Annexin V as a Probe of Aminophospholipid Exposure and Platelet Membrane Vesiculation: A Flow Cytometry Study Showing a Role for Free Sulfhydryl Groups

By Jeanne Dachary-Prigent, Jean-Marie Freyssinet, Jean-Max Pasquet, Jean-Claude Carron, and Alan T. Nurden

Annexin V, a protein with a high affinity and a strict specificity for aminophospholipids at physiologic calcium concentrations, was used to probe platelet activation and the development of procoagulant activity. Platelet secretion was studied in parallel using VH10, a murine monoclonal antibody specific for GMP-140, an α-granule membrane glycoprotein. Both proteins were labeled with fluorescein isothiocyanate and platelet activation was assessed by flow cytometry. Microparticles, which are shed from the platelet surface and also support procoagulant activity, were distinguished from platelets according to their associated light scattering signal. The relative ability of different inducers to trigger exposure of the procoagulant surface and microparticle formation was: ionophore A23187 > thrombin plus collagen > collagen > thrombin. The density of aminophospholipid on microparticles was higher than on remnant platelets. Platelet activation by these agonists was accompanied by GMP-140 exposure, both on platelets and microparticles. Here, thrombin was the most efficient agonist. The mechanisms responsible for the above processes were investigated using E-64-d, a specific membrane-permeable inhibitor of Ca^{2+}-activated protease (calpain); tetracaine, an activator of calpain; and N-ethylmaleimide and diamide, two sulfhydryl-reactive agents. These agents were added to platelets alone or before stimulation by agonists. Calpain activity was assessed by the hydrolysis of cytoskeletal proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Results showed that calpain activity is not essential for aminophospholipid translocation or for secretion. In contrast, although sulfhydryl-reactive agents alone can trigger procoagulant activity, they inhibit microvesicle formation and platelet secretion induced by the above agonists, suggesting that different mechanisms account for these phenomena. The use of annexin V in flow cytometry is a rapid method to assess procoagulant activity in platelets and the loss of phospholipid asymmetry in cell membranes.

THE PLATELET RESPONSE to agonists leads to the formation of the hemostatic plug and the development of procoagulant activity. The latter accelerates thrombin generation and the onset of coagulation. The procoagulant activity of platelets is linked to an increased exposure of phosphatidylserine on the outer leaflet of the plasma membrane,[1] a process that provides a catalytic surface where coagulation factors Va and Xa can bind and interact.[2] At the same time, enrichment of the outer leaflet of the platelet membrane in negatively charged phospholipids allows the assembly of the protein C-protein S system that has anticoagulant properties.[3] Changes in the phospholipid distribution during platelet activation are accompanied by the shedding of microparticles from the platelet membrane.[4] Recent evidence shows that the microparticles can support both procoagulant and anticoagulant activities.[5]

Studies on the development of procoagulant activity of platelets and, more generally, investigations into the mechanism involved in the transbilayer movement of phosphatidylserine, have included the measurement of prothrombinase activity,[1,6] digestion of phospholipids by specific phospholipases,[1,7] chemical labeling of phospholipids by membrane-impermeable agents,[8] and the direct application of physico-chemical methods such as electronic spin resonance (ESR)[9] and fluorescence.[10] Results from such studies have led to the hypothesis that a specific transfer protein, termed the aminophospholipid translocase, maintains amino phospholipids within the inner leaflet of the plasma membrane of resting cells.[11]

Recently, much attention has focussed on the detection of activation-dependent markers on platelets by flow cytometry. Such studies have used monoclonal antibodies (MoAbs) directed against determinants uniquely expressed on the activated platelet surface.[12] Studies have included the use of antibodies to GMP-140 (or PADGEM), a glycoprotein exclusive to the membranes of α-granules and expressed on the platelet surface after secretion and membrane fusion.[4,13,14] Others have used antibodies to conformation-dependent epitopes on glycoprotein (GP) IIb-IIIa complexes expressed as a primary response of platelets to physiologic agonists.[4,13,15] The procoagulant activity of platelets has been assessed using an MoAb against the light chain of factor Va.[4,13] One of the major advantages of flow cytometry is that platelet activation and microparticle formation can be assessed simultaneously.

In this study, we show that platelet activation leading to the development of procoagulant activity and microparticle shedding can be probed using annexin V, a placental protein with a high affinity and a strict specificity for aminophospholipids in the presence of physiologic calcium concentrations.[15-17] Platelet secretion was assessed in parallel using VH10, a murine MoAb specific for GMP-140. These probes were directly labeled with fluorescein-5-isothiocyanate (FITC) and used in flow cytometry. We have also examined the respective roles of aminophospholipid translocase and calpain

From the URA CNRS 1464, Hôpital Cardiologique, Pessac; the Laboratoire d'Immunologie, Hôpital Pellegrin, Bordeaux; and the Institut d'Hématologie et d'Immunologie, Faculté de Médecine, Strasbourg, France.

Submitted April 7, 1992; accepted December 18, 1992.

Supported by grants from INSERM (CRE no. 89003 to J.D.-P. and 910409 to J.-M.F.), the Conseil Régional d'Aquitaine, the Ministère de la Recherche et de la Technologie (contract no. 88.C.0575), the Université de Bordeaux II (DRED), and the Fondation de France.

Address reprint requests to Jeanne Dachary-Prigent, PhD, URA 1464 CNRS, Hôpital Cardiologique, Avenue du Magellan, 33604 Pessac, France.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" *in accordance with 18 U.S.C. section 1734 solely to indicate this fact.*

0006-4971/93/8110-0003$3.00/0

(calcium-dependent protease) in these processes. As both of these enzymes have been postulated to depend on a free thiol group at their active site,[11,18] we examined the action of sulfhydryl-reactive agents N-ethylmaleimide[19] and diamide,[20,21] alone or in the presence of agonists, on the platelet response. In addition, the effects of tetracaine, which is known to activate calpain and to promote procoagulant activity in platelets,[22] and of E-64-d, a specific membrane-permeable inhibitor of calpain,[23] were also studied. Although they confirm a role for free sulfhydryl groups in platelet function, our results suggest that the expression of procoagulant activity and microparticle production involve different processes; they also show that annexin V is an excellent probe for assessing aminophospholipid exposure on platelets.

MATERIALS AND METHODS

Materials. Prostaglandin E_1 (PGE_1), bovine serum albumin (BSA; fatty acid free), dimethyl sulfoxide (DMSO), apyrase (grade 1), diamide, tetracaine, and N-ethylmaleimide were purchased from Sigma Chemical Co (St Louis, MO). Human α-thrombin was obtained from Ortho Diagnostic System Inc (Raritan, NJ); collagen (equine tendon) from Hormon-Chemie (Munich, Germany); and ionophore A23187 from Calbiochem (La Jolla, CA). FITC was from Aldrich (Strasbourg, France). E-64-d was the generous gift of Dr K. Hanada (Taisho Pharmaceutical Co Ltd, Omiya, Japan). A23187 and E-64-d were dissolved in DMSO as stock solutions.

Preparation and fluorescence labeling of proteins. Annexin V was prepared from human placenta according to the procedure of Funakoshi et al,[15] and was shown to be monodisperse by neutron low-angle scattering. The purified protein migrated as a single band of apparent molecular weight of 36 Kd by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was further characterized by determining its isoelectric point (pI = 4.8), and by sequencing peptide 261-270 present only in placental anticoagulant protein I (annexin V).[24] The protein was stored at −70°C as a 2.2 mg/mL solution in 50 mmol/L Tris-HCl, 0.1 mol/L NaCl buffer, pH 7.5. In a typical labeling experiment, 16.7 nmol of annexin V, gel-filtered on a PD10 column (Pharmacia France, St Quentin-en-Yvelines, France) equilibrated in 20 mmol/L borate, 0.15 mol/L NaCl buffer, pH 9.5, was incubated overnight at 4°C with FITC at a molar ratio of 100 (FITC/annexin V). Unbound FITC was separated by gel-filtration on a PD10 column equilibrated in 50 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.4. The FITC-labeled protein was eluted in the void volume and dialyzed for 48 hours against the same buffer at 4°C. The MoAb VH10 was prepared in Bordeaux as a result of a collaboration between the URA 1464 CNRS and the Centre Régional de Transfusion Sanguine of Bordeaux. The procedures used will be reported elsewhere (V. Jallu, D. Pintigny, J. Chevaleyre, G. Vezon, and A. T. Nurden, manuscript in preparation). In brief, Balb/c mice were immunized by repeated injections of washed platelets previously activated by α-thrombin. Spleen cells were fused with the myeloma X63 Ag8 6.5.3 in the presence of polyethylene glycol. Antibody screening was performed by standard enzyme-linked immunosorbent assay (ELISA) and "dot blot" procedures using paraformaldehyde (PFA)-fixed thrombin-activated platelets. Selected wells were subcloned by limiting dilution and ascites fluid prepared using pristane-primed Balb/c mice. VH10 recognized thrombin-activated platelets, but failed to bind to unstimulated platelets. It bound to a protein of 140 Kd (unreduced) on Western blotting that was also recognized by S12, a well-characterized MoAb to GMP-140.[12] VH10 IgG were purified by affinity chromatography on protein A-sepharose CL-4B (Pharmacia). FITC-labeling of the purified IgG was performed as described above for annexin V, but at a FITC/protein molar ratio of 12 and with an incubation time of 4 hours at room temperature. The FITC/protein molar ratio was calculated using the following extinction coefficients: ϵ_{495} = 78,000 mol/L^{-1} cm^{-1} for FITC[15], $\epsilon^{1\%}_{280}$ = 14 for VH10. The molar concentration of annexin V was deduced from its amino acid composition. The FITC/protein molar ratio was 3.5 to 6.5 for annexin V and 2.4 for VH10.

Blood collection and preparation of washed platelets. In a typical experiment, venous blood (15 mL) was obtained from healthy volunteers and anticoagulated with National Institutes of Health (NIH) formula A acid-citrate-dextrose solution (1 part anticoagulant:6 parts blood). Platelet-rich plasma (PRP) was prepared by centrifugation at 180*g* for 15 minutes at room temperature. Platelets were isolated according to the procedure of Sims et al.[4] In brief, apyrase (25 μg/mL) and PGE_1 (0.1 μg/mL) were added to the PRP, which was then centrifuged for 15 minutes at 850*g*. The platelet pellet was resuspended in 145 mmol/L NaCl, 4 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 0.5 mmol/L sodium phosphate, 0.1% (wt/vol) glucose, 0.1% (wt/vol) BSA, 5 mmol/L PIPES, pH 6.8 (buffer I), containing apyrase and PGE_1. Platelets were then applied to 10 mL sepharose CL-2B (Pharmacia) equilibrated in buffer I. Platelets eluting in the void volume were adjusted to 5×10^7/mL in 137 mmol/L NaCl, 4 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 0.5 mmol/L sodium phosphate, 0.1% (wt/vol) glucose, 0.1% (wt/vol) BSA, 10 mmol/L HEPES, pH 7.4 (buffer II). On occasion, PRP was directly gel-filtered on sepharose CL-2B equilibrated in buffer II. Platelets eluting in the void volume were adjusted as described above.

Platelet activation and microparticle formation. Gel-filtered platelets, adjusted to 5×10^7 cells/mL in buffer II containing 2 mmol/L $CaCl_2$, were incubated without stirring for 10 minutes at 37°C in the presence of the following agonists: ionophore A23187 (1 to 4 μmol/L), thrombin (0.1 to 0.5 U/mL), collagen (5 or 10 μg/mL), or a combination of thrombin plus collagen (see text). With ionophore A23187, the final concentration of DMSO did not exceed 0.05% (vol/vol) and platelet lysis, as determined by lactate dehydrogenase release, was not observed. Agonist concentrations were chosen to give maximum platelet responses under the experimental conditions. Samples (100 μL) of stimulated or control platelets (5×10^6 cells) were then incubated with annexin V-FITC (140 nmol/L) or VH10-FITC (11 nmol/L) for 10 minutes at room temperature. The annexin V-FITC and VH10-FITC concentrations were predetermined from saturation curves obtained for (1) platelets and (2) shed microparticles after platelet activation by A23187 (for annexin V-FITC) or thrombin (for VH10-FITC). As some members of the annexin (lipocortin) family have been reported to be collagen-binding proteins,[25] 1 mL of 5×10^7 platelets incubated with the thrombin plus collagen combination, or collagen alone, were reapplied to 10 mL of sepharose CL-2B equilibrated in buffer II containing 2 mmol/L $CaCl_2$ and the gel filtration repeated to eliminate the agonist(s). Platelets were collected and labeled with annexin V-FITC or VH10-FITC. Binding was not different from that observed in the presence of the agonist(s). No labeling of platelets or microparticles with annexin V was detected in the presence of 2 mmol/L EDTA.

Flow cytometry. Samples were analyzed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Le Pont de Claix, France). The light scatter and the fluorescence channels were set at logarithmic gain. The forward angle light scatter setting was E00, with a threshold of 16. Figure 1 shows dot plots of side versus forward angle light scatter for nonactivated platelets and platelets exposed to the calcium-ionophore A23187. It can be clearly seen that a new population of smaller particles has appeared after activation. The forward angle light scatter versus fluorescence of bound annexin V-FITC is shown in Fig 2. Microparticles (R1) and platelets (R2) are distinguished according to their associated light scattering. The lower limit of the platelet gate was defined on the forward angle light scatter

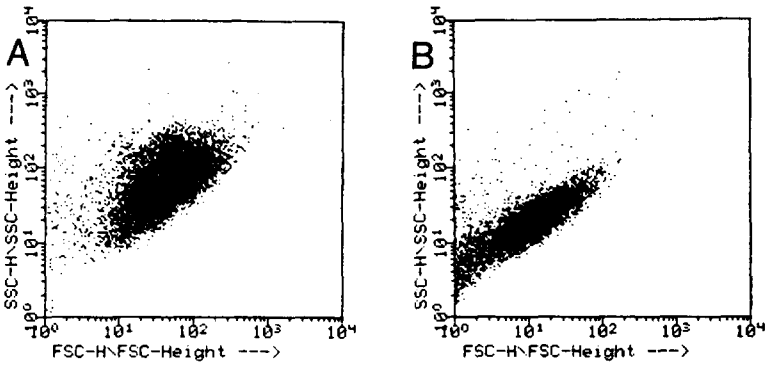

Fig 1. Dot plots of forward versus side light scatter for (A) nonactivated platelets and (B) platelets activated by 3 μmol/L ionophore A23187.

histogram of the ionophore-treated platelet suspension because these platelets have a smaller size compared with nonactivated control platelets (Figs 1 and 2). The microparticles and/or debris and machine noise calculated for nonactivated control platelets incubated at 37°C for 10 minutes or 1 hour (as a control when thiol-reactive agents and calpain inhibitor were studied, see below) did not exceed 1% to 2% of the total particles. Where possible, a total of 10,000 particles was analyzed in each of the defined regions to have a better definition of the mean fluorescence of platelets and microparticles, respectively. Mean fluorescence intensities were expressed in linear mode using LYSIS II software (Becton Dickinson).

Studies on the mechanisms leading to aminophospholipid exposure and microparticle formation. Unless stated otherwise, platelets were incubated with 5 mmol/L diamide, 5 mmol/L N-ethylmaleimide, or 2 mmol/L tetracaine for 1 hour at 37°C without stirring, conditions shown by others to give maximal procoagulant activity without significant platelet lysis.[19-22] When used, the calpain inhibitor E-64-d (0.150 mmol/L) was also preincubated with the platelet suspensions for 1 hour at 37°C either before the addition of the agonists or, in the occasional experiment, before the addition of tetracaine (see text or figure legends). The selected concentration of each of the above agents was that giving the maximum effect under our experimental

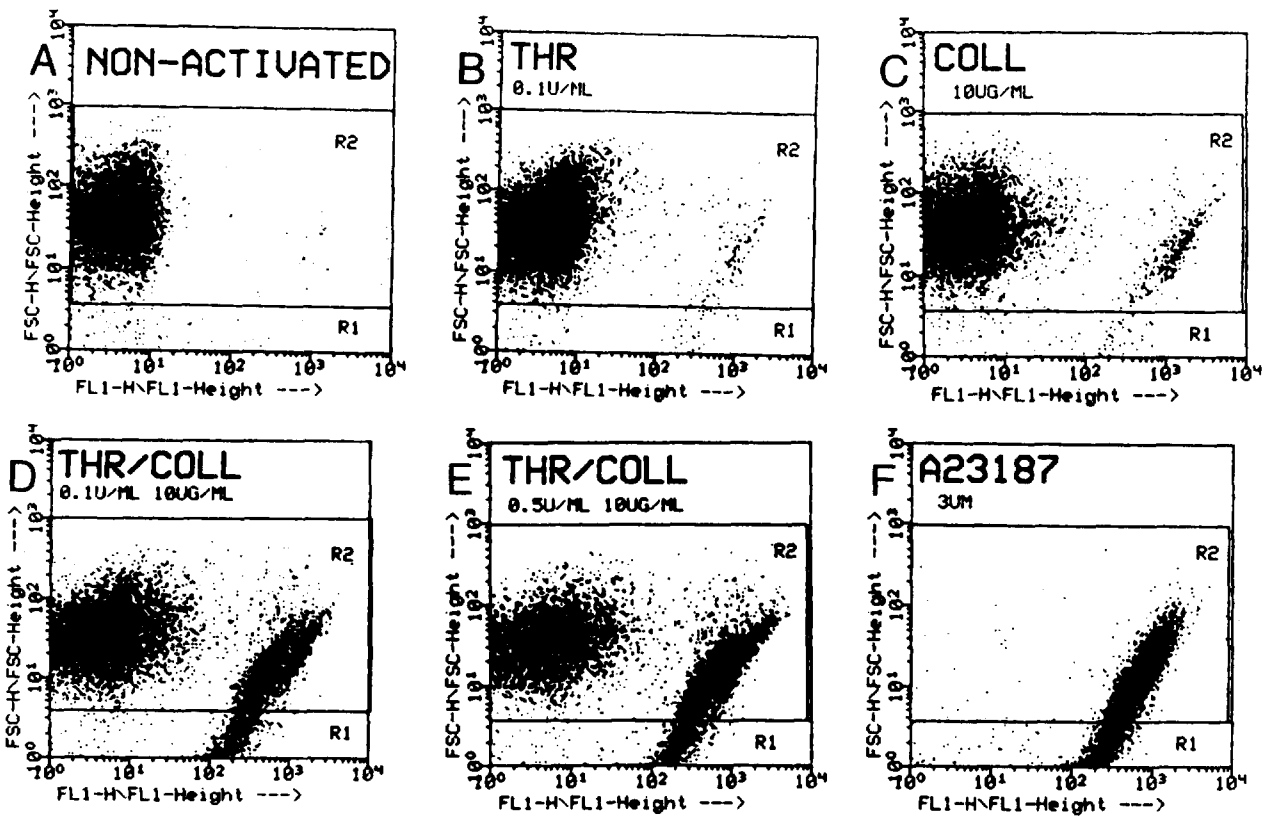

Fig 2. Dot plots of fluorescence versus forward light scatter for (A) nonactivated platelets, and platelets activated by 0.1 U/mL thrombin (B), 10 μg/mL collagen (C), 0.1 U/mL thrombin + 10 μg/mL collagen (D), 0.5 U/mL thrombin + 10 μg/mL collagen (E), or 3 μmol/L ionophore A23187 (F) and labeled with annexin V-FITC. The areas R1 and R2 correspond, respectively, to microparticles and to platelets, as explained in Materials and Methods. Note the large increase in the microparticle density after activation with ionophore A23187 and the thrombin + collagen combinations.

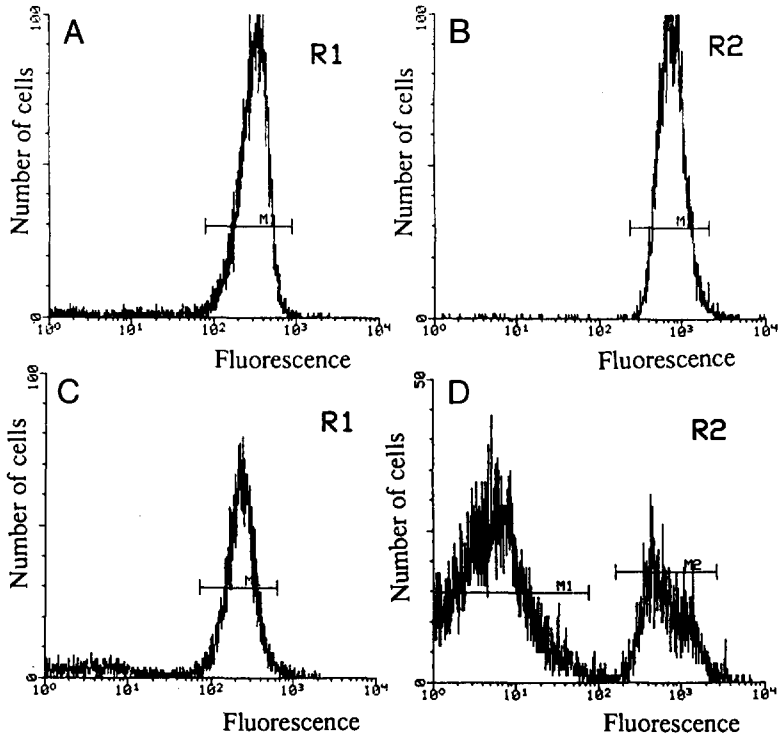

Fig 3. Flow cytometric analysis of the binding of annexin V-FITC to microparticles and platelets activated by 3 μmol/L ionophore A23187 (A and B) or 0.1 U/mL thrombin + 10 μg/mL collagen (C and D). Platelets (B and D) and microparticles (A and C) were discriminated by their associated light scattering properties, as shown in Fig 2.

conditions. In the case of E-64-d, the final concentration of DMSO was 0.02% (vol/vol). Parallel control incubations in the absence of the above agents were always performed. Incubations of platelets with agonists were performed as described above (see also Results).

Evaluation of calpain activity by SDS-PAGE. One milliliter of gel-filtered platelets (5×10^7 platelets) was incubated at 37°C with agonists or sulfhydryl-reactive agents or tetracaine or E-64-d as for the flow cytometry experiments. Then, 0.2 mL of 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 30 mmol/L EDTA, pH 7.4, was added and the platelets were pelleted by centrifugation. Each pellet was resuspended in 100 μL of 20 mmol/L Tris-HCl, 5 mmol/L EDTA, 5 mmol/L dithiothreitol, 2% (wt/vol) SDS, pH 7, and incubated for 1 hour at 37°C for denaturation and disulfide reduction to occur. Fifty microliters was loaded on 7% polyacrylamide slab gels and electrophoresis was performed according to procedures described by Nurden et al.[26] Proteins were revealed by Coomassie blue R250 staining.[27]

RESULTS

Use of annexin V-FITC to detect agonist-induced exposure of aminophospholipids on platelets and microparticles. Gel-filtered platelets were activated with different agonists at 37°C and aminophospholipid exposure assessed with annexin V-FITC. The fluorescence versus forward light scatter representation was selected because both cell size and annexin V-FITC fixation are assessed (Fig 2). For ionophore A23187, most of the platelets bound annexin V and maximal binding was seen with 2 to 3 μmol/L of this agonist (R2 in Fig 2F). Note how the fluorescence intensity has shifted to the right. Such a result suggests that appreciable aminophospholipid exposure has occurred. The corresponding fluorescence histogram is shown in Fig 3B. A sharp symmetrical profile is seen. When platelets were activated with the other agonists, the presence of a specific subpopulation expressing maximal binding of annexin V was again clearly observed (Fig 2). However, this subpopulation varied in density according to the sequence: thrombin + collagen > collagen > thrombin. The corresponding mean fluorescence intensities are given in Table 1. The remaining platelets showed less annexin V binding. Figure 3D illustrates the fluorescence histogram (R2) for platelets activated by the thrombin + collagen combination (0.1 U/mL and 10 μg/mL, respectively). The presence of two populations is clearly shown. The peak corresponding

Table 1. Mean Fluorescence Intensities of Annexin V-FITC Bound to Microparticles and to Platelets Activated by Different Agonists

Agonist	Microparticles	Platelets
Nonactivated platelets	ND	3.2*
Thrombin (0.5 U/mL)	313.4	749.9
Thrombin (0.1 U/mL) + collagen (10 μg/mL)	248.2	752.0
Thrombin (0.5 U/mL) + collagen (10 μg/mL)	243.6	749.3
A23187 (3 μmol/L)	296.9	749.8

Platelets were incubated for 10 minutes at 37°C with or without agonist. Samples were analyzed by flow cytometry as detailed in Materials and Methods. Microparticles and remnant platelets were discriminated by their associated light scatter signal, and aminophospholipid exposure was assessed by annexin V-FITC binding. Mean fluorescence intensity were calculated on 10,000 cells acquired in R1 and R2 as defined in Materials and Methods. Only the subpopulation expressing maximal annexin V binding was shown for activated platelets.

Abbreviation: ND, not determined.

* The mean fluorescence intensity of annexin V bound to nonactivated control platelets.

to the subpopulation expressing maximal annexin V binding is to the right of the profile. This peak is somewhat asymmetric. In fact, with the thrombin + collagen combinations and unlike A23187, careful examination of the profile for annexin V-labeled platelets suggests that two closely situated populations are present (Fig 2). This asymmetry makes it difficult to compare the mean fluorescence intensities with that of the narrow histogram for ionophore A23187-activated platelets.

The activated platelets shed microparticles that were also labeled with annexin V-FITC (R1 in Fig 2). The production of microparticles depended both on the nature of the agonists and on their concentrations. Figure 2 clearly shows that the sequence of agonist efficiency for the formation of microparticles was: ionophore A23187 > thrombin + collagen at all concentrations tested > collagen > thrombin. In Fig 3 are shown the fluorescence histograms of microparticles labeled with annexin V for platelets activated with ionophore A23187 and with the thrombin + collagen combination. The narrow and symmetrical histograms are suggestive of a homogenous population of microparticles labeled with annexin V-FITC. The mean fluorescence intensities are given in Table 1. It can be seen that the high dose of thrombin (0.5 U/mL) and A23187 (3 μmol/L) induced microparticles with the highest mean fluorescence intensity. However, microparticles derived from platelets stimulated with different amounts of thrombin + collagen showed only a limited variation in mean fluorescence intensities (Table 1). Although the number of microparticles varied somewhat (Fig 2D and E), the synergic effect of collagen and thrombin was such that the expression of aminophospholipid and the microparticle formation were always greater than with each reagent alone (Fig 2).

Detection of platelet secretion using the MoAb VH10-FITC to GMP-140. GMP-140 expression on the activated platelets and microparticles was analyzed with VH10-FITC. Figure 4 illustrates typical fluorescence histograms of VH10-FITC bound to microparticles (Fig 4A and C) and to platelets (Fig 4B and D) activated by the ionophore A23187 (3 μmol/L) and by thrombin (0.1 U/mL) + collagen (10 μg/mL). The histograms were again fairly symmetrical, and showed that the bulk of the activated platelets expressed GMP-140 on their surface. The relative efficiency of the agonists to induce GMP-140 expression as detected by the mean fluorescence intensities of the corresponding fluorescence histograms was of the order thrombin > thrombin + collagen > ionophore A23187 > collagen (Table 2). This is quite different from the results for annexin V-FITC binding, showing that secretion and aminophospholipid exposure are not coordinated events. Furthermore, platelet subpopulations not expressing GMP-140 were not encountered, except with collagen, which, under our experimental conditions (in the absence of stirring), induced less GMP-140 expression on platelets than the other agonists. As with annexin V, microparticles were also labeled by VH10-FITC (Fig 4A and C), showing that they too contain GMP-140 on their surface. Here, similar mean fluorescence intensities were seen whatever the agonist (Table 2).

Sulfhydryl-dependence of aminophospholipid exposure and secretion during platelet activation. Both aminophospholipid translocase and calpain activities have been shown to depend on a free thiol group, which can be alkylated by N-ethylmaleimide[19] or oxidized by diamide.[20,21] Calpain can also be activated by tetracaine, which has been shown to promote procoagulant activity in platelets.[22] Flow cytometry showed that incubation of platelets with each of these agents was followed by aminophospholipid exposure and significant binding of annexin V (Table 3). One explanation for these findings is that the aminophospholipid translocase was inhibited. Nonetheless, significant formation of microparticles did not occur (see below). Platelets incubated with diamide or tetracaine were not labeled with VH10-FITC, showing that secretion had not occurred (results not shown). N-ethylmaleimide was an exception, for here some labeling with VH10-FITC was seen, indicating at least a partial secretory response (results not shown).

The influence of these agents on the platelet response to agonists was then examined. Experiments were performed in which platelets were first incubated with N-ethylmaleimide, diamide, or tetracaine, and then activated with ionophore A23187. Preincubation of platelets with these agents prevented or dramatically reduced microparticle formation induced by ionophore A23187 (Fig 5). As a consequence, mean fluorescence intensities of annexin V-FITC bound to platelets after incubation with either of these agents (with or without ionophore A23187) tended to be greater than with the ionophore A23187 alone (Table 3). This may be expected if microparticle shedding involves the loss of surface-exposed aminophospholipids. Preincubation of platelets with diamide or tetracaine before activation by the various agonists did not result in VH10-FITC binding, suggesting that secretion was inhibited (results not shown). The fact that N-ethylmaleimide alone induced VH10-FITC binding prevented its use here.

SDS-PAGE of platelet proteins. The role of calpain in microparticle formation and the development of procoagulant activity was next assessed. As previously shown,[22,28] calpain activity resulted in the degradation of filamin (actin-binding protein), talin, and myosin with the appearance of a limited number of high molecular weight hydrolysis products when platelet proteins were analyzed by SDS-PAGE (Fig 6). Most hydrolysis was seen when platelets were incubated with ionophore A23187 in Ca^{2+}-containing buffer. The extent of hydrolysis was dependent on the agonist and decreased according to the sequence: ionophore A23187 > thrombin + collagen > collagen. No hydrolysis was observed with thrombin alone, confirming a finding previously reported for unstirred platelets by Fox et al.[28] Interestingly, no calpain activity was detected with any agonist when the platelets were incubated in buffer in which divalent cations were replaced by EGTA (Fig 6). This suggests that agonist-mediated influx of extracellular Ca^{2+} was responsible for calpain activation.

When platelets were incubated with tetracaine, the three cytoskeletal proteins were degraded, both when platelets were suspended in the presence of Ca^{2+} (Fig 7) or EGTA (result not shown). Such a result suggests either a direct action of tetracaine on the enzyme and/or the mobilization of internal pools of Ca^{2+}. The remaining experiments were performed on platelets incubated in buffer II in the presence of calcium. No change in the protein pattern was observed with N-ethyl-

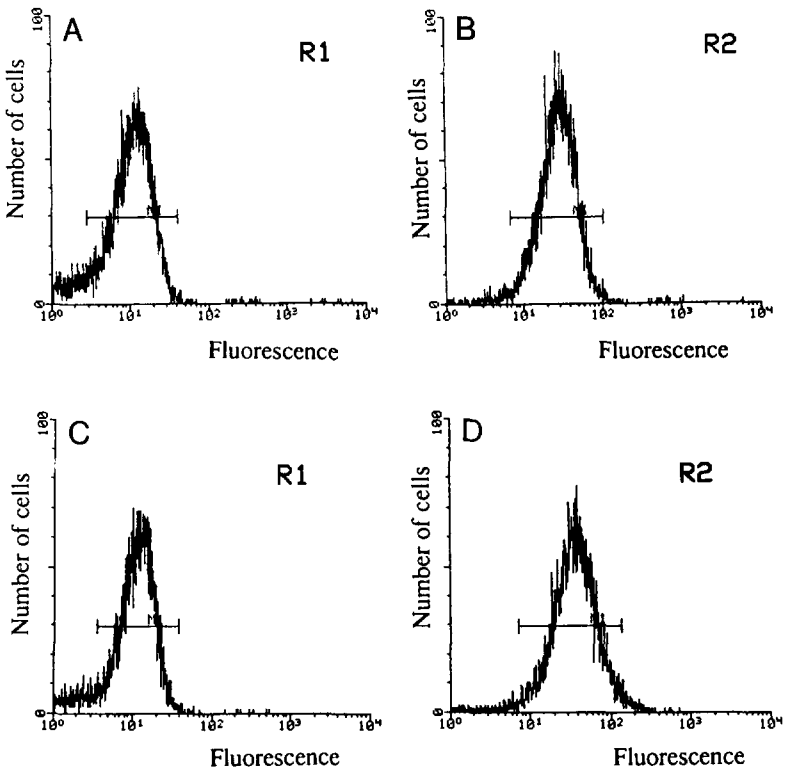

Fig 4. Flow cytometric analysis of GMP-140 expression on platelets and microparticles as assessed using FITC-labeled MoAb VH10. As in Fig 3, platelets were activated by 3 μmol/L ionophore A23187 (A and B) or 0.1 U/mL thrombin + 10 μg/mL collagen (C and D). Platelets (B and D) and microparticles (A and C) were discriminated by their associated light scattering properties, as shown in Fig 2.

maleimide (Fig 7). When added to platelet suspensions, diamide induced extensive cross-linking of cytoskeletal proteins. This occurs through the oxidation of sulfhydryl groups and the formation of disulfides. Despite the fact that electrophoresis was performed under reducing conditions, some cross-linked high molecular weight polymers remained undissociated when diamide-treated samples were analyzed (Fig 7, see the additional band close to the top of the gel). This high molecular weight band was stronger under nonreducing conditions when the three high molecular weight cytoskeletal proteins were no longer present (result not shown). When platelets preincubated with diamide were further activated by ionophore A23187, or thrombin (0.1 U/mL) + collagen (10 μg/mL), calpain was no longer activated, as shown by the absence of any change in the protein pattern and by the absence of degraded forms of filamin, talin, and myosin (Fig 7).

Calpain-dependence of the aminophospholipid exposure and secretion. E-64-d, a specific membrane-permeable inhibitor of calpain,[23] was next used. E-64-d is derived from E-64, a potent inhibitor of cysteine proteases, and has been shown to react irreversibly with the thiol group at the active site.[29] SDS-PAGE of platelet proteins confirmed that incu-

Table 2. Mean Fluorescence Intensities of VH10-FITC Bound to Microparticles and to Platelets Activated by Different Agonists

Agonist	Microparticles	Platelets
Nonactivated platelets	ND	2.0
Thrombin (0.1 U/mL)	ND	45.3
Thrombin (0.5 U/mL)	12.5	43.3
Collagen (10 μg/mL)	ND	3.3
Thrombin (0.1 U/mL) + collagen (10 μg/mL)	11.5	35.5
Thrombin (0.5 U/mL) + collagen (10 μg/mL)	12.6	36.8
A23187 (3 μmol/L)	11.4	27.6

Platelets were incubated with different agonists, flow cytometry was performed, and secretion was assessed by the binding of the MoAb VH10-FITC to GMP-140, both on platelets and on microparticles. Ten thousand cells were acquired in R1 and R2 as defined in Materials and Methods. Most of the activated platelets bound VH10-FITC, except when collagen alone is the agonist, for which the mean fluorescence is close to that corresponding to nonactivated, control platelets.

Abbreviation: ND, not determined.

Table 3. Effect of Thiol-Reactive Agents and of Tetracaine on Anionic Phospholipid Exposure

Platelets Incubated With	Without A23187 (mean fluorescence intensity)	With A23187 (mean fluorescence intensity)
Control	2.8	441.5
NEM	723.3	732.4
Diamide	743.8	691.2
Tetracaine	649.2	588.7

Platelets were incubated for 1 hour at 37°C with N-ethylmaleimide (NEM; 5 mmol/L), diamide (5 mmol/L), or tetracaine (2 mmol/L), followed by 10 minutes of incubation with or without ionophore A23187 (4 μmol/L). Samples were analyzed by flow cytometry as detailed in the legend to Table 1. Aminophospholipid exposure was assessed by annexin V-FITC binding. Mean fluorescence intensities were determined on 10,000 cells acquired in R2 as defined in Materials and Methods.

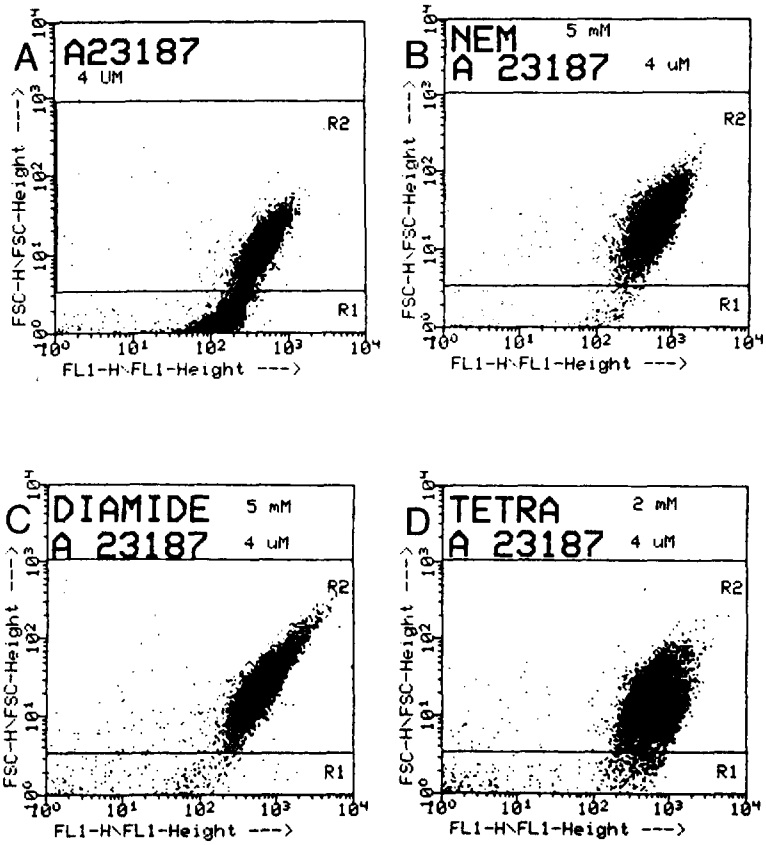

Fig 5. Dot plots of fluorescence versus forward light scatter for platelets activated with 4 μmol/L A23187 in the absence (A) or in the presence of 5 mmol/L N-ethylmaleimide (NEM) (B), 5 mmol/L diamide (C), or 2 mmol/L tetracaine (D) and labeled with annexin V-FITC. R1 and R2 are defined as in Fig 2.

bation of platelets with E-64-d (1 hour, 150 μmol/L, 37°C) prevented calpain hydrolysis of filamin, talin, and myosin as induced by A23187, thrombin + collagen, collagen, and tetracaine (Fig 8). Parallel studies showed that incubation of platelets with E-64-d before activation with ionophore A23187 3 μmol/L, dramatically reduced the extent of microparticle formation (Fig 9). Despite this inhibition, the ionophore A23187-treated platelets bound annexin V-FITC and the corresponding histogram exhibited a higher mean fluorescence intensity than in the absence of E-64-d (932 instead of 820 for this experiment). Thus, E-64-d was clearly inhibiting microparticle formation, but not the surface expression of aminophospholipid. With respect to secretion, flow cytometry showed that a subpopulation of platelets was not labeled with VH10-FITC, suggesting a partial inhibition of the secretory response (results not shown).

DISCUSSION

Procoagulant activity in platelets and in other cells arises from the increased exposure of anionic phospholipids at the outer leaflet of the plasma membrane after cell stimulation or cell damage.[30] The loss of membrane asymmetry might result from local perturbation of the bilayer structure after membrane fusion, accompanied by scrambling of the amino species to the outer leaflet of the plasma membrane.[11] Alternatively, the transfer has been associated with enzymatic activity and a role for both calpain and aminophospholipid translocase has been proposed.[11,31] However, as aminophospholipid translocase has yet to be isolated, its potential role can only be studied by probing or quantifying anionic phospholipids on the outer leaflet of the plasma membrane. Total or partial inhibition of the enzyme should result in increased exposure of amino species, whereas their disappearance would reflect an active enzyme.[32]

Recently, annexin V was shown to bind to activated platelets.[33] Moreover, in the same study, it was shown that (1) phospholipid vesicles containing phosphatidylserine completely inhibited binding of annexin V to platelets, whereas those containing phosphatidylinositol were without effect; and (2) annexin V completely blocked the binding of ^{125}I-labeled factor Xa to activated platelets. Studies from our laboratory also show that annexin V inhibits factor Xa-Va activity on prothrombin in the prothrombinase assay (J. Dachary-Prigent and J.-M. Pasquet, unpublished data). Overall, the available evidence strongly suggests a competition between annexin V and the factor Xa-Va complex for the negatively charged phospholipids exposed on the platelet membrane after activation. Our present study shows that, when coupled to FITC, annexin V can be used to probe procoagulant platelets and derived microparticles by fluorescence-gated flow cytometry. We have compared different agonists and different agonist concentrations with respect to their ability to induce platelet procoagulant activity as detected by annexin V-FITC binding to the activated platelet surface. Experiments were performed in parallel with the MoAb VH10 directed against GMP-140 secreted from the α-granules. VH10 provided another in-

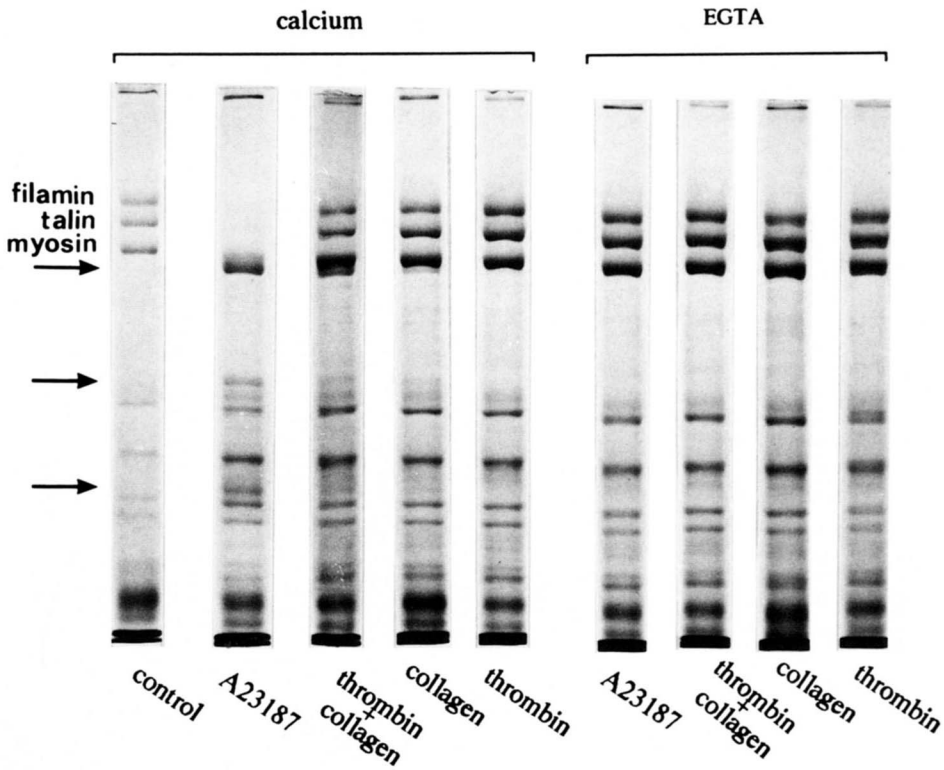

Fig 6. Calpain-induced degradation of high molecular weight platelet proteins as assessed by SDS-PAGE. Gel-filtered platelets were suspended in buffer II containing 2 mmol/L $CaCl_2$ (calcium) or in buffer II, in which the $CaCl_2$ was replaced with 2 mmol/L EGTA (EGTA). Samples were then incubated for 10 minutes with the stated agonists before sedimentation and solubilization with SDS, as described in Materials and Methods. Samples were electrophoresed after disulfide reduction and proteins detected by Coomassie blue R250 staining. Platelets were nonactivated or stimulated with ionophore A23187 (4 μmol/L); thrombin (0.1 U/mL) + collagen (10 μg/mL); collagen (10 μg/mL); or thrombin (0.1 U/mL). Arrows show major degradation products of 190, 135, and 93 Kd resulting from the calpain hydrolysis of filamin, talin, or myosin.

dependent probe of platelet activation. It was found that both procoagulant microparticles and platelets can be detected and labeled with annexin V and VH10. The sequence of agonist efficiency to induce annexin V binding to platelets was: ionophore A23187 > thrombin + collagen > collagen > thrombin (Fig 2). The same order has been found for agonist-induced generation of platelet prothrombinase activity.[1] In our hands, 2 to 3 μmol/L of ionophore A23187 were necessary to achieve maximal expression of aminophospholipids; under these conditions, all platelets bound annexin V-FITC with maximal fluorescence.

Microparticle formation was also shown to be agonist dependent (Fig 2). The ionophore A23187 was the strongest inducer and the mean fluorescence intensity of the corresponding microparticles suggests that these particles contained a higher density of aminophospholipid than those induced

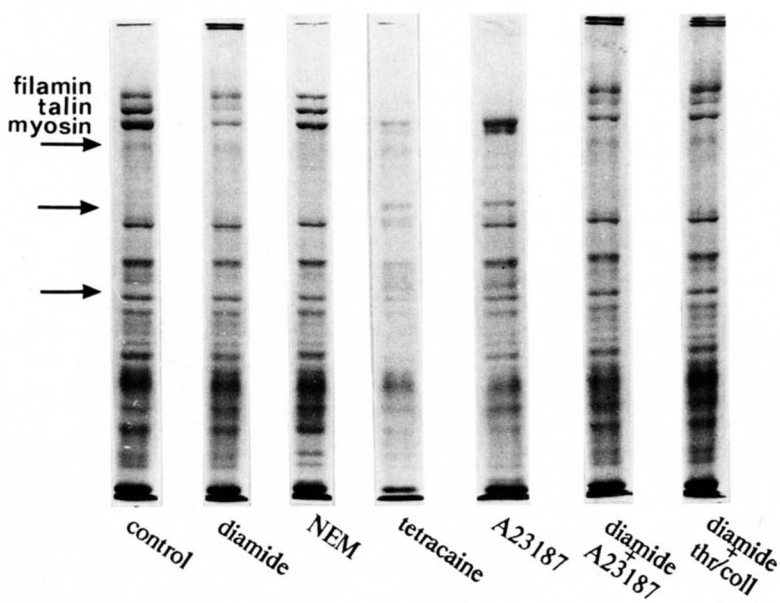

Fig 7. SDS-PAGE of proteins from platelets incubated with sulfhydryl-reactive agents and with tetracaine (1 hour at 37°C), followed or not by activation with ionophore A23187 or thrombin + collagen (10 minutes at 37°C). Left to right shown are nonactivated (control) platelets and platelets incubated with diamide (5 mmol/L); N-ethyl maleimide (NEM) (5 mmol/L); tetracaine (2 mmol/L); ionophore A23187 (4 μmol/L); diamide (5 mmol/L) followed by ionophore A23187 (4 μmol/L); and diamide (5 mmol/L) followed by thrombin (0.1 U/mL) + collagen (10 μg/mL).

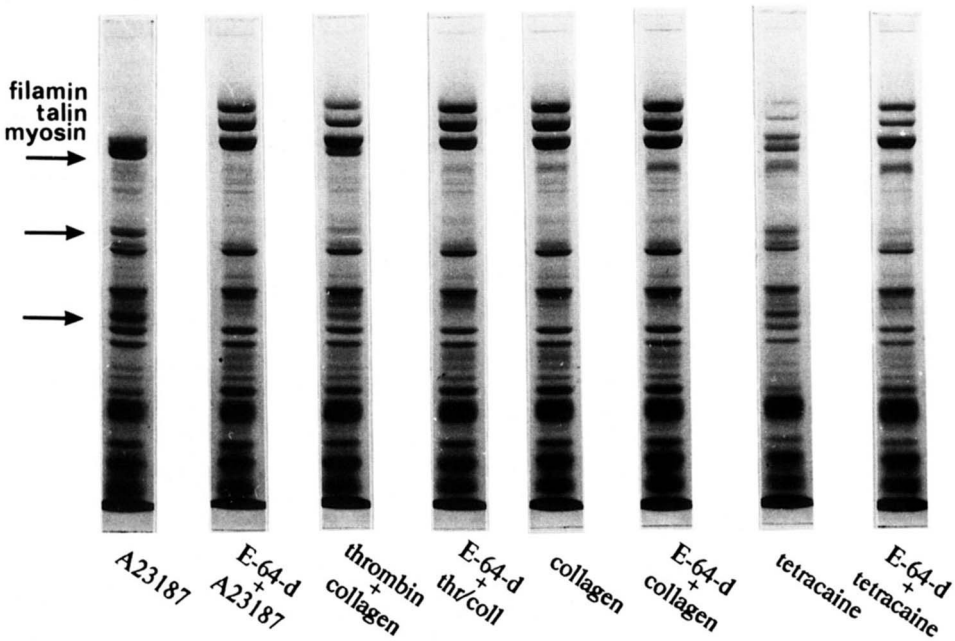

Fig 8. SDS-PAGE of platelet proteins showing the inhibition of the hydrolysis of filamin, talin, and myosin by the membrane-permeable calpain inhibitor E-64-d. Platelets were activated after incubation for 1 hour at 37°C in the absence or presence of E-64-d (150 μmol/L). Agonists used were A23187 (4 μmol/L); thrombin (0.1 U/mL) + collagen (10 μg/mL); collagen (10 μg/mL); and tetracaine (2 mmol/L).

by the thrombin + collagen combination. Interestingly, thrombin at 0.5 U/mL also generated microparticles that were highly labeled by annexin V-FITC, even through the number of particles was much less than when thrombin was combined with collagen. Thus, it appeared that microparticles derived from platelets stimulated with different agonists showed variation both in their number and in aminophospholipid expression. Considering that the ratio of the mean fluorescence intensity of platelets versus that of microparticles is about 2.5 to 3 (Table 1), whereas the ratio of their sizes may be reasonably postulated to be about 5 to 10 because most of the remnant platelets were in the second decade of the forward light scatter axe and microparticles were situated in the first half of the first decade (Fig 2), it is highly probable that the density of aminophospholipid on microparticles is higher than that on remnant platelets.

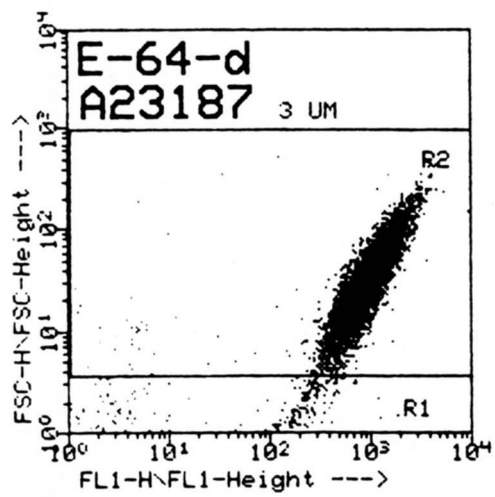

Fig 9. Dot plots of fluorescence versus forward light scatter for platelets activated with A23187 (3 μmol/L) after incubation with E-64-d (150 μmol/L) for 1 hour at 37°C and labeled with annexin V-FITC. R1 and R2 are defined as in Fig 2.

The amounts of VH10-FITC that bound to activated platelets were also agonist dependent, but here the order was thrombin > thrombin + collagen > A23187 > collagen (Table 2). This sequence may be explained in part by the fact that thrombin, which alone induced only limited microparticle production, is a highly efficient inducer of α-granule secretion. In contrast, collagen-induced release may require stirring and platelet to platelet contact. The ionophore A23187 induced the formation of the greatest amount of microparticles. Therefore, the relative lack of GMP-140 expression on ionophore-activated platelets is interesting. It may be that as the number of microparticles formed from each platelet increases, the less residual GMP-140 remains on the platelet surface, and consequently the less the platelets are labeled by the antibody. Alternatively, the ionophore A23187 has been shown to be a relatively poor inducer of the release reaction when platelets are stimulated under nonstirred conditions.[34] Thus, the extent of VH10 binding to both platelets and microparticles will depend both on the degree of microparticle production and on the extent of the release reaction. It is of considerable interest that the microparticles contain GMP-140. Although the precise mechanism responsible for their formation is unknown, the presence of GMP-140 implies that their formation continues during and/or after secretion.

The use of annexin V and VH10 as probes for aminophospholipid exposure and secretion allowed further insights into the mechanisms involved in these platelet responses. Platelets that had been incubated with N-ethylmaleimide, diamide, or tetracaine were labeled with annexin V-FITC (Table 3), suggesting that a translocation of aminophospholipid to the platelet surface had occurred. Such a result is in

agreement with other studies.[21,22] This transfer may be due to direct inhibition of the aminophospholipid translocase or, alternatively, to disruption of the bilayer structure of the membrane after the insertion of these agents. Significantly, the translocation of aminophospholipids, which was of the same order if not greater than that induced by the ionophore A23187, was not accompanied by a large-scale formation of microparticles. In addition, when ionophore A23187 was added to platelets preincubated with these agents, the normal ionophore-induced formation of microparticles was dramatically reduced (Fig 5). At the same time, the annexin V-labeling remained fairly unchanged as compared with that of the controls (Table 3). These results clearly show that aminophospholipid exposure is not necessarily associated with microparticle shedding. Interestingly, SDS-PAGE of platelet proteins confirmed that calpain was no longer activated by ionophore A23187 (Fig 7). Therefore, it is possible that calpain was inhibited by these agents, or that they were interfering with the processes leading to its activation.

When platelets were preincubated with E-64-d, a specific membrane-permeable calpain inhibitor, it was shown by SDS-PAGE that ionophore A23187-induced degradation of cytoskeletal proteins no longer occurred. A similar finding was observed for the thrombin + collagen combination (Fig 8). Interestingly, microparticle formation was strongly inhibited when platelets were preincubated with E-64-d and activated with A23187 (Fig 9), a finding confirming previous observations from prothrombinase activity measurements.[35] This suggests a role for calpain in the production of microparticles. At the same time, ionophore A23187-treated platelets continued to be labeled with annexin V-FITC (Fig 9), indicating that aminophospholipid exposure was not changed, whereas calpain was inhibited. Comfurius et al[32] have previously stated that calpain was not involved in the translocation of anionic phospholipids on the basis of prothrombinase activity measurements. Occasionally, we observed that E-64-d alone could induce annexin V binding to a fraction of the platelet population (results not shown), implying that the aminophospholipid translocase can be inhibited by this agent.

Tetracaine behaved differently from ionophore A23187 under our experimental conditions in that it induced the degradation of cytoskeletal proteins when incubated with platelets resuspended in EGTA-containing medium. This suggests that it was more effective than ionophore A23187 in mobilizing intracellular stores of Ca^{2+} under our experimental conditions, or that it had a direct effect on calpain. However, tetracaine was also an inhibitor of ionophore-induced shedding of microparticles. This showed that activation of calpain and degradation of cytoskeletal proteins did not automatically lead to microparticle production. Another, tetracaine-sensitive step, appears to be required. The stimulatory effect of tetracaine on calpain was inhibited by E-64-d (Fig 8). Tetracaine is already known to promote procoagulant activity in platelets,[22] probably by direct perturbation of the lipid order with consecutive changes in the orientation of membrane phospholipids.[36] An increase in calcium uptake has been described for another local anesthetic, dibucaine,[37] suggesting that aminophospholipid exposure induced by these agents may arise from the combined action of changes in membrane fluidity and aminophospholipid translocase inhibition.

Thus, our results show that microparticle formation induced by the agonists was inhibited by sulfhydryl-reactive agents, including E-64-d, a specific calpain inhibitor. A concomitant inhibition of calpain degradation of cytoskeleton proteins by these agents was demonstrated. So, it is tempting to correlate both activities, as was done by Fox et al.[28,35] However, it has been shown that platelets and erythrocytes from a patient with the Scott syndrome (an inherited defect of platelet procoagulant activity), in which calpain activities are normal compared with control cells, also have a defective Ca^{2+}-induced microparticle formation.[4,38] This suggests an alternative hypothesis, that microparticle formation may also depend on the integrity of another cysteine-dependent enzyme(s) sensitive to thiol-reactive agents (and tetracaine), for such inhibitors may not exhibit an absolute specificity towards calpain.[39]

Platelets preincubated with N-ethylmaleimide, diamide, or tetracaine can no longer undergo secretion in response to agonists, as was shown by the absence of binding of VH10-FITC, an MoAb to GMP-140. The antagonist effect of diamide and of local anesthetics on platelet aggregation and secretion has already been reported.[36,40] A partial inhibition of agonist-induced secretion by platelets preincubated with 0.15 mmol/L E-64-d observed in this study is in agreement with previous results showing inhibition of thrombin-induced platelet aggregation and secretion by E-64-d at a high concentration (1 mmol/L).[41] Such an effect was assigned to inhibition of calpain hydrolysis of protein kinase C, because it was shown that P47 and P20 were no longer phosphorylated. In contrast, Fox et al[28] found no effect of calpain inhibition by calpeptin on phosphorylation of myosin light chain and P40. Furthermore, new synthetic inhibitors specific for calpain had little or no effect on platelet aggregation and secretion.[42] These results may be explained by the different specificities and mechanisms of inhibition of calpain.[39] Moreover, such compounds could also react with a crucial free thiol group of other cysteine-dependent enzymes involved in the secretion process because it has been reported that platelet activation by ionophore A23187, or the combined action of thrombin + collagen, results in a significant reduction of the total free sulfhydryl content of the platelet.[21] One of these proteins might be aminophospholipid translocase because it probably has a free SH group(s) susceptible to react with the above agents[11] and it has been proposed that it might participate in the fusion mechanism leading to secretion.[43]

In conclusion, annexin V is a valuable probe to investigate the capacity of platelets to shed microparticles and to exhibit procoagulant activity. It offers a readily obtainable alternative to the light chain of factor Va that is currently used for this purpose.[4] As aminophospholipids are ubiquitously distributed, annexin V can be used to study other cell types and species and pathologic situations in which these reactions may be expected to occur.[14] Thus, flow cytometry with annexin V-FITC could be a rapid method to assess prethrombotic states without requiring the use of species- and activation-specific antibodies. Recently, the three-dimensional structure of annexin V has been resolved,[44,45] and structural

features of its interaction with a phospholipid monolayer determined from two-dimensional crystal lattices.[46] Potentially, this molecule could be used to quantitate the phospholipid dependence of blood coagulation reactions at the platelet or microparticle surface under circumstances in which the proportion of phosphatidylserine may be rate limiting. The knowledge of the nature of the phospholipid exposed during the dose-dependent response to various platelet agonists is of fundamental importance for a better understanding of the specific catalytic role of phospholipids in hemostasis and thrombosis. The pharmacologic modulation of aminophospholipid exposure or their neutralization by engineered derivatives of the annexin family can be anticipated to be as efficient as antivitamin K, but much faster.

ACKNOWLEDGMENT

We thank Prof J.H. Bezian (Université de Bordeaux II) for making available the Becton Dickinson FACScan flow cytometer used in this study and F. Parrot (Laboratoire de Biochimie, Hôpital Pellegrin) for the amino acid composition of annexin V-FITC. We also thank Dr Paquita Hourdillé and her staff for making available the facilities in their laboratory.

REFERENCES

1. Bevers EM, Comfurius P, Zwaal RFA: Changes in membrane phospholipid distribution during platelet activation. Biochim Biophys Acta 736:57, 1983

2. Mann KG, Nesheim ME, Church WR, Haley P, Kishnaswamy S: Surface-dependent reactions of the vitamin K-dependent enzyme complexes. Blood 76:1, 1990

3. Walker FJ: Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. J Biol Chem 258: 11128, 1981

4. Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil S: Assembly of the platelet prothrombinase complex is linked to vesiculation of platelet plasma membrane. Studies in Scott syndrome: An isolated defect in platelet procoagulant activity. J Biol Chem 264:17049, 1989

5. Tans G, Rosing J, Thomassen MCLGD, Heeb MJ, Zwaal RFA, Griffin JH: Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. Blood 77: 2641, 1991

6. Baldwin JM, O'Reilly R, Whitney M, Lucy JA: Surface exposure of phosphatidylserine is associated with the swelling and osmotically-induced fusion of human erythrocytes in the presence of Ca^{++}. Biochim Biophys Acta 1028:14, 1990

7. Bevers EM, Comfurius P, Zwaal RFA: The nature of the binding site for prothrombinase at the platelet surface as revealed by lipolytic enzymes. Eur J Biochem 122:81, 1982

8. Hale JE, Schroeder F: Asymmetric transbilayer distribution of sterol across plasma membranes determined by fluorescence quenching of dehydroergosterol. Eur J Biochem 122:649, 1982

9. Seigneuret M, Devaux PF: ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: Relation to shape-change. Proc Natl Acad Sci USA 81:3751, 1984

10. Bevers E, Verhallen PFJ, Visser AJWG, Comfurius P, Zwaal RFA: Bidirectional transbilayer lipid movement in human platelets as visualized by fluorescent membrane probe 1-(4-(trimethylammonio)phenyl)-6-phenyl-1,3,5-hexatriene. Biochemistry 29:5132, 1990

11. Devaux PF: Static and dynamic lipid asymmetry in cell membranes. Biochemistry 30:1163, 1991

12. Abrams C, Shattil SJ: Immunological detection of activated platelets in clinical disorders. Thromb Haemost 65:467, 1991

13. Sims PJ, Faioni EM, Wiedmer T, Shattil SJ: Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. J Biol Chem 263: 18205, 1988

14. Abrams CS, Ellison N, Budzynski AZ, Shattil SJ: Direct detection of activated platelets and platelet-derived microparticles in humans. Blood 75:128, 1990

15. Funakoshi T, Heimark RL, Hendrickson LE, McMullen BA, Fujikawa K: Human placental anticoagulant protein: Isolation and characterization. Biochemistry 26:5572, 1987

16. Reutelingsperger CPM, Hornstra G, Hemker HC: Isolation and partial purification of a novel anticoagulant from arteries of human umbilical cord. Eur J Biochem 151:625, 1985

17. Tait JF, Gibson D, Fujikawa K: Phospholipid binding properties of human placental anticoagulant protein-I, a member of the lipocortin family. J Biol Chem 264:7944, 1989

18. Murachi T, Tanaka K, Hatanaka M, Murakami T: Intracellular Ca^{2+}-dependent protease (calpain) and its high molecular-weight endogenous inhibitor (calpastatin). Adv Enzyme Regul 19:407, 1980

19. Morrot G, Herve P, Zachowski A, Fellmann P, Devaux PF: Aminophospholipid translocase of human erythrocytes: Phospholipid substrate specificity and effect of cholesterol. Biochemistry 28:3456, 1989

20. Middelkoop E, Van der Hoek EE, Bevers EM, Comfurius P, Slotboom AJ, Op den Kamp JAF, Lubin BH, Zwaal RFA, Roelofsen B: Involvement of ATP-dependent aminophospholipid translocase in maintaining phospholipid asymmetry in diamide-treated human erythrocytes. Biochim Biophys Acta 981:151, 1989

21. Bevers EM, Tilly RHJ, Senden JMG, Comfurius P, Zwaal RFA: Exposure of endogenous phosphatidylserine at the outer surface of stimulated platelets is reversed by restoration of aminophospholipid translocase activity. Biochemistry 28:2382, 1989

22. Verhallen PFJ, Bevers EM, Comfurius P, Zwaal RFA: Correlation between calpain-mediated cytoskeletal degradation and expression of platelet procoagulant activity. A role for the platelet membrane-skeleton in the regulation of membrane lipid asymmetry? Biochim Biophys Acta 903:206, 1987

23. McGowan EB, Becker E, Detwiler TC: Inhibition of calpain in intact platelets by the thiol protease inhibitor E-64-d. Biochem Biophys Res Commun 158:432, 1989

24. Tait JF, Sakata M, McMullen BA, Miao CH, Funakoshi T, Hendrikson LE, Fujikawa K: Placental anticoagulant proteins: Isolation and comparative characterization of four members of the lipocortin family. Biochemistry 27:6268, 1988

25. Wirl G, Swarch-Albiez R: Collagen-binding proteins of mammary epithelial cells are related to Ca^{++} and phospholipid-binding annexins. J Cell Physiol 144:511, 1990

26. Nurden AT, Dupuis D, Pidard D, Kunicki T, Caen JP: Analysis of the glycoprotein and protein composition of Bernard-Soulier platelets by single and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J Clin Invest 67:1431, 1991

27. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

28. Fox JEB, Reynolds CC, Austin CD: The role of calpain in stimulus-response coupling: Evidence that calpain mediates agonist-induced expression of procoagulant activity in platelets. Blood 76: 2510, 1990

29. Tamai M, Matsumoto K, Omura S, Koyama I, Ozawa Y, Hanada K: In vitro and in vivo inhibition of cysteine proteinases by EST, a new analog of E-64. J Pharmacobiodyn 9:672, 1986

30. Zwaal RFA, Bevers EM, Comfurius P, Rosing J, Tilly RHJ, Verhallen PFJ: Loss of membrane phospholipid asymmetry during activation of blood platelets and sickled red cells; mechanism and physiological significance. Mol Cell Biochem 91:23, 1989

31. Bevers EM, Comfurius P, Zwaal RFA: Platelet procoagulant activity: Physiological significance and mechanisms of exposure. Blood Rev 5:146, 1991

32. Comfurius P, Senden JMG, Tilly RHJ, Schroit AJ, Bevers EM, Zwaal RFA: Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase. Biochim Biophys Acta 1026:153, 1990

33. Thiagarajan P, Tait JF: Binding of annexin V/placental anticoagulant protein I to platelets. J Biol Chem 265:17420, 1990

34. Hourdillé P, Heilmann E, Combrie R, Winckler J, Clemetson KJ, Nurden AT: Thrombin induces a rapid redistribution of GP Ib-IX complexes within the membrane systems of activated platelets. Blood 76:1503, 1990

35. Fox JEB, Austin CD, Reynolds C, Steffen PK: Evidence that agonist-induced activation of calpain causes the shedding of procoagulant-containing microvesicles from membrane of aggregating platelets. J Biol Chem 266:13289, 1991

36. Seeman P: The membrane actions of anesthetics and tranquilizers. Pharmacol Rev 24:583, 1972

37. Peerschke EIB: Platelet membrane alteration induced by the local anesthetic dibucaine. Blood 68:463, 1986

38. Bevers EM, Wiedmer T, Comfurius P, Shattil SJ, Weiss HJ, Zwaal RFA, Sims PJ: Defective Ca^{2+}-induced microvesiculation and deficient expression of procoagulant activity in erythrocytes from a patient with a bleeding disorder: A study of the red blood cells of Scott syndrome. Blood 79:380, 1992

39. Medhi S: Cell-penetrating inhibitors of calpain. Trends Biochem Sci 16:150, 1991

40. Patscheke H, Wörner P: Sequential effects of the thiol-oxidizing agent, diamide, on human platelets. Thromb Res 12:609, 1978

41. Ishii H, Kuboki M, Fujii J, Hirashi S, Kazama M: Thiol-protease inhibitor, EST, can inhibit thrombin-induced platelet activation. Thromb Res 57:847, 1990

42. Anagli J, Hagman J, Shaw E: Investigation of the role of calpain as a stimulus-response mediator in human platelets using new synthetic inhibitors. Biochem J 274:497, 1991

43. Tilly RHJ, Senden JMG, Comfurius P, Bevers EM, Zwall RFA: Increased aminophospholipid translocase activity in human platelets during secretion. Biochim Biophys Acta 1029:188, 1990

44. Huber R, Römisch J, Paques E-P: The crystal and molecular structure of human annexin V an anticoagulant protein that binds to calcium and membranes. EMBO J 9:3867, 1990

45. Huber R, Schneider M, Mayr I, Römisch J, Paques E-P: The calcium binding sites in human annexin V by crystal structure analysis at 2.0 A resolution. FEBS Lett 275:15, 1990

46. Mosser G, Ravanat C, Freyssinet J-M, Brisson A: Sub-domain structure of lipid-bound annexin-V resolved by electron image analysis. J Mol Biol 217:241, 1990

1993 81: 2554-2565

Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups

J Dachary-Prigent, JM Freyssinet, JM Pasquet, JC Carron and AT Nurden

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.