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 physiological 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 generation and the onset of coagulation. The procoagulant activity of platelets is linked to an increased exposure of amino phospholipids on microparticles. 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 Ca2+-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.

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(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, we examined the action of sulf-hydryl-reactive agents N-ethylmaleimide and diamide, 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, and of E-64-d, a specific membrane-permeable inhibitor of calpain, 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 amidophospholipid 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 Funkoshi et al, 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) and sequence peptide 261–270 present only in placental anticoagulant protein I (annexin V). 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, Saint Quentin-en-Yvelines, France) equilibrated in 20 mmol/L buffer I containing 2 mmol/L CaCl$_2$ and the calcium-ionophore A23187, or 10 pg/ml of annexin V, was labeled with annexin V–FITC or 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, 1 L of 5 × 10$^6$ platelets incubated with the thrombin plus collagen combination, or collagen alone, were reapplied to 10 mL of sepharose CL-2B equilibrated in buffer II. Platelets eluting in the void volume were adjusted to 10$^6$ 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 mmol/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, 1 L of 5 × 10$^6$ 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 Clairs, France). The light scatter and the fluorescence channels were set at logarithmic gain. The forward angle light scatter setting was E06, 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) were distinguished according to their associated light scattering. The lower limit of the platelet gate was defined on the forward angle light scatter...
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).

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

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.16-22 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.
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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 × 10⁷ platelets) was incubated at 37°C with agonists or sulphydryl-reactive agents or tetracaine or E-64-d as for the flow cytomtery 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. Proteins were revealed by Coomassie blue R250 staining.

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

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Microparticles</th>
<th>Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonactivated platelets</td>
<td>ND</td>
<td>3.2*</td>
</tr>
<tr>
<td>Thrombin (0.5 U/mL)</td>
<td>313.4</td>
<td>749.9</td>
</tr>
<tr>
<td>Thrombin (0.1 U/mL) + collagen (10 μg/mL)</td>
<td>248.2</td>
<td>752.0</td>
</tr>
<tr>
<td>Thrombin (0.5 U/mL) + collagen (10 μg/mL)</td>
<td>243.6</td>
<td>749.3</td>
</tr>
<tr>
<td>A23187 (3 μmol/L)</td>
<td>296.9</td>
<td>748.8</td>
</tr>
</tbody>
</table>

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 was 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 synergistic 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).

Sulphydryl-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 or oxidized by diamide. Calpain can also be activated by tetracaine, which has been shown to promote procoagulant activity in platelets. 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, 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 Ca2+-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 unstimulated platelets by Fox et al. 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 Ca2+ 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 Ca2+ (Fig 7) or EGTA (results not shown). Such a result suggests either a direct action of tetracaine on the enzyme and/or the mobilization of internal pools of Ca2+. 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-
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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, 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. SDS-PAGE of platelet proteins confirmed that incubation with E-64-d prevented the exposure of annexin V.

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

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Microparticles</th>
<th>Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonactivated platelets</td>
<td>ND</td>
<td>2.0</td>
</tr>
<tr>
<td>Thrombin (0.1 U/mL)</td>
<td>ND</td>
<td>45.3</td>
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<tr>
<td>Thrombin (0.5 U/mL)</td>
<td>12.5</td>
<td>43.3</td>
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<tr>
<td>Collagen (10 μg/mL)</td>
<td>ND</td>
<td>3.3</td>
</tr>
<tr>
<td>Thrombin (0.1 U/mL) + collagen (10 μg/mL)</td>
<td>11.5</td>
<td>35.5</td>
</tr>
<tr>
<td>Thrombin (0.5 U/mL) + collagen (10 μg/mL)</td>
<td>12.8</td>
<td>36.8</td>
</tr>
<tr>
<td>A23187 (3 μmol/L)</td>
<td>11.4</td>
<td>27.6</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Platelets Incubated With</th>
<th>Without A23187</th>
<th>With A23187</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(mean fluorescence intensity)</td>
<td>(mean fluorescence intensity)</td>
</tr>
<tr>
<td>Control</td>
<td>2.8</td>
<td>441.5</td>
</tr>
<tr>
<td>NEM</td>
<td>723.3</td>
<td>732.4</td>
</tr>
<tr>
<td>Diamide</td>
<td>743.8</td>
<td>691.2</td>
</tr>
<tr>
<td>Tetracaine</td>
<td>649.2</td>
<td>598.7</td>
</tr>
</tbody>
</table>

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.
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. 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. Alternatively, the transfer has been associated with enzymatic activity and a role for both calpain and aminophospholipid translocase has been proposed. 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.

Recently, annexin V was shown to bind to activated platelets. 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 125I-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 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.
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 CaCl₂ (calcium) or in buffer II, in which the CaCl₂ 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.

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).

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. 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
by the thrombin + collagen combination. Interestingly, thrombin at 0.5 U/mL also generated microparticles that were highly labeled by annexin V-FITC, even though 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.

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 stimulating 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. 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. 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 dramat-
ically reduced (Fig 5). At the same time, the annexin V-la-
beling remained fairly unchanged as compared with that of
the controls (Table 3). These results clearly show that ami-
rophospholipid 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 cal-
pain was inhibited by these agents, or that they were inter-
fering 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 cyto-
skeletal 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 obser-
vations from prothrombinase activity measurements. This
suggests a role for calpain in the production of microparticles.
At the same time, ionophore A23187-treated platelets con-
tinued to be labeled with annexin V-FITC (Fig 9), indicating
that aminophospholipid exposure was not changed, whereas
calpain was inhibited. Comfurius et al have previously stated
that calpain was not involved in the translocation of anionic
phospholipids on the basis of prothrombinase activity measure-
ments. Occasionally, we observed that E-64-d alone could
induce annexin V binding to a fraction of the platelet pop-
ulation (results not shown), implying that the aminophos-
pholipid translocase can be inhibited by this agent.

Tetracaine behaved differently from ionophore A23187
under our experimental conditions in that it induced the de-
gradation 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 under our experimental con-
ditions, or that it had a direct effect on calpain. However, tetr-
caine 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-sensi-
tive step, appears to be required. The stimulatory effect of
tetracaine on calpain was inhibited by E-64-d (Fig 8). Tet-
caine is already known to promote procoagulant activity in
platelets, probably by direct perturbation of the lipid order with consecutive changes in the orientation of mem-
brane phospholipids. An increase in calcium uptake has
been described for another local anesthetic, dibucaine, sug-
gesting that aminophospholipid exposure induced by these
agents may arise from the combined action of changes in
membrane fluidity and aminophospholipid translocase in-
hibition.

Thus, our results show that microparticle formation in-
duced by the agonists was inhibited by sulfhydryl-reactive
agents, including E-64-d, a specific calpain inhibitor. A con-
comitant 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. 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-induced microparticle formation. This suggests an
alternative hypothesis, that microparticle formation may also
depend on the integrity of another cysteine-dependent en-
zyme(s) sensitive to thiol-reactive agents (and tetracaine),
for such inhibitors may not exhibit an absolute specificity towards
calpain.

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 di-
amide and of local anesthetics on platelet aggregation and
secretion has already been reported. 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 con-
centration (1 mmol/L). Such an effect was assigned to in-
hibition of calpain hydrolysis of protein kinase C, because
it was shown that P47 and P20 were no longer phosphorylated.
In contrast, Fox et al found no effect of calpain inhibition
by calpeptin on phosphorylation of myosin light chain and
P40. Furthermore, new synthetic inhibitors specific for cal-
pain had little or no effect on platelet aggregation and secre-
tion. These results may be explained by the different spec-
ficities and mechanisms of inhibition of calpain. 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. One of these
proteins might be aminophospholipid translocase because
it probably has a free SH group(s) susceptible to react with the
above agents and it has been proposed that it might partici-
pate in the fusion mechanism leading to secretion.

In conclusion, annexin V is a valuable probe to investigate
the capacity of platelets to shed microparticles and to ex-
itiate procoagulant activity. It offers a readily obtainable al-
ternative to the light chain of factor Va that is currently used for
this purpose. As aminophospholipids are ubiquitously distri-
buted, annexin V can be used to study other cell types and
species and pathologic situations in which these reactions
may be expected to occur. Thus, flow cytometry with ann-
exin V-FITC could be a rapid method to assess prethrom-
botic states without requiring the use of species- and acti-
vation-specific antibodies. Recently, the three-dimensional
structure of annexin V has been resolved and structural
features of its interaction with a phospholipid monolayer determined from two-dimensional crystal lattices. 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.

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

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