of these particular signaling components can blunt the effects of GP VI. The collagen field has been considerably enhanced by the introduction of receptor specific agonists. In particular, convulxin, isolated from the venom of Crotalus durissus terrificus (tropical rattlesnake) is a GP VI specific agonist which has greatly facilitated analysis of this receptor (4).

Numerous studies have examined the effect of dual stimulation with one strong (thrombin, collagen or thromboxane A_2) and one weak agonist (ADP, epinephrine or vasopressin) (41, 42) or with two weak agonists (43, 44). A synergism has been observed between agonists utilized at sub-optimal concentrations (42, 44, 45) and within 10-20 seconds of each other (43, 45). These reports offer the conclusion that stimulation with one agonist can "prime" the platelet for a second agonist by mechanisms which are incompletely understood (45). Even more interesting is the observation that a combination of agonists can elicit a specific phosphorylation response that neither reagent by itself can promote (41). While little is known concerning dual stimulation with two strong agonists, one would anticipate that this offers the opportunity for extreme activation of the platelet, particularly if the agonists utilize fundamentally different signaling pathways.

The literature on soluble adhesive proteins interacting with resting and activated platelets is truly voluminous (46, 47). Clearly the interactions of GP IIb/IIIa with Fbg (46) and vWF with GP Ib (48) occupy central positions in the current understanding of adhesive protein interactions, and each interaction has been dissected in great detail. Two hallmarks of these adhesive protein/platelet interactions are the saturable, reversible binding of adhesive proteins to their respective receptors and the need to "activate" the receptor or adhesive protein. In the case of GP IIb/IIIa, platelet activation causes a conformational change in the receptor switching it from a quiescent, non-binding form into an activated binding form (46). For vWF, an adjunct factor such as shear stress or ristocetin is required to promote an interaction with GP Ib, although vWF can also bind to activated GP IIb/IIIa (48, 49).

Factor V

In addition, activated platelets accelerate thrombin generation by providing a surface that promotes two procoagulant reactions, conversion of factor X to Xa and production of thrombin from prothrombin (2, **8**). These prohemostatic reactions are performed by homologous, membrane-bound, Ca^{2+}-dependent complexes: the tenase complex, consisting of the serine protease IXa and the nonenzymatic cofactor FVIIIa (50), and the prothrombinase complex, composed of the serine protease factor Xa and the nonenzymatic cofactor FVa (50). Furthermore, activated platelets also control at least one anticoagulant reaction, inactivation of factor Va by activated protein C (51). Approximately 20% of factor V contained in whole blood is found in the α-granules of platelets (52) and can be secreted after platelet activation (53). There is clinical and experimental evidence suggesting that platelet-derived FV plays a critical role in maintaining physiologic hemostasis. Factor V Quebec was originally described as an autosomal dominant bleeding disorder characterized by mild thrombocytopenia, fully functional plasma FV but defective platelet FV (54), suggesting that platelet-derived FV might be more important than plasma-derived FV. This concept is reinforced by the description of two patients with acquired inhibitors of FV. A patient with non-Hodgkin's lymphoma and gastrointestinal bleeding was found to have a FV inhibitor directed against both plasma and platelet FV (55), whereas a second patient with a neutralizing inhibitor active only against plasma-derived FV presented no bleeding tendency, despite surgical challenge (56). In addition, it has recently been shown that platelets can protect platelet-derived but not plasma-derived FV from proteolytic

inactivation by APC (57). Together, these observations indicate that platelet-derived, membrane-bound FV has a pivotal role in promoting and maintaining hemostasis at sites of vascular damage. The ability of platelets to sustain assembly and activity of the tenase and prothrombinase complexes depends on the type of agonist used (58, 59), and this correlates with the agonists' ability to induce expression of negatively charged membrane phospholipids (60). The most effective agonists are the Ca^{2+} ionophore A23187, the complement membrane attack complex C5b-9, and the combined stimuli of collagen and thrombin (58, 59). Therefore, the most important physiologic stimulus able to induce procoagulant activity at sites of endothelial damage would be the combined action of thrombin and collagen (58).

Transglutaminases

Transglutaminases (TG) catalyze intermolecular cross-linking of proteins by formation of an amide bond between a glutamine residue on one protein and a lysine residue on another (61). Several TG genes have been cloned from keratinocytes, epidermus, prostate and endothelium (62). For the purposes of this thesis, two TG isoforms are particularly relevant: type II TG is referred to as tissue TG and is present in the cytosol of erythrocytes, endothelial cells, and hepatocytes; the second form is factor XIII involved with coagulation (62, 66). While there are many uncertainties regarding the function of TG enzymes, particularly the cytoplasmic forms, hepatocytes and endothelial cells are documented to have TG on their extracellular surface (63).

Two transglutaminase activities have been described in human platelets. One is regulated by calmodulin and is rather resistant to thiol reagents (63). Its role in platelet function is unknown, and it is assumed to be a type II enzyme. The second activity is factor XIII. Fifty percent of all FXIII A chains present in blood are located in the cytoplasm of platelets (64). While plasma FXIII is activated by thrombin mediated cleavage of a 4500 Da peptide from the amino terminus of the A chain (65, 66), A chains in the platelet cytoplasm are fully active once they encounter calcium levels sufficient to facilitate catalysis. The substrate specificity of FXIII for glutamine (gln, Q) donors as well as lysine (lys, K) acceptors has been extensively examined; Fbg, Fn, α2-AP, FV, Tsp and vWF are all known to serve as either Q donors or K recipients for FXIII (65, 67).

The role of platelet FXIII A chain is not well understood. While over 90% of this A chain is present in the cytoplasm and does not appear to be secreted during platelet activation, early studies indicated that control platelets could normalize clotting of FXIII deficient plasma (64). Furthermore, activation of gel filtered platelets results in FXIII-like cross-links between α_2-AP and Fbg (102) as well as between FV and actin (68). These apparent contradictions have not been resolved, although two reports which indicate that platelets also have a pool of α-granule FXIII, (69, 70) may provide some insight. Alpha granule FXIII is reported to be A2B2 tetramers (70), possibly incorporated into α-granules by endocytosis similar to other plasma proteins (71). In addition, binding of FXIIIa to activated platelets has been described in several reports (72-74).

Platelet FcγIIA receptors

The platelet low-affinity Fc receptor (FcγRIIA or CD32) is a single-chain integral membrane glycoprotein of 40 kd present at about 1500 copies per cell (**8**, 75). It is the only Fc receptor found on platelets. Soluble immune complexes and certain platelet-activating antibodies are able to activate platelets through Fc receptor engagement (75), and numerous autoimmune disorders associated with circulating immune complexes or antiplatelet autoantibodies exhibit vascular lesions and thrombotic complications (76-78). Anti-platelet antibodies that activate platelets are thought to bridge their respective antigens and Fc receptors (79-82), whereas immune complexes may activate platelets by directly cross-linking Fc receptors (83). One of the antigens commonly involved in antibody-dependent platelet activation in the laboratory is CD9, a member of the tetraspanin family of transmembrane proteins (84). With either method of stimulation, tyrosine phosphorylation of the platelet Fc receptor appears to be an early signaling event, followed by PLCγ2 activation and calcium mobilization (85, 86). The ability of agonists other than thrombin and collagen/convulxin to promote COAT-platelet formation has not been investigated, but it has been assumed that receptors that recapitulate the same intracellular messengers as seen with collagen and thrombin might allow COAT-platelet formation. Because the glycoprotein VI collagen receptor shares several signaling pathways with the platelet Fc receptor (87), it was logical to examine the ability of Fc receptors to promote COAT-platelet production.

MAIN OBJECTIVES AND SPECIFIC AIMS

Platelets co-stimulated with collagen and thrombin reveal a sub-population of cells which express high levels of surface-bound, functional factor V. This sub-population represents ~30% of all platelets and is referred to as COAT-platelets. While the exact role of COAT-platelets in hemostasis is yet to be determined, this thesis suggests that COAT-platelets offer a new mechanism for providing concentrated, pro-hemostatic factors at a site of maximal platelet activation. To produce COAT-platelets, it is hypothesized that coincident α-granule secretion, aminophospholipid exposure and transglutaminase availability are required. The following will address the exact mechanism of COAT-platelet formation:

1. Additional proteins on the surface of COAT-platelets:
COAT-platelets will also express von Willebrand factor, fibrinogen, α_2-antiplasmin, thrombospondin, and fibronectin on their surface. COAT-platelets are observed upon dual activation with thrombin plus collagen type I, type V, type VI or convulxin, an agonist specific for the collagen receptor glycoprotein VI; however, no single agonist examined will able to produce COAT-platelets.

2. The role of microparticles:
In order to define the relative contributions of platelets and platelet-derived microparticles to the observed procoagulant activity, the level of residual procoagulant activity after separation of platelets and microparticles by centrifugation will be assessed.

3. Transglutaminase activity and COAT platelet formation:
COAT-platelet formation, as monitored by FV, vWF, α_2-AP, Fbg or Fn binding, will inhibited by dansyl cadaverine, putrescine and acetyl-casein, all inhibitors of transglutaminases

4. Fibrinogen on COAT-platelets:
The binding of Fbg to dual-agonist stimulated platelets will be investigated to determine if Fbg is bound to the GP IIb/IIIa receptor on COAT-platelets

5. Formation of COAT-platelets in platelet rich plasma:

Studies detailed above have utilized gel-filtered platelets, an experimental system which has many advantages but does not reflect the natural environment for platelets. Therefore we will investigate whether COAT-platelets can be formed in PRP upon activation with two agonists.

6. FcRT- platelets:

The collagen receptor GP VI shares several signaling pathways with the Fc receptor (FcγRIIa; CD32) present on human platelets. As a result, the ability of FcγRIIa engagement to facilitate generation of COAT-platelets will be tested.

7. Identification of casein peptide cross-links in COAT-platelets:

Preliminary data indicate that a fluorescently labeled β-casein derived peptide (Fl-CP_{15}) is coupled to a small, hydrophobic molecule in the platelet membrane during COAT-platelet formation. This hydrophobic anchor will be identified with a combination of enzymatic digestion, reverse phase HPLC and mass spectrometry.

8. Identification of membrane attachment site for transglutaminase reaction:

The reactivity of biotinylated-CP_{15} with COAT-platelets offers an opportunity to determine if a membrane protein was serving as the lysine acceptor for transglutaminase immobilization of the α-granule born proteins. The advantage of a low molecular weight marker like biotinylated-CP_{15} is that it would not significantly affect the SDS-PAGE migration of the membrane protein to which it was coupled. However, SDS-PAGE/Western blot analysis of biotin-CP_{15} derivatized COAT-platelets did not reveal any protein labeled with biotin. As a result of this negative Western blot experiment, the possibility that a small molecule is serving as the anchor for CP_{15} will be investigated.

MATERIALS AND METHODS

Materials

Sepharose CL-2B, Fc-specific goat-anti-mouse-IgG (±FITC), goat-anti-human-IgG, L-cysteine, iodoacetic acid, ficin, bovine thrombin, bovine casein, dansylcadaverine, 5-HT, 5-hydroxyindoleacetic acid, goat antihuman fibrinogen, monoclonal-anti-fibrinogen (85D4), rabbit-antihuman von Willebrand factor, rabbit-antihuman fibronectin, goat-antihuman albumin, guinea-pig liver transglutaminase, Gly-Pro-Arg-Pro-amide, A23187, BSA, MES and HEPES buffers were purchased from Sigma Chemical Corp (St Louis, Mo).

Anti-CD32 mAb (IV.3) was obtained from Medarex Inc (Annandale, NJ). Anti-CD9 mAbs (ALB-6 and ML-13) were from Immunotech (Marseille, France) and PharMingen (San Diego, CA), respectively. PE-labeled streptavidin was provided by Molecular Probes (Eugene, OR). PE-labeled annexin-V was purchased from PharMingen (San Diego, CA). Thiazole orange was from Becton Dickinson (San Jose, CA). Convulxin was purified as previously described (4). Monoclonal Ab (mAb) HFV-237 against the light chain of human factor V was a gift of Dr C.T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK), mAbs S12 and G5 recognizing P-selectin and TAB, raised against glycoprotein IIb/IIIa was provided by Dr R.P. McEver (University Oklahoma Health Sciences Center, Oklahoma City, OK). Monoclonal Ab ED4H1 against α2-AP was a gift from Dr. P.A. McKee (University of Oklahoma, Health Sciences Center, Oklahoma City, OK), LIBS-6 a monoclonal antibody that recognizes glycoprotein IIb/IIIa with bound ligand (104) was from Dr. M.H. Ginsberg (Ariad Pharmaceuticals Inc., Cambridge, Massachusetts), sheep polyclonal against human FXIII was obtained from Biodesign International (Kennebunk, ME), rabbit-anti-human-thrombospondin Ab was obtained from CalBiochem, (La Jolla, CA), monoclonal Ab 9C11, inhibiting thrombin activation of factor XIII (101), monoclonal Ab CUB7402 and AB-1 against tissue transglutaminases and monoclonal Ab C6.7 against thrombospondin were purchased from Neomarkers (Fremont, CA). R2 a purified, polyclonal antibody raised against plasma factor XIII that inhibits the transglutaminase activity of factor XIIIa (102) was a generous gift of Dr. G.L. Reed (Harvard School of Public Health, Boston, MA); goat antihuman factor XIII for flow cytometry was from Biodesign International; rabbit antihuman thrombospondin was

from Calbiochem (San Diego, CA). SU732 (1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride; compound I) (100) and DMP-802 a glycoprotein IIb-IIIa inhibitor (105) was a generous gift of Dr. A. Stern (DuPont Pharmaceuticals Company, Wilmington, DE).

Acetylated casein was produced by a published procedure (88). Fab2 ragments of HFV-237 and S12 were prepared by incubating 1 mg of biotinylated antibody in PBS with 7.5 mmol/L cysteine and 1:50 ratio (wt/wt) of ficin for 90 minutes at 37°C (89). Iodoacetic acid (15 mmol/L) was added to inhibit further proteolysis. Digests were dialyzed against PBS, and Fc fragments, as well as undigested antibodies, were removed with a gammaBind G Sepharose column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Conjugation of anti-fibrinogen and anti-von Willebrand factor antibodies with FITC was performed as previously described (89).

Buffers

ACD consisted of 38.1 mmol/L citric acid, 74.8 mmol/L Na_3 citrate, and 136 mmol/L glucose. BSGC consisted of 129 mmol/L NaCl, 13.6 mmol/L Na_3 citrate, 11.1 mmol/L glucose, 1.6 mmol/L KH_2PO_4, and 8.6 mmol/L NaH_2PO_4 pH adjusted with NaOH to 7.3. PBS consisted of 150 mmol/L NaCl and 10 mmol/L NaH_2PO_4, pH 7.4. Saline consisted of 150 mmol/L NaCl. HEPES consisted of 100 mmol/L HEPES, pH 7.5. HEPES/saline consisted of 10 mmol/L HEPES and 140 mmol/L NaCl, pH 7.5.

Preparation of human gel-filtered platelets

Informed consent was obtained from donors in accordance with local institutional review board guidelines. Five milliliters of whole blood was drawn from the cubital vein into 0.5 mL of ACD with a 20-gauge needle and a plastic syringe. PRP was prepared immediately with a 1:2 dilution of whole blood with room temperature (RT) BSGC, pH 7.3, and centrifugation in 12×75 mm plastic tubes at 170g for 8 minutes at room temperature. Gel filtration of platelets was performed by layering 2 mL of PRP onto a 25×60 mm (30 mL) column of Sepharose CL-2B equilibrated with saline. GFPs were quantitated with a System 9000 cell counter from Serono-Baker Diagnostics (Allentown, PA) and normalized to a concentration of 4×10^7 platelets/mL in BSGC, pH 7.3.

Platelet activation for flow-cytometric studies

Reactions were performed in 12×75 mm round-bottom plastic culture tubes. GFPs were activated in 100 μL reactions containing 40 μL of HEPES/saline with 5 mmol/L $CaCl_2$ and 2.5 mmol/L $MgCl_2$, 40 μL of HEPES/saline with 1 mg/mL BSA, 10 μL of GFPs (4×10^7 platelet/mL), appropriate mAb, and 10 μL of agonist. For inhibitory studies, 1 mg/mL acetylated casein or 200 μmol/L dansylcadaverine was added immediately before activation. After 10 minutes at 37°C, the reaction was stopped with 200 μL of 1.5% formalin (wt/vol) in HEPES/saline, and platelets were fixed for 20 minutes at room temperature. Four milliliters of 1 mg/mL BSA in PBS (BSA/PBS) was added, and samples were centrifuged at 1500g for 15 minutes. The pelleted platelets were resuspended in 200 μL of BSA/PBS and stained with the appropriate secondary reagents. After 30 minutes at room temperature, platelets were washed with 4 mL of BSA/PBS, centrifuged at 1500g for 15 minutes, and resuspended in 350 μL of BSA/PBS for flow cytometry.

Platelet activation with convulxin plus thrombin

Gel-filtered platelets were activated in 100 μl reactions containing 40 μl of HEPES/saline with 5mM $CaCl_2$ and 2.5 mM $MgCl_2$; 40 μl of HEPES/saline with 1 mg/ml BSA, 10 μl of agonist, inhibitor and/or antibody, and 10 μl of gel-filtered platelets; standard convulxin and thrombin concentrations were 500 ng/ml and 5nM final, respectively. After 10 min of activation at 37 ^{o}C, 200 μl of 1.5% (wt/vol) formalin in PBS was added to stop the reaction and fix the platelets. After 20 min of fixation at room temperature, 4 μl of 1 mg/ml BSA in PBS were added, and the samples were centrifuged at 1,500g for 15 min. We resuspended platelets in 200 μl of BSA/PBS and stained them with the appropriate secondary reagents. Several antibodies were included during platelet activation, including anti-FV (HFV237), anti-α_2-AP (ED4H1), LIBS-6 and anti-tissue transglutaminase (CUB-7402). Other antibodies were incubated with platelets after activation and fixation. For inhibition studies, we pre-incubated platelets with inhibitory antibodies against factor XIII (9C11 or R2) for 15 min at room temperature before platelet activation.

Platelet activation by cross-linking FcγRIIA

Preliminary studies demonstrated that 1 μg/mL IV.3 completely saturated FcγRIIA receptors, as determined by incubating resting platelets with increasing concentrations of IV.3 for 20 minutes at RT and detecting bound antibody with FITC-labeled GAMG. No α-granule release was ever observed by coating platelets solely with IV.3. In a separate set of preliminary experiments, platelets coated with 1 μg/mL IV.3 were incubated for 10 minutes at 37°C with increasing concentrations of Fc-specific GAMG. The GAMG concentration resulting in maximal platelet activation, as detected by P-selectin expression, was thereby determined. For FcRT-platelet experiments, GFPs were preincubated with 1 μg/mL IV.3 for 20 minutes at RT along with 15 μg/mL B-(Fab)237 or B-(Fab)S12. Platelets were then activated by cross-linking IV.3 with 40 μg/mL Fc-specific GAMG plus 5 nmol/L thrombin for 10 minutes at 37°C. After fixing and washing, samples were stained for flow cytometry as detailed above.

Platelet activation with anti-CD9 antibodies

Preliminary experiments were performed to characterize the platelet-activating properties of ALB-6 and ML-13. By using graded concentrations of the two mAbs, maximal exposure of P-selectin was observed with 10 μg/mL ALB-6 or ML-13. Gel-filtered platelets, along with biotinylated HFV- 237 or biotinylated S12, were activated with 5 nmol/L thrombin plus either 10 μg/mL ALB-6 or ML-13 for 10 minutes at 37°C. After fixing and washing, platelets were stained for surface-bound P-selectin, factor V, fibrinogen, or von Willebrand factor, as described above.

Platelet activation in PRP and in whole blood

Five milliliters of whole blood were drawn into ACD and diluted 1:2 with HEPES/saline. One millimole per liter of Gly-Pro-Arg-Pro was added to inhibit fibrin polymerization, along with 1 μmol/L DMP802 to prevent glycoprotein IIb/IIIa-mediated platelet aggregation. A portion of the blood was centrifuged in 12×75 mm plastic tubes at 170g for 8 minutes at room temperature to produce PRP. The remainder of the anticoagulated whole blood was diluted 1:14 in HEPES/saline along with 1 mmol/L Gly-Pro-Arg-Pro and 1 μmol/L DMP802. Both PRP and whole blood were activated in the presence of 10 μg/mL biotinylated HFV-237, 8 mmol/L $CaCl_2$, and 4 mmol/L $MgCl_2$ with 5 nmol/L thrombin plus either 500 ng/mL convulxin or 10 μg/mL ALB-6 for 10 minutes at 37°C. For FcγRIIA crosslinking experiments, diluted whole blood or

PRP was preincubated with 1 μg/mL IV.3 for 20 minutes at RT, and platelets were then activated by cross-linking with 40 μg/mL Fc-specific GAMG plus 5 nmol/L thrombin for 10 minutes at 37°C. For inhibitory studies, 1 mg/mL acetylated casein or 200 μmol/L dansylcadaverine was added before activation.

Reticulated platelet studies

Thiazole orange, a fluorescent dye that binds to remnant RNA present in reticulated platelets, was used to allow identification of the youngest platelets in the circulation (90-92). Platelets were activated and processed as described above, except that an additional staining step was introduced. After staining and washing with secondary reagents, platelets were resuspended in 500 μL of TO reagent, as provided by the manufacturer, and incubated for 30 minutes at room temperature before flow cytometry.

Detection of exposed aminophospholipids

Annexin-V was used as a probe for aminophospholipid exposure (93). Gel-filtered platelets were activated in the presence of PE-labeled annexin-V, as detailed previously. After a 10-minute incubation at 37°C, the 100 μL reaction mix was diluted with 500 μL of HEPES/ saline containing 5 mmol/L $CaCl_2$ and 2.5 mmol/L $MgCl_2$ and promptly assayed by means of flow cytometry. In dual labeling experiments PE-labeled annexin-V and FITC-labeled anti-fibrinogen or FITC-labeled anti-von Willebrand factor were used. After 10 minutes of incubation at 37°C, the reaction mix was diluted with 500 μL of BSA/PBS with 5 mmol/L $CaCl_2$ and 2.5 mmol/L $MgCl_2$ (3). Flow-cytometer parameters were set to avoid crossover fluorescence between FL1 (FITC-labeled anti-fibrinogen, FITC-labeled anti-von Willebrand factor) and FL2 (PE-labeled annexin-V).

Casein peptide

A 15mer peptide (SVLSLSQSKVLPVPE; CP_{15}) representing residues 161-175 of β-casein (94) was synthesized by the Molecular Biology Resource Facility of the University of Oklahoma, Health Sciences Center. The peptide was produced with its N-terminus either biotinylated (biotin-CP_{15}), fluorescein labeled (fl-CP_{15}) or underivatized (CP_{15}). Acetylated casein was prepared by published procedures (94).

CP_{15} adduct analysis

Gel-filtered platelets ($2x10^9$ platelet/ml) were activated with A23187 plus thrombin in the presence of 50 μM Fl-CP_{15}; five volumes of methanol were added to stop the reaction, and the sample was centrifuged at 2000g for 15 min. The supernatant was diluted with water to achieve 10% (v/v, final) methanol, and was applied to a 10x25 mm C-4 column (Vydac) at a flow rate of 5 ml/min. The column was eluted at 1 ml/min with a 10 to 50% gradient of acetonitrile in 10mM triethylamine/acetic acid (pH~7). One ml fractions were collected and analysed for fluorescence (485 nm excitation/530 nm emission) with a Biotek FL600 fluorometer. Two product peaks (P1 and P2) were identified and the relevant fractions were applied to a SPE/C-4 desalting column (J. T. Baker) and eluted with 50% acetonitrile in water. The fractions were then lyophilized.

Electrospray mass spectrometry was performed with a Sciex QSTAR hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems) equipped with an articulated ion spray source operated in the positive ionmode. Two μl of sample dissolved in 0.5% acetic acid in methanol/water (1:1) was loaded into a nanospray capillary, which was then mounted in the nanospray source (MDS Protana). The capillary was opened and positioned 2 mm from the orifice of the mass spectrometer. A potential of 1.375 V was applied to the needle to initiate the nanospray process. Samples were scanned over an m/z range of 400 to 3000 Da, with a step size of 0.4 Da and a dwell time of 1.5 ms per step.

Synthesis of serotonin adducts

Biotin-CP_{15} and Fl-CP_{15} (400 μM) were incubated with 10 mM serotonin, 5 mM $CaCl_2$, 0.1 mM dithiothreitol, 200 mM HEPES (pH 7.5) and 0.2 mU/ml guinea-pig liver tissue transglutaminase for 30 min at 37 ^oC. This reaction mixture was extracted with methanol and purified over HPLC as outlined above. We have synthesized BSA-(5-HT)$_n$ by reacting BSA (5 mg/ml) in 25 mM serotonin, 200 mM MES (pH 5.8) with 20 mM ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) for 3 h at 37 ^oC. We dialysed the product against HEPES/saline (pH 7.5), and measured the absorbance at 275 nm (A_{275}) to calculate the level of 5-HT substitution. BSA-(5-hydroxyindoleacetic acid)$_n$ was synthesized similarly.

RESULTS

Platelet activation with thrombin and convulxin induces high levels of factor V surface expression in a discrete fraction of platelets

Thrombin and convulxin, a specific agonist for the collagen receptor GP VI (4), are able to promote surface expression of α-granule FV. Figure 1 shows the capacity of thrombin (panel A) or convulxin (panel B) as single agonists to induce expression of FV on the platelet surface. The 5 nM thrombin and 500 ng/mL convulxin employed in this experiment are well above the concentrations required to induce maximal α-granule degranulation as detected with a monoclonal antibody (mAb) against P-selectin (92,93). Panel C shows that dual stimulation with both thrombin and convulxin in the presence of both Mg^{2+} and Ca^{2+} results in a dramatically different pattern of FV distribution. A fraction of the platelets express very high levels of factor V (region M2), while the remainder still express factor V but at lower levels than that observed with convulxin alone (region M1 minus M2; referred to as low-level FV). Those cells with the high level of surface FV expression, shown in region M2, are identified as COAT-platelets; the mean level of COAT-platelets in 10 normal individuals was 30.7 ± 4.7 % (± 1SD).

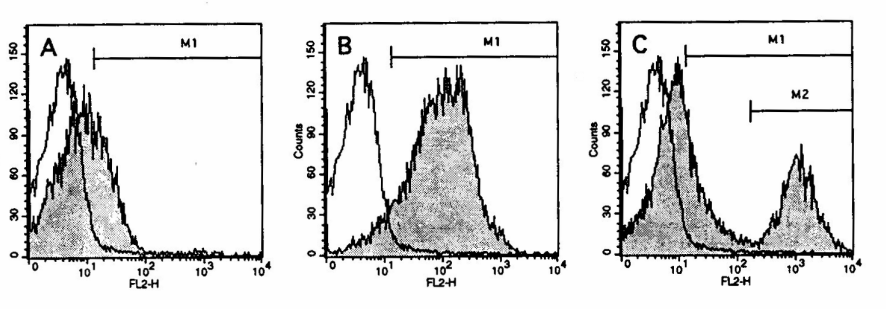


Figure 1 - Factor V binding to activated platelets
Platelets were activated with 5 nM thrombin (panel A), 500 ng/mL convulxin (panel B) or thrombin plus convulxin (panel C). Surface bound FV was detected immunologically (FL_2). Line histograms are control platelets; shaded histograms are stimulated cells. Region M1 represents all cells binding FV; region M2 represents cells binding very high levels of FV, referred to as COAT-platelets.

Costimulation of platelets with ionophore plus thrombin or ionophore plus convulxin also generates a COAT-platelet population, although with these nonphysiologic agonist combinations essentially all platelets express high levels of surface bound FV. Figure 2 summarizes the ability of single agonists to express α-granule FV on the surface of activated platelets. Although these individual agonists are capable of stimulating FV expression on up to 80% of all platelets, none of them generate COAT-platelet (Figure 2A). On the other hand, the combined action of thrombin and convulxin results in a lower overall level of FV-positive events, even though this dual stimulation results in COAT-like FV expression (Figure 2B). Furthermore, when thrombin is maintained at 5 nmol/L, there is a clear dose-response to convulxin for generation of COAT-platelet (Figure 2B). When the convulxin concentration is fixed at 500 ng/mL, there is a dose-dependent increase in COAT-platelet formation between 0.1 and 1 nmol/L thrombin, whereas with higher thrombin concentrations, the percentage of COAT-platelet remains constant (data not shown).

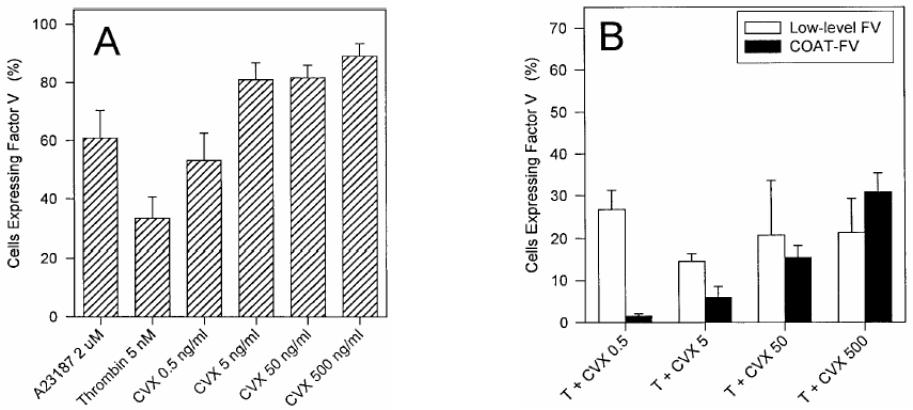


Figure 2 - Analysis of surface FV expression elicited by various agonists
Platelets were stimulated with various agonists at the concentrations depicted on the abscissa. In Panel A single agonists were used similar to experiments in Figure 1; the percentage of cells with surface FV is shown on the ordinate (mean ± 1 SD; n = 3-8). In Panel B, dual agonist stimulation was performed with thrombin held constant at 5 nmol/L and convulxin varied from 0.5 to 500 ng/mL. Two parameters are reported: COAT-platelets and low-level FV corresponding to region M2 and region M1 minus M2, respectively (Fig. 1). Note that in Panel B the percentage of cells with low-level binding remains relatively constant, whereas the number of cells with COAT-platelets increases with increasing convulxin concentration (n = 3-12).

COAT-platelets can also be generated by combined activation of thrombin and collagen. Table 1 summarizes the results obtained with collagen types I, III, V and VI. Utilizing all collagens at 20 μg/mL, a concentration which induces maximal α-granule degranulation (96), types I, V and VI are able to promote COAT-platelet formation to varying degrees while collagen type III only induces low-level FV expression.

Collagen type	Low-level factor V	COAT-platelet
I	14.5 ± 5.6	36.4 ± 6.9
III	45.7 ± 13.8	-
V	19.4 ± 7.5	30.9 ± 9.6
VI	10.1 ± 3.9	61.6 ± 6.2

Table 1 - Effect of dual activation with thrombin and collagen on factor V expression on platelets
To activate human platelets, 5 nmol/L thrombin and 20 µg/mL of collagen were used. Surface-bound FV was detected with monoclonal antibody HFV-237 (see "Materials and methods"), and the percentage of platelets expressing FV was then determined by flow cytometry. COAT-platelets are defined in Figure 2 as events in region M2; low-level FV represents cells in region M1 minus those in region M2 of Figure 2. Data represent mean ± 1 SD, n = 4.

The exposure of negatively charged membrane phospholipids parallels the expression of COAT-platelets but is not sufficient for its generation

The generation of COAT-platelets in response to thrombin plus collagen (or convulxin) is restricted to a subpopulation of platelets similar to previous reports on the exposure of aminophospholipids by activated platelets (60). To investigate whether these events might be associated, exposure of negatively charged membrane phospholipids upon platelet activation was monitored with fluorochrome-conjugated annexin V. Figure 3 shows representative flow cytometric dot plots of annexin-V binding promoted by ionophore A23187 and the combined action of thrombin plus convulxin. Ionophore resulted in essentially all platelets binding annexin V while thrombin plus collagen generated only a sub-population of annexin-positive cells. Two color flow cytometry demonstrated that the annexin-positive and COAT-platelet populations were one in the same.

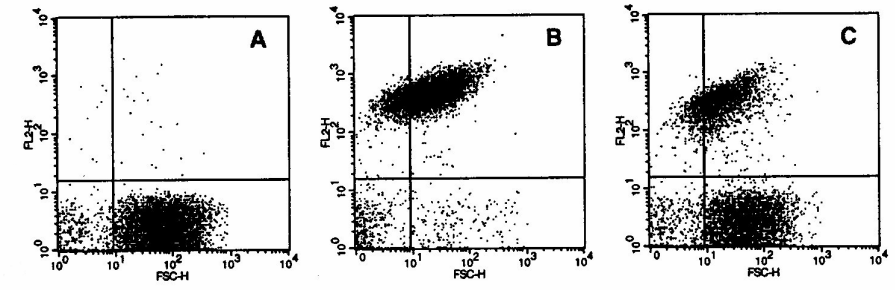


Figure 3 - Annexin V binding to stimulated platelets.
Platelets were stimulated with either 2 µM A23187 (panel B) or 5 nM thrombin plus 500 ng/mL convulxin (panel C) in the presence of PE-labeled annexin V (FL_2). Panel A represents resting platelets. Note that thrombin plus convulxin results in only a fraction of the platelets binding annexin V.

Results from several individuals are represented in Figure 4. No single agonist except for A23187 elicits significant numbers of annexin-positive cells; however, even though ionophore elicits annexin-positive cells, it does not produce COAT-platelets. On the other hand, dual stimulation of platelets with 5 nM thrombin and increasing concentrations of convulxin elicited significant levels of annexin binding. A combination of these data with earlier data on COAT-platelet formation (3) indicate a close relationship between these two endpoints (Figure 4 inset).

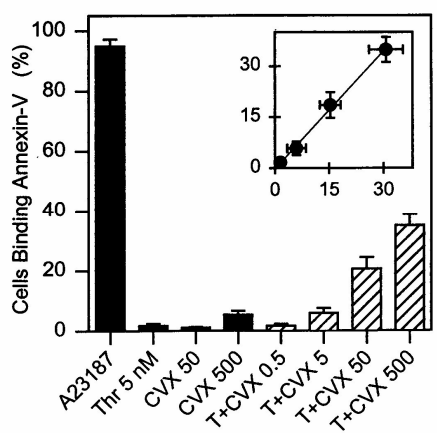


Figure 4 - Annexin V binding to platelets stimulated with single or dual agonists
Platelets were activated with the agonists indicated on the abscissa, and the binding of PE-labeled annexin V was monitored. All convulxin (CVX) concentrations represent ng/mL and T indicates 5 nM thrombin. The inset plots the percentage of cells binding annexin (ordinate) and the percentage of COAT-platelets observed (abscissa) for each of the thrombin and convulxin combinations shown in the hatched bars of the main graph (n=6).

Both exposure of aminophospholipids and COAT platelet expression are increased among reticulated platelets

Since only a portion of platelets express both high levels of FV and negatively charged membrane phospholipids in response to the combined action of thrombin and convulxin, the impact of platelet age was determined. To this purpose, thiazole orange, a fluorescent dye which binds to the remnant RNA still contained in reticulated platelets was employed to allow identification of the youngest platelets in the circulation (87-89). Figure 5 shows the exposure of negatively charged membrane phospholipids in response to 5 nM thrombin and 500 ng/mL convulxin for a representative experiment. When TO-negative (i.e., older) platelets are examined, 24.2 ± 7.0 % (mean ± 1SD; n=6) of the cells bind annexin-V versus 73.1 ± 4.5 % ($p < 0.001$) for the TO-positive (younger) platelets. Similarly, the percentage of COAT-platelets is enriched among the reticulated platelets: only 12.6 ± 3.1 % (n=5) of TO-

negative platelets become COAT-platelets in response to thrombin plus convulxin versus 65.6 ± 6.3 of the TO-positive platelets ($p < 0.001$).

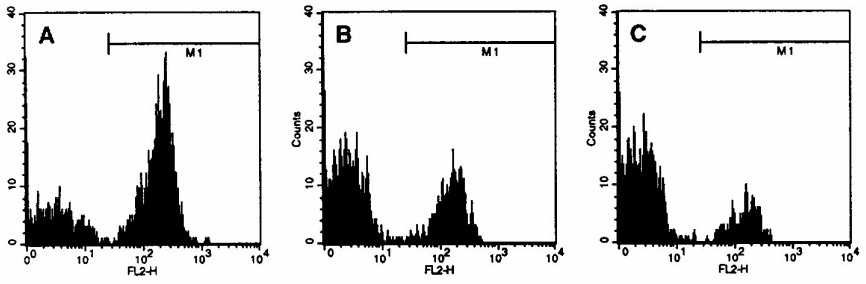


Figure 5 - Annexin V binding to TO+ and TO- platelets stimulated with thrombin plus convulxin
Platelets were stimulated with thrombin plus convulxin and then stained with an anti-FV mAb and thiazole orange. Panel A: TO+ platelets; Panel B: entire population; Panel C: TO - platelets.

Factor V peak surface expression is functionally relevant

In order to assess the functional relevance of COAT-platelets, three different approaches were utilized. First, all COAT-platelets bind exogenous FXa (Table 2), a requirement for functional prothrombinase. Second, the combined activation by thrombin plus convulxin was a more potent inducer of platelet FV-activity than is any single agonist examined (3). Third, the ability of different platelet agonists to promote platelet-dependent prothrombin activation was quantitated (Figure 6). Gel filtered platelets were activated for 10 minutes with the agonists indicated, prothrombin and FXa were then added, and the initial rate of prothrombin activation was determined. Convulxin stimulated the platelet activity in a concentration-dependent fashion although the combination of thrombin plus convulxin generated more prothrombinase activity than would be predicted for a simple summation of the individual agonists (Figure 6).

Platelets	FXa positive	FL1
Control	0.8 ± 0.4	1.7 ± 0.3
CVX	19.5 ± 1.7	4.8 ± 1.1
C + T (low level)	18.5 ± 1.0	4.4 ± 1.0
CVX + T (COAT)	82.8 ± 5.9	20.1 ± 4.8

Table 2 - FV peak surface expression is functionally relevant
Plateletes were activated with 500 ng/mL convulxin (CVX or C) alone or in combination with 5 nM thrombin (T) in the presence of 5 nM FXa, FITC-anti-HFXa and biotin-anti-HFV237. Cells with surface bound FV were identified as indicated in Figure 1. The % of cells positive for FXa as well as their mean FL_1 fluorescence is presented (n=3).

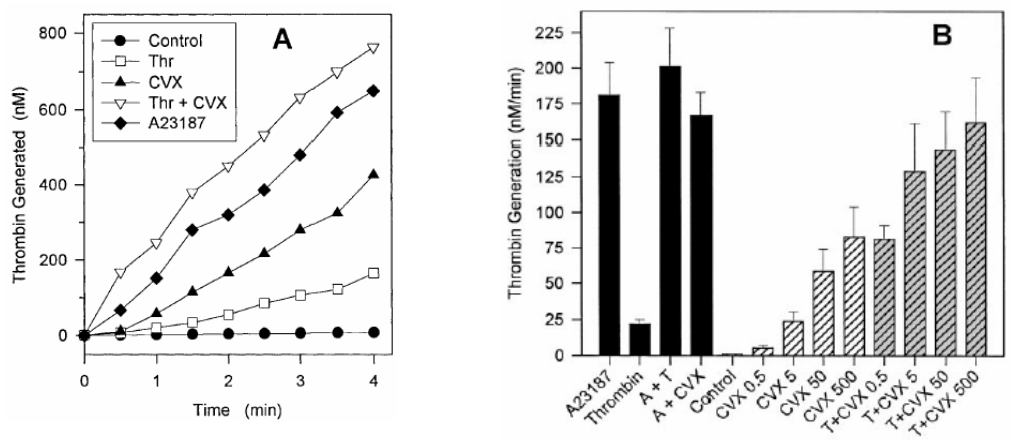


Figure 6 - Prothrombinase activity generated by single and dual agonists
GFPs were stimulated with various single- and dual-agonist combinations for 10 minutes as described in "Materials and methods." Platelets were then added to exogenous FXa and prothrombin, and the initial rate of prothrombin activation was determined. Panel A depicts a representative experiment and demonstrates that the initial rates of thrombin generation were linear for up to 4 minutes. Agonist concentrations were 5 nmol/L thrombin, 500 ng/mL convulxin, and 2 µmol/L A23187. Panel B represents the prothrombinase activity (nmol/L thrombin generated per minute) for various agonists. Ionophore (A) was 2 µmol/L; thrombin (T), 5 nmol/L; and convulxin (CVX) concentration was 500 ng/ml unless specifically designated (ng/mL) otherwise. Bars represent mean 6 ± SD; n = 3.

The role of microparticles

In order to define the relative contributions of platelets and platelet-derived microparticles to the observed procoagulant activity, the level of residual procoagulant activity after separation of platelets and microparticles by centrifugation was assessed (51). At any given time the relative contribution of microparticles to the measured procoagulant activity was less than 20% for thrombin plus convulxin; while the contribution of microparticles for A23187 induced prothrombinase activity reaches 40%. These data plus additional experiments (3) indicate that microparticles are not a significant factor in the prothrombinase activity of COAT-platelets.

Additional α-granule proteins on the surface of COAT-platelets

COAT-platelets were initially observed to have high levels of FV (3); subsequently a number of additional α-granule proteins were observed to be expressed in a similar manner. Figure 7 demonstrates the presence of Fbg, vWF, Fn, α2-AP and Tsp on thrombin plus convulxin activated platelets. In each instance, a fraction of platelets, similar to that observed with anti-FV antibodies, demonstrates increased levels of these proteins. Dual color flow cytometry indicated that FV and these other adhesive proteins

are present on the same sub-population of cells (data not shown). Antisera against IgG and albumin, also α-granule proteins, did not exhibit this "COAT-like" population upon dual stimulation (Figure 7). It is worth noting that all of the adhesive and procoagulant proteins identified above (FV, Fbg, Fn, vWF, α2-AP, Tsp) are substrates for transglutaminases (65).

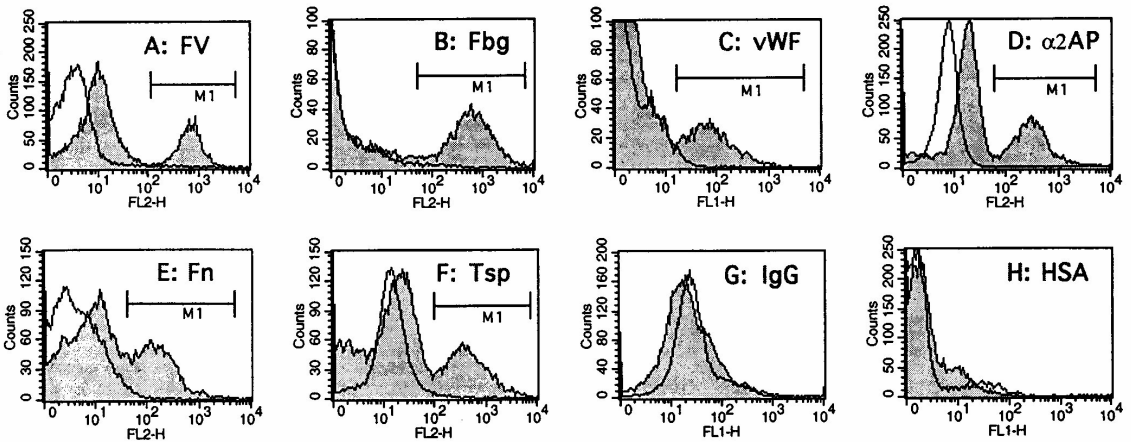


Figure 7 - Binding of additional α-granule proteins to the surface of COAT-platelets
GFPs were activated with convulxin and thrombin for 10 min at 37°C. After formalin fixation and washing, cells were stained with 8 different antibodies. For each antibody, control cells (line histograms) and activated cells (shaded histograms) are shown. Six of the antibodies demonstrate a "COAT-like" peak (region M1) which is inhibited by dansyl cadaverine (see next section); only anti-IgG and anti-HSA do not have this peak. FV: factor V; Fbg: fibrinogen; vWF: von Willebrand factor; α2-AP:α2-antiplasmin; Fn: fibronectin; Tsp: thrombospondin; HSA: human serum albumin.

Transglutaminase activity and COAT platelet formation

Since FV, Fbg, Fn, vWF, α_2-AP and Tsp are all substrates for transglutaminases (65, 97), the ability of transglutaminase inhibitors to affect COAT-platelet formation was investigated. Figures 8 and 9A demonstrate the effect of dansyl cadaverine, a competitive amino acceptor for transglutaminases (67), on the formation of FV-enriched COAT-platelets; a concentration-dependent inhibition was observed. In addition, dansyl

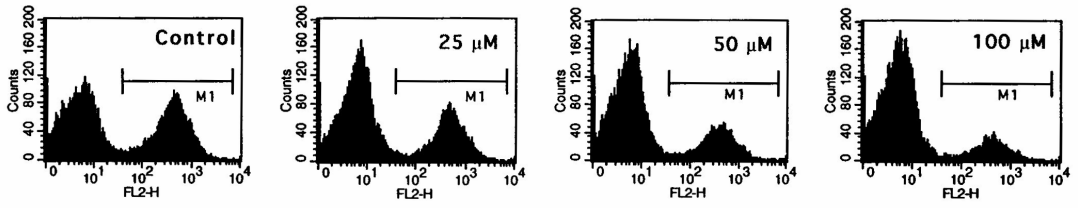


Figure 8 - Inhibition of COAT-platelet formation by dansyl cadaverine
Platelets were stimulated with convulxin and thrombin in the presence of increasing concentrations of dansyl cadaverine (25-100 μM). Surface-bound FV was stained as in Figure 1. Note the decrease in M1 peak as dansyl cadaverine concentration increases.

cadaverine inhibited the appearance of vWF, α_2-AP and Fbg on COAT-platelets (Figure 9B). Interestingly, each concentration of dansyl cadaverine tested inhibited all four proteins to essentially the same extent suggesting that dansyl cadaverine may be competing against a common amino acceptor shared by all four proteins. Similar studies with another transglutaminase inhibitor, acetyl-casein (98), showed a different pattern (Figure 10). In this instance, inhibition by acetyl-casein varies with each protein examined. Since acetyl-casein is a competitor of the gln-donor half of the transglutaminase reaction, it is competing against each individual transglutaminase substrate (i.e. Fbg, FV, etc.) and its particular affinity for the transglutaminase relative to that of Fbg, FV, etc. determines the degree of inhibition. In addition, SU732 (99, 100), a low molecular weight inhibitor of transglutaminases, was also found to eliminate COAT-platelet formation (data not shown).

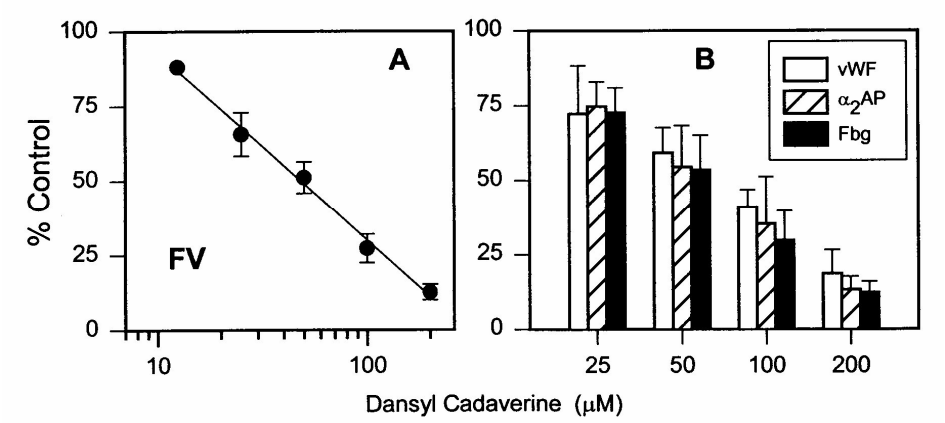


Figure 9 - Inhibition of COAT-platelet formation by dansyl cadaverine.
Panel A: COAT-platelet inhibition shown in Figure 8 is plotted as % initial COAT production versus dansyl cadaverine (μM). Panel B: Appearance of three other proteins in COAT-platelets was monitored as a function of dansyl cadaverine inhibition. von Willebrand factor: blank bars; α2-antiplasmin: hatched bars; fibrinogen: solid bars. Note the similar inhibition for all four proteins at a given dansyl cadaverine concentration.

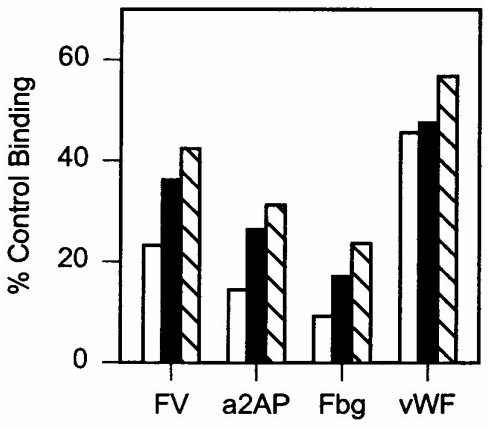


Figure 10 - Inhibition of COAT-platelet formation by acetyl-casein.
COAT platelet production was monitored as a function of acetyl-casein concentration: open bars: 1.5 mg/ml; solid bars: 1.0 mg/ml; and hatched bars: 0.5 mg/ml. Inhibition is shown as % initial COAT production. Note the different inhibition for each protein at a given acetyl-casein concentration.

To further investigate a role for transglutaminase(s) in the formation of COAT-platelets, a 15 residue peptide corresponding to the gln-donor sequence of casein (65) was synthesized. This peptide, SVLSLSQSKVLPVPE (CP_{15}) was prepared either biotinylated or fluorescein labeled. When gel filtered platelets were activated with convulxin and thrombin in the presence of 50 μM biotinylated-CP_{15}, a biotin labeled population of cells was observed (Figure 11) which was similar in percentage of cells to the COAT-platelets stained with anti-HFV. Higher concentrations of CP_{15} were found to be inhibitory to FV incorporation into COAT-platelets, but this 50 μM level of CP_{15} does not significantly interfere. Incorporation of biotinylated-CP_{15} into convulxin plus thrombin stimulated platelets was inhibited by dansyl cadaverine (Figure 11) and acetyl-casein (data not shown).

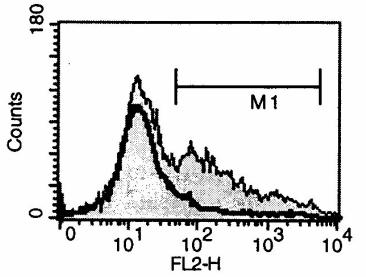


Figure 11 - Biotin-CP_{15} attachment to COAT-platelets. Platelets were activated with thrombin plus convulxin in the presence of 50 μM biotin-CP_{15}. After fixation and washing, the platelets were stained with PE-streptavidin. Activated platelets are indicated with the shaded histogram; the line histogram represents a dansyl cadaverine inhibited reaction. Platelets in the M1 region correspond to COAT-platelets.

Platelets have significant levels of the factor XIIIa subunit in their cytoplasm (119) as well as a tissue transglutaminase (120). Therefore we also tested two antibodies that inhibit factor XIII for their effect on COAT-platelets. Monoclonal antibody 9C11 inhibits thrombin activation of factor XIII (101), and R2 inhibits factor XIIIa activity (102). These antibodies did not affect α-granule secretion or phosphatidylserine exposure (data not shown); however, both antibodies prevented, dose-dependently, the appearance of FV on COAT-platelets (Figure 12). Accumulation of fibrinogen and von Willebrand factor on these cells was also inhibited by 9C11 and R2 (data not shown). Although the high concentrations of antibody required for inhibition might raise doubts about their specificity, similar levels were required for inhibiting factor XIII in whole plasma (101).

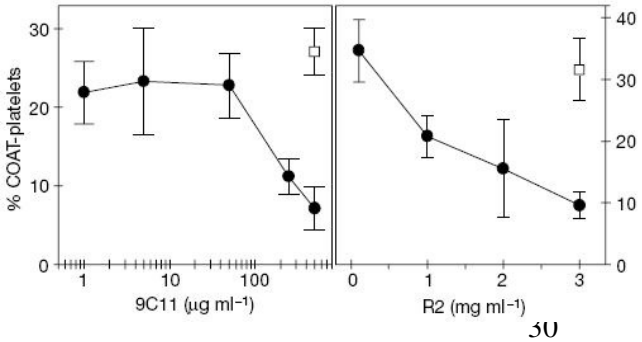


Figure 12 - Inhibition of FXIII effect on COAT-formation. Effect of anti-FXIII antibodies 9C11 and R2, respectively, on COAT-platelet synthesis as measured by FV incorporation (mean±s.d., n = 3±7); open symbols represent control antibodies.

COAT-platelets were also stained with two anti-transglutaminase antisera to identify surface-bound transglutaminases. Unexpectedly, experiments with antibodies against tissue transglutaminase and factor XIII indicated that both transglutaminases are present on the surface of COAT-platelets (Figure 13). The first is AB-1, a mAb against tissue transglutaminase type II, and the second is a sheep polyclonal against human FXIII. The tissue transglutaminase stains the same COAT population as does anti-FV or any of the other COAT-defining protein. Anti-FXIII also stains COAT-platelets, but to a lesser and more variable extent (Figure 13). Further experiments will be needed to delineate the roles of these transglutaminases in COAT-platelet formation.

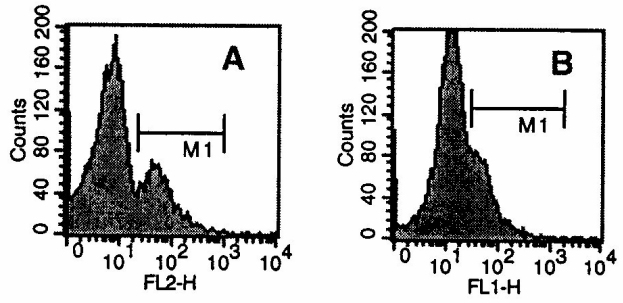


Figure 13 - Labeling of COAT-platelets with anti-transglutaminase antibodies.
Gel filtered platelets were activated with convulxin plus thrombin and then stained with either anti-tissue transglutaminase mAb (Panel A) or anti-FXIII polyclonal antibody (Panel B). The M1 region for Panel A corresponds to COATplatelets independently identified with anti-FV mAb.

Fibrinogen on COAT-platelets

The binding of Fbg to dual-agonist stimulated platelets was further investigated to determine if Fbg is bound to the GP IIb/IIIa receptor on COAT-platelets. Specifically, the binding of FITC-PAC-1 (103) to convulxin plus thrombin activated platelets was examined. PAC-1 binds only to activated GP IIb/IIIa, but not in the presence of a bound ligand such as Fbg (103). As shown in Figure 14 (panel B), two peaks of PAC-1 binding were observed after activation with convulxin plus thrombin, and dual staining with an anti-FV mAb indicated that the lower level PAC-1 peak (M1) represented the COAT-platelets. Upon dual stimulation in the presence of dansyl cadaverine, the lower fluorescence peak (M1) was eliminated (data not shown). Staining for surface glycoprotein IIb/IIIa after thrombin plus convulxin activation showed that all platelets had similar receptor density (data not shown). We also stained the dual-activated platelets with LIBS-6, a monoclonal antibody that recognizes glycoprotein IIb/IIIa with bound ligand (104). COAT-platelets, identified by bound fibronectin, also react with LIBS-6 (Figure 14, panel D). These results show that the glycoprotein IIb/IIIa receptors on COAT-platelets are occupied by ligand.

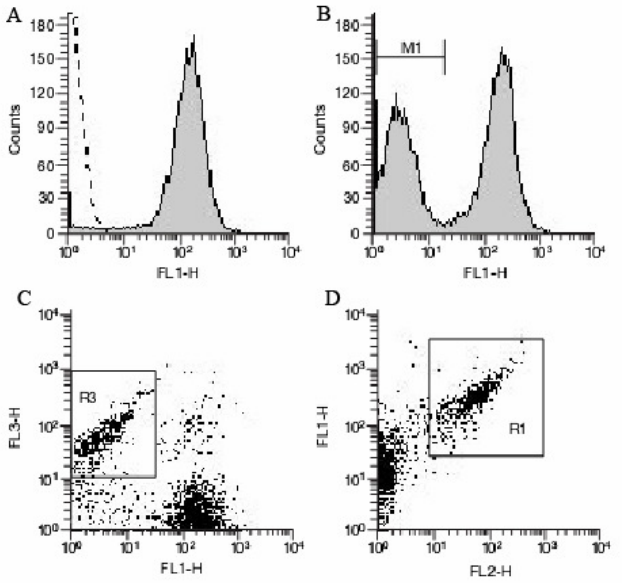


Figure 14 - COAT-platelets bind low levels of PAC-1.
Platelets activated with thrombin alone (panel A; filled histogram) or convulxin plus thrombin (panel B) in the presence of FITC-PAC-1. Analysis of both FV (ordinate) and FITC-PAC-1 on convulxin plus thrombin activated platelets shows that COAT-platelets (R3) bind low levels of PAC-1 (panel C). Cells activated by convulxin plus thrombin and stained with LIBS-6 (FL1) and anti-fibronectin (FL2); cells in R1 are positive for both probes (panel D).

It is noteworthy that PAC-1 cannot displace ligands from COAT-platelets. Considering that PAC-1 has a 50-fold higher affnity for activated glycoprotein IIb/IIIa than has fibrinogen (103), and that bound fibrinogen and fibronectin on COAT-platelets are also not displaced by DMP-802 (data not shown), another strong glycoprotein IIb/IIIa antagonist (105), it is reasonable to suggest that glycoprotein IIb/IIIa receptors on COAT-platelets are retaining adhesive proteins by an unknown mechanism.

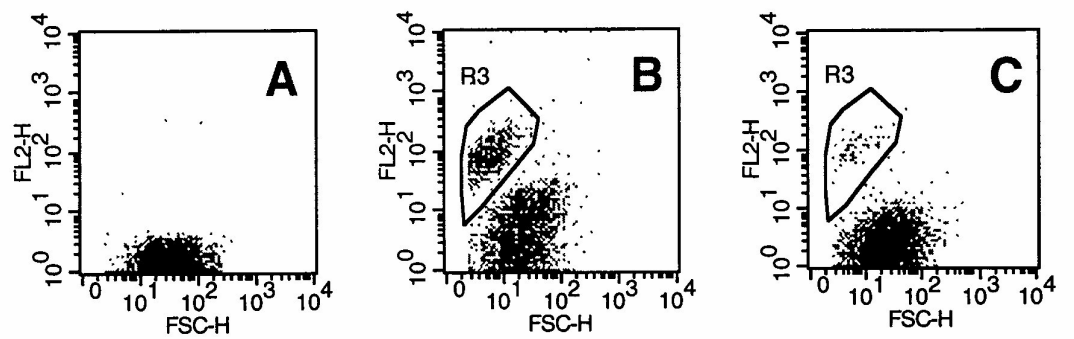
Next, the effect of GP IIb/IIIa inhibitors on the Fbg binding of COAT-platelets was also investigated. Specifically, 7E3, an anti-GP IIb/IIIa mAb which blocks Fbg binding (106) and DMP-802, a high affinity, low molecular weight GP IIb/IIIa inhibitor (105), were utilized to try to inhibit Fbg incorporation into COAT-platelets. Neither inhibitor affected COAT-platelet formation or Fbg incorporation despite control experiments which documented the inhibitors' ability to block PAC-1 binding.

Formation of COAT-platelets in platelet rich plasma

Studies detailed above have utilized gel-filtered platelets, an experimental system which has many advantages but does not reflect the natural environment for platelets. We, therefore, investigated whether COAT-platelets can be formed in PRP upon activation with two agonists. Whole blood was drawn in the presence of DEGR-CK, an inhibitor of factors IXa and Xa (107), and then diluted with HEPES/saline, containing $CaCl_2$, $MgC1_2$, gly-pro-arg-pro and DMP-802, a GP IIb/IIIa antagonist (105). PRP was

prepared and added with minimal dilution to tubes containing agonists, inhibitors, and relevant antibodies. After a 10 minute incubation at 37°C, formalin fixation and washing, platelets were analyzed by flow cytometry for bound FV. Figure 15 demonstrates that a population similar to COAT-platelets is generated upon dual agonist activation of PRP. Confirmation that these are COAT-platelets is provided in Panel C of Figure 15 where dansyl cadaverine is shown to inhibit formation of this population, the current gold-standard test for COAT-platelets. These PRP-derived COAT-platelets have less surface FV than normal COAT-platelets, and they represent only 60-70% of the normal COAT percentage observed with gel filtered platelets.

Figure 15 - Generation of COAT-platelets in PRP.



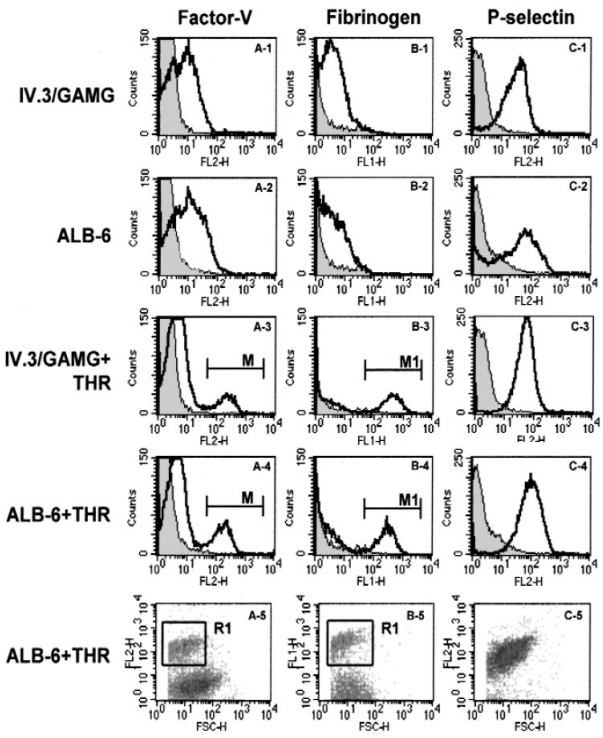
Platelet rich plasma, anti-coagulated with DEGR-CK, was stimulated with convulxin plus thrombin. Surface bound FV was identified with mAb 237/PE-GAMG (FL2). Panel A represents platelets from control PRP; Panel B depicts PRP activated with convulxin plus thrombin; Panel C indicates the effect of 200 μM dansyl cadaverine on convulxin/thrombin activation. COAT-platelets in region R3 represent 17 % of total in Panel B and 2 % in Panel C.

FcRT- platelets

The collagen receptor GP VI shares several signaling pathways with the Fc receptor (FcγRIIa; CD32) present on human platelets (108). As a result, the ability of FcγRIIa engagement to facilitate generation of COAT-platelets was tested. Figure 16 demonstrates that Fc receptor activation can substitute for collagen or convulxin stimulation in the production of COAT-platelets. For example, ALB-6, an anti-CD9 mAb that activates platelets by means of FcγRIIA engagement (84), together with thrombin resulted in COAT platelet formation, as monitored with either factor V (panel A-4, region M) or fibrinogen (panel B-4, region M1). However, platelets stimulated with ALB-6 alone resulted in α-granule release (panel C-2) but no COAT platelet production (panels A-2 and B-2). Similar observations were made with ML-13, another anti-CD9 mAb (data not shown). Generation of COAT-platelets with anti-CD9 mAbs was completely blocked with IV.3, a mAb against FcγRIIA, whereas IV.3 did not affect

COAT platelet formation in convulxin plus thrombin-stimulated platelets (data not shown). Cross-linking of platelet Fc receptors together with thrombin stimulation also resulted in FcRT platelet formation. Specifically, platelets coated with saturating concentrations of IV.3 and subsequently incubated with thrombin and Fc-specific GAMG demonstrated FcRT platelets (regions M and M1, panels A-3 and B-3). However, stimulation with IV.3/GAMG alone resulted in α-granule release only (panel C-1) but no FcRT platelet generation (panels A-1 and B-1). Figure 17 demonstrates that the same subpopulation of dual-activated platelets is positive for factor V, fibrinogen, and von Willebrand factor with both COAT-platelets and FcRT platelets. Additional experiments verified that FcRT platelets are also enriched for thrombospondin, α_2-antiplasmin, and fibronectin (data not shown).

Figure 16 - Factor V, fibrinogen, and P-selectin expression on activated platelets.



Human GFPs were activated by cross-linking IV.3-coated Fc receptors with Fc-specific GAMG (row 1), by addition of anti-CD9 mAb ALB-6 (row 2), by coactivation with thrombin (THR) plus IV.3/GAMG (row 3), or by coactivation with thrombin plus ALB-6 (rows 4 and 5). Surface-bound factor V was detected with mAb HFV-237 (column A), surface-bound fibrinogen was detected with FITC-labeled goat anti-human fibrinogen (column B), and α-granule secretion was monitored with mAb against P-selectin (column C). In each panel control platelets are indicated by a shaded histogram and stimulated cells by a line histogram. Cells in regions M and M1 binding high levels of factor V or fibrinogen are referred to as FcRT platelets (see text). Row 5 demonstrates a dot plot of forward scatter versus fluorescence for platelets activated by ALB-6 plus thrombin and stained with the same indicator antibody used for each column. Region R1 represents FcRT platelets.

As transglutaminase inhibitors are effective in preventing COAT platelet formation (**5**) we examined the effect of two transglutaminase inhibitors, dansylcadaverine and acetyl-casein. Figure 18 demonstrates that both dansylcadaverine and acetylated casein prevent the formation of FcRT platelets observed with thrombin plus ALB-6 or thrombin plus FcγRIIA cross-linking. Control experiments indicated that platelets stimulated with Fc

receptor agonists in the presence of transglutaminase inhibitors still release α-granules (data not shown), as it is shown with COAT-platelets.

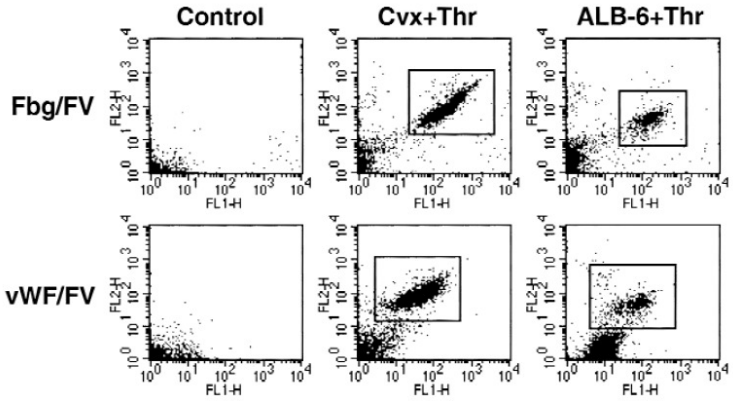


Figure 17 - Two-color analysis of COAT and FcRT platelets.
GFPs were activated with convulxin plus thrombin or ALB-6 plus thrombin, as described in Methods, to generate COAT-platelets and FcRT platelets, respectively. Cells in the first row were stained for fibrinogen (FL1, abscissa) and factor V (FL2, ordinate), and those in the second row were stained for von Willebrand factor (FL1) and factor V (FL2). Note that cells in boxes are positive for both monitored molecules, factor V versus fibrinogen and factor V versus von Willebrand factor. Platelets stimulated with IV.3/GAMG plus thrombin gave similar results (data not shown).

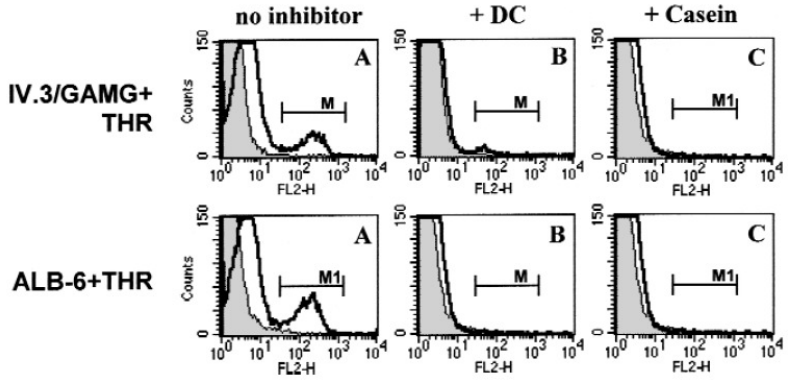


Figure 18 - Inhibition of FcRT platelet formation.
Platelets were costimulated with IV.3/GAMG plus thrombin (row 1) and ALB-6 plus thrombin (row 2). Factor V binding was monitored as detailed in Figure 14. Platelets were activated without inhibitor (column A), with 200 µmol/L dansylcadaverine (DC; column B), and with 1 mg/mL acetyl-casein (column C).

Figure 19, panel A, summarizes data on COAT-platelet and FcRT-platelet production by normal blood donors. In all cases the level of COAT-platelets formed with convulxin plus thrombin is higher than the percentage of FcRT platelets produced with thrombin

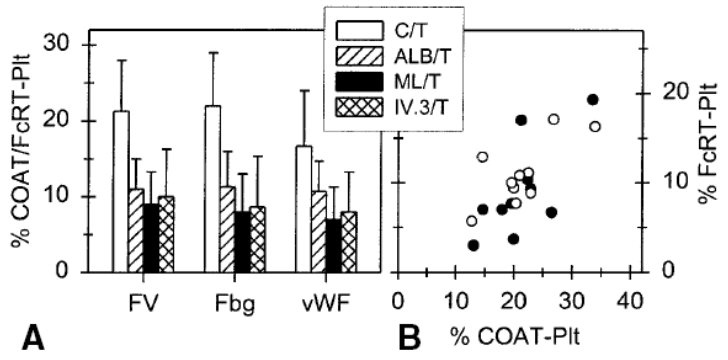


Figure 19 - FcRT platelet formation with different agonist combinations.
Platelets were coactivated with either 500 ng/mL convulxin plus 5 nmol/L thrombin (C/T; COAT-platelets) or with FcγRIIA receptors plus 5 nmol/L thrombin (FcRT platelets), as indicated on the legend of panel A. The percentage of cells with surfacebound α-granule factor V, fibrinogen, or von Willebrand factor produced by different agonist combinations (ie, region M1 of Figure 1) is shown on the ordinate (mean ± 1 SD; n = 11-44). Panel B plots the COAT platelet and FcRT-platelet production for 10 different donors; factor V was monitored. FcRT platelets were generated by either ALB-6 plus thrombin (open circles) or by FcγRIIA cross-linking plus thrombin (filled circles).

plus Fc receptor stimulation, regardless of the indicator molecule used (factor V, fibrinogen, or von Willebrand factor) or the method of Fc receptor engagement. For factor V, all three methods for FcRT-platelet production provide, on average, 46.5% ± 4.7% (± SD; n = 87) of the COAT-platelets observed with convulxin plus thrombin. Furthermore, Figure 19, panel B, demonstrates the relationship between COAT-platelet and FcRT-platelet production for 10 individuals. These data indicate both the strong correlation between COAT platelet and FcRT platelet formation (r = 0.7) and the fact that no individual produced more FcRT platelets than COAT-platelets.

Two hallmarks of COAT-platelets are exposure of surface aminophospholipids and an enrichment among the youngest cells (3). Dual labeling of anti-CD9 plus thrombin activated platelets with PE-labeled annexin-V (93). and FITC-labeled anti-human von Willebrand factor revealed that the FcRT platelets expressing high levels of α-granule von Willebrand factor are also positive for annexin-V (Figure 20).

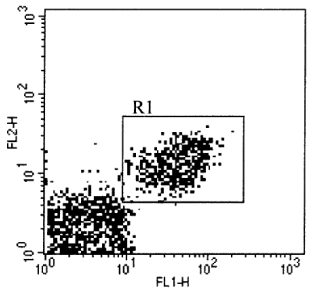


Figure 20 - FcRT platelets and aminophospholipid exposure.
GFPs were activated with 10 µg/mL ALB-6 plus 5 nmol/L thrombin. Negatively charged phospholipids were monitored with PE-labeled annexin-V (FL2), and surface-bound von Willebrand factor was detected with FITC-labeled goat anti-human von Willebrand factor (FL1). Cells expressing high levels of von Willebrand factor (region R1) are also positive for annexin-V. Analysis of fibrinogen and factor V binding versus annexin-V revealed similar results.

To examine the age dependence of FcRT-platelet formation, we used TO, a fluorescent dye that binds to remnant RNA still present in reticulated platelets (90-92). Figure 21 demonstrates that TO-positive cells are more capable of producing FcRT platelets than are aged cells (TO-negative cells) for all agonists examined. Aminophospholipid exposure by Fc receptor plus thrombin-stimulated platelets revealed similar age-dependent characteristics (data not shown). Although the absolute number of COAT-platelets formed is greater than the number of FcRT-platelets, the relative enrichment among the different age groups is similar regardless of the agonist (Figure 21, panel C), suggesting that the lower observed percentage of FcRT-platelets is not a function of even younger cells being required for their production.

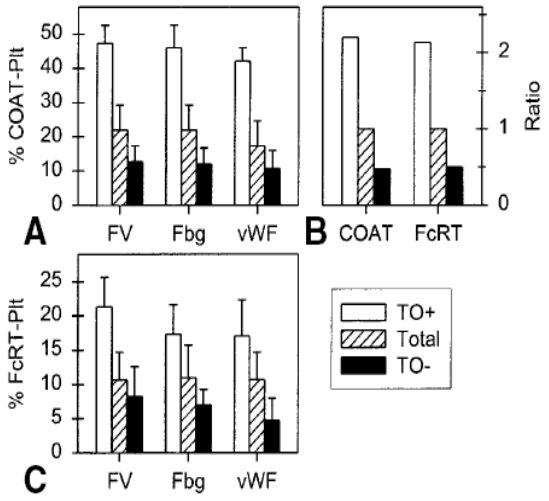


Figure 21 – FcRT-platelet formation and cell age.
Platelets were stimulated with convulxin plus thrombin (A) or with ALB-6 plus thrombin (B). Surface-bound factor V, fibrinogen, and von Willebrand factor were monitored as indicated on the abscissa, and reticulated platelets were identified with TO. The percentage of COAT-platelets and FcRT-platelets produced by TO-positive cells (open bars), by all cells (hatched bars), and by TO-negative cells (filled bars) is shown on the ordinate (mean ± 1 SD; n = 4-5). C depicts the relative enrichment or depletion for COAT-platelet and FcRT-platelet production among TO-positive and TO-negative cells, respectively.

We have also investigated the effect of other agonist combinations. Costimulation of platelets with convulxin and an FcγRIIA agonist induced full activation, as detected by P-selectin expression, but no FcRT platelets. In addition, no synergistic effect was found when both convulxin and thrombin were used in combination with FcγRIIA engagement (data not shown). Finally, we have not observed generation of COAT-platelets or FcRT platelets with any single agonist.

Additionally we have performed several parallel experiments in PRP and whole blood to determine whether FcRT platelets are also produced in the presence of plasma. For example, Figure 22 demonstrates the generation of FcRT platelets in PRP and whole blood by thrombin plus ALB-6; binding of factor V and fibrinogen was monitored. The formation of FcRT platelets in PRP and whole blood is affected by the same inhibitors

used in Figure 8 (data not shown). Also, the absolute levels of factor V and fibrinogen on the FcRT platelets in Figure 22 are less than those observed with GFPs (Figure 14). The explanation for this is not clear; however, because FcRT platelet formation requires a transglutaminase activity, plasma may provide alternative transglutaminase substrates that attenuate the levels of factor V and fibrinogen as it has been showed that fibrinogen in plasma are capable of serving as transglutaminase substrates during COAT platelet synthesis.

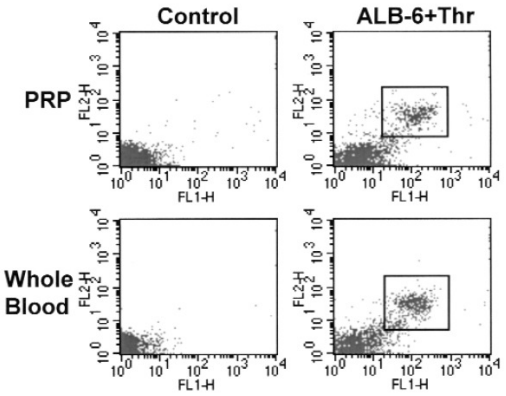


Figure 22 - FcRT platelets in PRP and whole blood.
PRP (row 1) and whole blood (row 2) were activated with ALB-6 plus thrombin, as described in Methods. Cells were monitored for surface-bound fibrinogen (FL1, abscissa) and factor V (FL2, ordinate). Boxes indicate FcRT-platelets positive for fibrinogen and factor V. PRP and whole blood stimulated with IV.3/GAMG plus thrombin gave similar results (data not shown).

Identification of membrane attachment site for transglutaminase reaction

The reactivity of biotinylated-CP_{15} with COAT-platelets offered an opportunity to determine if a membrane protein was serving as the lysine acceptor for transglutaminase immobilization of the proteins identified in Figure 7. The advantage of a low molecular weight marker like biotinylated-CP_{15} is that it would not significantly affect the SDS-PAGE migration of the membrane protein to which it was coupled. However, SDS-PAGE/Western blot analysis of biotin-CP_{15} derivatized COAT-platelets did not reveal any protein labeled with biotin (data not shown). Control experiments utilizing low concentrations of biotinylated, reference proteins demonstrated sufficient sensitivity to detect the expected level of biotin-CP_{15}. As a result of this negative Western blot experiment, the possibility that a small molecule is serving as the anchor for CP_{15} was investigated.

COAT-platelets were formed in the presence of 5(6)-carboxy-fuorescein-CP_{15} (fl-CP_{15}), extracted with methanol, and analysed on a C-4 reverse phase high performance liquid chromatography (HPLC) column with gradient elution. We observed two peaks (P1 and P2) that were not present in the starting material (Figure 23), and analysed these by mass spectrometry. Peak P2 had a molecular weight of 1970, 30 Da greater than the

starting material at 1940 g/mole. However, earlier experiments had suggested that CP_{15} was subject to carboxy terminal proteolysis during incubation with platelets, raising the possibility that the 1970 weight represented an adduct of a peptide sorter than CP_{15}. For example, if the peptide were CP_{14} the 1970 mass would represent the peptide plus 160 Da. Additional expreiments occasionally demonstrated more extensive carbopeptidase activity with an observed weight of 1677 dalton for peak P2 which is compatible with a CP_{11}-peptide plus 160 Da. Considering the stoichiometry of the transglutaminase reaction, the 160 mass translates into a transglutaminase substrate of 176 Da when the released amino group was taken into account. Serotonin (5-hydroxytryptamine), an abundant platelet constituent with an M_r of 176, is a recognized transglutaminase substrate (109). Peak P1 represents a methylamine derivative of fl-CP_{15}.

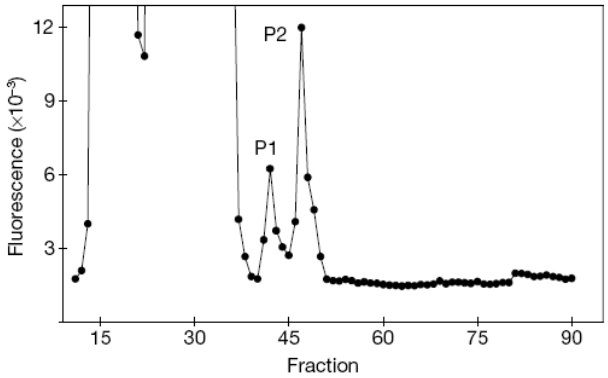


Figure 23 - HPLC analysis of Fl-CP_{15} product.
COAT-platelets were formed in the presence of 5(6)-carboxy-fuorescein-CP_{15} (fl-CP_{15}), extracted with methanol, and analysed on a C-4 reverse phase high performance liquid chromatography (HPLC) column with gradient elution. Two peaks has been observed (P1 and P2) that were not present in the starting material and analysed these by mass spectrometry. P2 had a molecular weight of 1970, 30 Da greater than the starting material at 1940 g/mole. P1 represents a methylamine derivative of fl-CP_{15}.

To further investigate the possible role of serotonin in transglutaminase reaction fibrinogen was immunopurifed from control and COAT-platelets and hydrolysed with mercaptoethane sulphonic acid to release conjugated serotonin (110). We then used reverse phase HPLC, in combination with electrochemical detection, to quantify the amount of serotonin. Fibrinogen from COAT-platelets contained conjugated serotonin, with an average of 7.6 ± 3.4 serotonin molecules per fibrinogen (mean ± SD; n = 4). Serotonin was only observed after acid hydrolysis, indicating that it does not represent non-covalently bound material. Unexpectedly, fibrinogen from control platelets also had 4.0 ± 1.8 serotonin molecules per fibrinogen, but this is significantly less than observed

after activation ($P < 0.04$). Fibrinogen purified from plasma did not have detectable amounts of bound serotonin.

To further confirm the conjugation of serotonin to procoagulant proteins, we fractionated total soluble proteins from control and COAT-platelets using an HPLC molecular sieve column, and analysed the high molecular weight fractions for conjugated serotonin as above. Both control and COAT-platelet extracts had serotonin conjugated to the macromolecular fraction. COAT-platelets averaged 1.8 ± 0.3 times (n = 3) more serotonin, roughly the same fold increase observed with purified fibrinogen. Fibrinogen-serotonin conjugates in resting platelets may either represent derivatization occurring as fibrinogen is packaged into α-granules or, alternatively, selective uptake of very low amounts of fibrinogen-serotonin adducts from the plasma.

We enzymatically synthesized an authentic serotonin adduct of CP_{15}, biotin-CP_{15}-Q^7-5'-HT (B-CP_{15}-(5-HT)), using guinea pig liver tissue transglutaminase utilizing biotin-CP_{15} and serotonin. The product was purified by HPLC and its mass verified by mass spectrometry (1968 Daltons). Platelets activated with convulxin plus thrombin bound B-CP_{15}-(5-HT), whereas resting cells did not (Figure 24A and B). However, increasing concentrations of B-CP_{15}-(5-HT) indicated that up to 70% of all activated platelets would bind this probe (Figure 24C). Since the mean COAT-platelet level for the experiments in Figure 25 was 23.8 ± 3.8%, these data indicate that the presence of the binding site, which required for the proposed model below (see in discussion), is not the limiting factor in determining which cells become COAT-platelets.

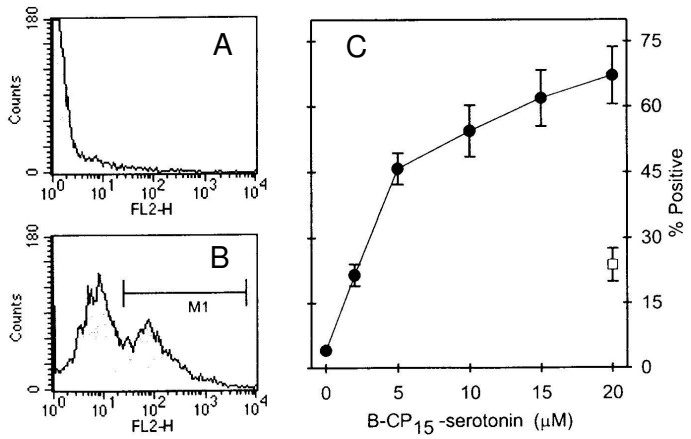


Figure 24 – Binding of B-CP_{15}-(5-HT) to platelets.
Ten μM B-CP_{15}-(5-HT) was incubated with resting (Panel A) or convulxin plus thrombin activated (Panel B) platelets and then stained with PE-SA. Various concentrations of B-CP_{15}-(5-HT) were tested, and the percentage of cells in region M1 is shown in the line graph (Panel C). The open square indicated the mean level of COAT-platelets (HFV-237 monitored) for these expreiments (23.8 ± 3.8%; n=3).

Additional support for the role of serotonin in COAT-platelet formation was provided by inhibition studies with albumin derivatized with serotonin. A multivalent serotonin adduct of bovine serum albumin, biotin-BSA-(5-HT)$_6$, was synthesized chemically and tested for its ability to bind to COAT-platelets. Biotin-BSA-(5-HT)$_6$ bound to dual agonist-activated platelets in a concentration-dependent manner, and maximal binding closely approximated the percentage of COAT-platelets (Figure 25A). Furthermore, BSA-(5-HT)$_6$ inhibited the retention of α-granule proteins on COAT-platelets (Figure 25B). Factor V, fibrinogen and fibronectin concentrations on COAT-platelets were dose-dependently attenuated by BSA-(5-HT)$_6$; the IC_{50} for inhibition of FV was 1.8 mg/ml or roughly 26 nM. Furthermore, a serotonin transport inhibitor, fluoxetine (111), inhibited the retention of FV and fibrinogen by COAT-platelets (data not shown).

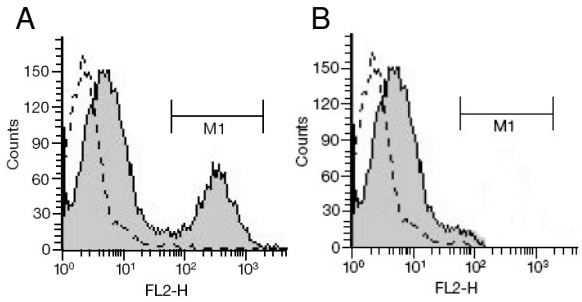


Figure 25 - Binding of biotin-BSA-(5-HT)$_6$ to activated platelets.
Resting (dashed line) and activated (filled histogram) platelets are shown. Panel A: GFPs were activated with convulxin plus thrombin in the presence of 5 µg/ml biotin-BSA-(5-HT)$_6$; cells were analysed for bound biotin. Note the "COAT-like" population (M1). Panel B: Platelets were activated with convulxin plus thrombin in the presence of biotin-HFV237 and 5 µg/ml BSA-(5-HT)$_6$; cells were analysed for biotin-HFV237. Production of COAT-platelets (region M1) is blocked.

DISCUSSION

We have demonstrated that the combined action of two physiologic agonists, thrombin and collagen, is able to promote high levels of α-granule born prohemostatic protein expression on the surface of a discrete fraction of platelets; we have referred to this population as COAT-platelets. Convulxin, a specific agonist for the collagen receptor GPVI (4) can substitute for collagen in this reaction. We have also shown that generation of COAT-platelets parallels the exposure of negatively charged membrane phospholipids, although aminophospholipid exposure is not sufficient to generate these subpopulation of activated cells. Similarly, α-granule release is required but not sufficient for COAT-platelet generation, since we observe a dose-dependent increase in formation of COAT-platelets with agonist concentrations well above that necessary to induce P-selectin expression in more than 95% of all platelets (95). In addition, platelets expressing FV, in particular COAT-pletelets, are functionally relevant and quantitatively more important under these conditions than platelet-derived MP in promoting procoagulant activity. These results also demonstrate that COAT-platelet formation is enriched in reticulated platelets, suggesting that young platelets are more likely than aged ones to undergo this transformation. Previous studies from our laboratory have demonstrated that aging platelets lose reactivity toward thrombin (95) and as well as collagen/convulxin (112). It is therefore conceivable that these age-related changes in reactivity toward single agonists are especially critical for an activation endpoint (COAT-platelet formation), which relies on both of these agonists.

1. Additional proteins on the surface of COAT-platelets:

COAT-platelet has been initially observed and characterised regarding of FV bindig to dual-agonist activated platelets (3). We have here subsequently demostrated that COAT-platelets also express von Willebrand factor, fibrinogen, α_2-antiplasmin, thrombospondin, and fibronectin on their surface (**5**). COAT-platelets were observed upon dual activation with thrombin plus collagen type I, type V, type VI or convulxin, an agonist specific for the collagen receptor glycoprotein VI; however, no single agonist examined were able to produce COAT-platelets (3, **5**).

2. The role of microparticles:

In order to define the relative contributions of platelets and platelet-derived microparticles to the observed procoagulant activity, the level of residual procoagulant activity after separation of platelets and microparticles by centrifugation was assessed (51). We have also shown that dual stimulation with thrombin plus collagen, platelet-derived MP appear to contribute less than 20% of the prothrombinase activity in the absence of exogenous Va (3). The difference between ours and previous studies (59) in the relative contribution of platelets and platelet microparticles to prothrombinase activity may reflect that the latter study was performed in the presence of exogenous factor Va. Despite the fact that MP generated in vivo can stimulate coagulation (115) and that MP-related procoagulant activity has been implicated in pathologic prothrombotic states (116), our results agree with previous observations (53, 117-118) and are consistent with the concept that under physiologic conditions an adequate hemostatic response must be rapid and localized to the site of vascular injury. This concept is supported by the observation that platelet FV appears to be uniquely important to hemostasis even in patients with near normal levels of plasma FV. Additionally our clinical experience supports the importance of another coagulation factor, serum FVII activity as well, in severely bleeding patients with low platelet count (**22**).

3. Transglutaminase activity and COAT platelet formation:

One characteristic shared by all the α-granule proteins present on COAT-platelets is that each is a known transglutaminase substrate, and this seemed to offer a plausible explanation for the strong affinity of these proteins for the platelet surface. We therefore examined the impact of transglutaminase inhibitors on COAT-platelet formation and found an attenuation (**5**). More importantly, it was also demonstrated that a synthetic transglutaminase substrate CP_{15}, a 15-residue peptide serving as a glutamine donor, was incorporated into COAT-platelets (**5**), thereby providing positive evidence that a transglutaminase was active during COAT-platelet formation.

The identity of the transglutaminase responsible for coupling serotonin to α-granule proteins is still uncertain. Platelets have significant levels of the factor XIIIa subunit in their cytoplasm (119) as well as a tissue transglutaminase (120). Immunochemical studies of COAT-platelets identified both transglutaminases on the surface of these cells

(**5**), and an anti-FXIII antibody was able to inhibit COAT-platelet formation (**5**). However, Jobe et al. (121) report in an abstract that FXIIIa knockout mice are unaffected in their ability to produce COAT-platelets. While this point remains unresolved, it is noteworthy that Walther et al. (122) have recently demonstrated that small, cytoplasmic GTP-ases are derivatized with serotonin via a transglutaminase activity during platelet activation; this derivatization has been termed serotonylation.

4. Fibrinogen on COAT-platelets:

The binding of Fbg to dual-agonist stimulated platelets has been investigated to determine if Fbg is bound to the GP IIb/IIIa receptor on COAT-platelets. The unusual retention of these α-granule proteins on COAT-platelets was demostrated by the inability of PAC-1 to displace or prevent the binding of fibrinogen (**5**). PAC-1 is a monoclonal antibody which recognizes the activated conformation of GP IIb/IIIa with an affinity 50 times greater than does fibrinogen, and PAC-1 can actually be used as a GP IIb/IIIa antagonist to prevent platelet aggregation (103). Even though experiments with LIBS-6 indicated that GP IIb/IIIa molecules on COAT-platelets were occupied (**5**) these observations led to the conclusion that fibrinogen, and perhaps other α-granule proteins, were being retained on the platelet surface with an exceptional affinity.

5. Formation of COAT-platelets in platelet rich plasma:

We have performed additional experiments utilizing PRP and demostrated that COAT-platelets can also be formed in an experimental system which reflect more natural environment for platelets. The current gold-standard test for COAT-platelets, transglutaminas inhibitors, were shown to inhibit formation of this specific population of activated platelets, allowing us to demonstrate that these cells are similar to those observed utilizing GFPs. These PRP-derived COAT-platelets have less surface FV than normal COAT-platelets, and they represent only 60-70% of the normal COAT percentage observed with gel filtered platelets.

6. FcRT- platelets:

We have also demonstrated that COAT-platelet production is not restricted to activation with thrombin plus collagen (or convulxin); these unusual platelets can also be generated by engagement of the Fc receptor (FcγRIIA) in combination with thrombin (**7**). The distinct subpopulation of activated platelets observed with this latter method is

referred to as FcRT-platelets. We have observed that FcRT-platelets share a number of properties with COAT-platelets, including high levels of several α-granule proteins on their surface, exposure of negatively charged platelet phospholipids, susceptibility to transglutaminase inhibitors, and increased prevalence among young cells (**5**). FcγRIIA, a low-affinity receptor for IgG, is the only Fc receptor present on platelets, and it can be clustered physiologically by immune complexes (83) resulting in platelet activation. In addition, numerous anti-platelet antibodies are known to activate platelets through engagement of the Fc receptor, a mechanism mimicked here by ALB-6 and ML-13, two anti-CD9 mAbs (84). Considering that the platelet collagen receptor glycoprotein VI shares many signaling pathways with FcγRIIA (87), it is not unexpected that Fc receptor stimulation together with thrombin can generate a "COAT-platelet"-like population. However, none of these agonists alone was sufficient to induce COAT-platelet or FcRT-platelet production. One surprising discrepancy between COAT-platelets and FcRT-platelets is the absolute percentage of cells produced; there were approximately half as many FcRT-platelets as COAT-platelets generated for a given donor. This difference may be a function of the absolute number of glycoprotein VI versus FcγRIIA molecules present on the membrane. Although the number of FcγRIIA molecules is approximately 1500 copies per cell (75), the number of glycoprotein VI molecules has not been reported, although it is likely to be significantly higher because it is observable with simple protein staining of sodium dodecylsulfate–polyacrylamide gel electrophoresis gels (37), and FcγRIIA is not. The absolute number of receptors available for stimulation may affect the temporal or quantitative distribution of intracellular second messengers generated on engagement and thereby differentially affect complex activation events required for COAT-platelet or FcRT-platelet production.

7. Identification of casein peptide cross-links in COAT-platelets:

For the purpose of identification of transglutaminase cross-links in COAT-platelets we have synthetised a 15-residue peptide derived from β-casein (CP_{15}) which included a transglutaminase-active glutamine (**5**). Preliminary data has indicated that a fluorescently labeled casein peptide (Fl-CP_{15}) is coupled to a small, hydrophobic molecule in the platelet membrane during COAT-platelet formation. This transglutaminase substrate was also used to address the question of which platelet component(s) served as the amino-donor in this transglutaminase reaction. While the

anticipated result was that a platelet membrane protein would be the anchoring site for these transglutaminase substrates, the actual molecule conjugated to CP_{15} has turned out to be serotonin (**5**). This unexpected finding was corroborated by the demonstration that fibrinogen recovered from COAT-platelets had covalently bound serotonin (**5**).

8. Identification of membrane attachment site for transglutaminase reaction:
To identify the possible binding site for these proteins on the platelet mebrane CP_{15} was prepared either biotinylated (biotin-CP_{15}) or fluorescein labeled (Fl-CP_{15}). When biotin-CP_{15} was included in a COAT-platelet assay, the biotin label was incorporated into COAT-platelets, and this incorporation could be blocked with transglutaminase inhibitors. SDS-PAGEanalysis of biotin-CP_{15} incorporated COAT-platelets revealed no high molecular weight platelet membrane proteins. Therefore Fl-CP_{15}-labeled COAT-platelets were extracted with ethanol and analyzed on reverse phase HPLC. The protuct than was analyzed by mass spectrometry and found to be the starting material Fl-CP_{15} plus 176 Da. The most common constituent in platelets with a molecular weight of 176 Da is serotonin. Direct evidence of serotonin's role was provided by identifying conjugated serotonin on fibrinogen isolated from COAT-platelets.

Two different mechanisms appear to control surface binding of procoagulant protein released from α-granules. Low-level surface expression of these proteins can be induced by all agonists examined and is independent from the exposure of negatively charged membrane phospholipids, confirming the existence of a binding site other than aminophospholipids (113). COAT-platelet generation is only induced by the combined stimulus of two agonists, requires the presence of extracellular calcium, and parallels the exposure of aminophospholipids, although the latter is not sufficient for its generation. Moreover, only platelets expressing high leveles of surface bound FV are able to maximally bind exogenous FXa. This is reminiscent of the model recently proposed by Bouchard et al. For effector cell protease receptor 1 (EPR-1) mediated binding of FXa (114).

COAT-platelets, coinciding with aminophospholipid exposure and the highest ability to bind FXa, theoretically represents the most efficient substrate for prothrombinase complex assembly. When two stimuli inducing similar amounts of negatively charged phospholipids are compared, the stimulus able to induce high levels os surface bound

FV (COAT-platelets) is more efficient in promoting thrombin generation. Our observations demonstrate that COAT-platelets expressing high levels of surface bound FV, even though it is present in just a minority of activated cells, is functionally more relevant than low-level FV expressing non-COAT-platelets.

These data allowed a preliminary model of COAT-platelet formation and structure to be proposed. This model is based upon four primary findings: several α-granule proteins are coordinately retained on the COAT-platelet surface; fibrinogen is bound to the COAT-platelet with an exceptional affinity; serotonin is conjugated to at least some of these α-granule proteins; and fibrinogen and thrombospondin have binding sites for serotonin-derivatized proteins. The proposed model (Figure 26) addresses all four findings. With this model, α-granule proteins bind to their traditional receptors, e.g. fibrinogen to GP IIb/IIIa and FVa to PS. In addition, serotonin-derivatized α-granule proteins are also able to interact with serotonin binding sites on neighboring fibrinogen and/or thrombospondin molecules. For example, serotonin-derivatized fibrinogen on COAT-platelets is not only bound to GP IIb/IIIa, but also to neighboring fibrinogen or thrombospondin molecules as a result of serotonin-dependent interactions. The consequence of these multivalent interactions is stabilization of the surface bound complex.

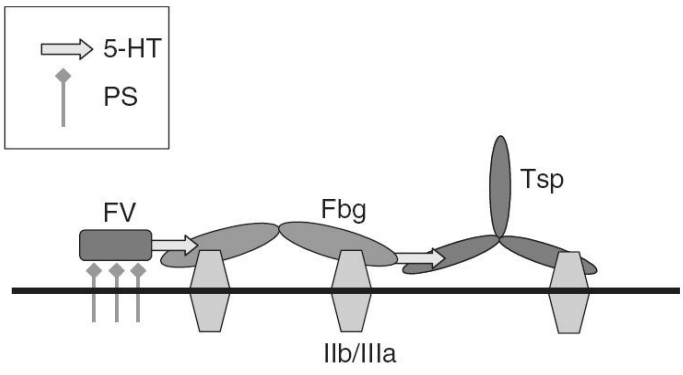


Figure 26 - Model of proposed structure for COAT-platelets.
This model reflects four key findings relevant to the generation of COAT-platelets. Procoagulant proteins bind to their traditional receptors (e.g. FV to PS, Fbg to GP IIb/IIIa). Second, α-granule proteins on COAT-platelets are covalently derivatized with serotonin (arrow) via a transglutaminase reaction. In this figure both FV and Fbg are derivatized with serotonin. Third, Fbg and thrombospondin (Tsp) have binding sites that recognize conjugated serotonin; in this figure, the arrowhead is binding to serotonin binding sites on Fbg and Tsp. Fourth, the multiple interactions of α-granule proteins with surface-bound molecules (e.g. Fbg-serotonin with GP IIb/IIIa and Fbg or Tsp) result in a stabilized network of proteins on the COAT-platelet surface.

As a result, COAT-platelets represent a unique component of hemostasis. The requirement for dual stimulation indicates that COAT-platelets will only be formed under circumstances of extreme haemostatic need, such as immobilization of platelets on the collagen surface of a ruptured vessel in the presence of continuous thrombin generation. Moreover, the physiological significance of COAT-platelets is yet unknown, although inspection of the prothrombotic proteins present on the surface of these cells leads to speculation that they could be significant contributors to thrombotic processes. Most remarkable are the presence of an active prothrombinase complex and the availability of surface PS. While there are observations which suggest an important role for COAT-platelets, hard data on this subject are still lacking.

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