Evidence That Agonist-induced Activation of Calpain Causes the Shedding of Procoagulant-containing Microvesicles from the Membrane of Aggregating Platelets*

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One of the responses of platelets to stimulation is activation of intracellular calpain (the Ca$^{2+}$-dependent protease). Previously, we have shown that activation of calpain in platelets is involved in the generation of platelet procoagulant activity. Because procoagulant activity is present on the microvesicles that are shed from activated platelets, in this study we examined whether calpain is involved in the shedding of microvesicles. Platelets were incubated with the physiological agonists collagen or thrombin. The extent of activation of calpain correlated positively with the amount of procoagulant-containing microvesicles that formed, and the shedding of procoagulant-containing microvesicles was inhibited by calpeptin, MDL, and EST (E-64-d), three membrane-penetrating inhibitors of calpain. The protein composition of the microvesicles shed from aggregating platelets was similar to that of microvesicles shed by platelets in which the association of the membrane skeleton with the plasma membrane had been disrupted by incubation of platelets with dibucaine or ionophore A23187. Furthermore, like microvesicles shed from dibucaine- or ionophore A23187-treated platelets, those shed from the aggregating platelets possessed procoagulant activity. These results are consistent with the possibility that activation of calpain in aggregating platelets causes the shedding of procoagulant-containing microvesicles. We suggest that the shedding of microvesicles results from the calpain-induced hydrolysis of the platelet membrane skeleton.

Calpain (the Ca$^{2+}$-dependent protease) is present in many cell types, yet its function is understood poorly. One cell in which calpain has been shown to become activated as a consequence of stimulation of the cell with physiological agonists is the blood platelet (1, 2). Activation of calpain within platelets results in the hydrolysis of a few selective proteins (3–5). These include actin-binding protein, talin, and spectrin, three cytoskeletal proteins that have been implicated as mediators of actin-membrane interactions (for review, see Ref. 6). However, perhaps because only small amounts of these proteins are hydrolyzed and hydrolysis is not detected until after platelets have changed shape, secreted their granule contents, and aggregated, the physiological function of calpain activation within platelets has not been clear.

Calpain can be activated if platelets are incubated with dibucaine (7) or the Ca$^{2+}$ ionophore A23187 (8). Using these two agents, we recently provided evidence that activation of calpain resulted in shedding of portions of the plasma membrane from the platelet in the form of microvesicles (9). The shedding of the microvesicles correlated positively with the calpain-induced hydrolysis of the membrane skeleton. The microvesicles had binding sites for the prothrombinase complex on their surface and therefore could accelerate the formation of thrombin from prothrombin. Because procoagulant-containing microvesicles are shed from platelets that are stimulated with physiological agonists, we suggested that the shedding might be one consequence of calpain activation in platelets aggregating in response to a physiological agonist. However, there is evidence that microvesicles are shed from platelets that have been incubated with physiological agonists under conditions in which calpain is not likely to have been activated (10, 11). Further, the shedding of procoagulant-containing microvesicles induced by the complement proteins C5b-9 was not inhibited if the calpain inhibitor leupeptin was incorporated into the cytosol (11).

Because leupeptin does not cross the plasma membrane readily in the absence of the complement proteins, it has not been possible to use this inhibitor to determine whether activation of calpain in platelets stimulated with physiological agonists is responsible for the shedding of the procoagulant-containing microvesicles from these platelets. Recently, however, several other inhibitors of calpain have been synthesized (12–14). Unlike leupeptin, these agents freely cross the plasma membrane and inhibit intracellular calpain. Further, unlike leupeptin, they do not inhibit the proteolytic action of serine proteases such as thrombin (Ref. 15, and data not shown). We have used three of these inhibitors (MDL (12), EST (E-64-d) (13), and calpeptin (14)) to investigate the role of calpain in the stimulus-response pathway in platelets. In a recent study (15), we have shown that although many platelet responses proceed normally under conditions in which all detectable hydrolysis of actin-binding protein is inhibited, the agonist-induced generation of procoagulant activity does not.

In the present study, we show that activation of calpain in platelets aggregating in response to physiological agonists causes the shedding of procoagulant-containing microvesicles. Because the microvesicles shed when calpain was activated by a physiological agonist were similar in composition and functional activity to those shed when the membrane skeleton...
is disrupted, we suggest that they may result from the calpain-induced disruption of actin-membrane interactions.

MATERIALS AND METHODS

Incubation and Fractionation of Platelet Suspensions—Platelets were isolated, their surface glycoproteins [H]-labeled, and suspensions maintained at 37 °C in a Tyrode’s buffer as described (16). Platelets were incubated with collagen (Horme, Munich, West Germany), thrombin (provided by Dr. J. W. Fenton II of the New York Department of Health, Albany, NY), or a combination of the two agonists. Unless otherwise stated, incubations were performed while the suspension was stirred. In some experiments, platelets were preincubated for 5 min with calpeptin (14) or for 10 min with MDL (12) or EST (13). Calpeptin, MDL, or EST was added in a final concentration of 0.1% (v/v) dimethyl sulfoxide. Calpeptin was the generous gift of Dr. T. Tsujinaka of Osaka University School of Medicine (Osaka, Japan); MDL was from Dr. S. Mehdi of Merrell Dow (Cincinnati, OH); and EST was from Dr. M. Tamai of Taisho Pharmaceutical (Saitama, Japan).

Microvesicles were isolated from the supernatant that remained when intact platelets were removed from suspension. This supernatant was obtained by centrifuging the platelet suspension at 15,600 × g for 1 min at ambient temperatures. EGTA (200 mM, pH 9.0) was added to a final concentration of 5 mM just before centrifugation. The addition of EGTA was especially important in experiments performed to determine whether microvesicles were shed from platelets that were activated in the absence of stirring (a condition in which calpain is not activated), because it minimized the activation of calpain that otherwise occurred during the centrifugation of these activated platelet suspensions; presumably the EGTA acted by preventing the aggregation of these platelets during centrifugation. The resulting platelet-free supernatant contained any microvesicles that had been shed from the platelets, as well as proteins that had been secreted from the platelet α-granules. The microvesicles were separated from other proteins by filtration of the 15,600 × g supernatant through polycarbonate filters (0.2 μm, Millipore, Bedford, MA) or by centrifugation of the supernatant at 100,000 × g for 2.5 h. For analysis of the protein composition of the microvesicles, the material sedimented at 100,000 × g was solubilized in a sodium dodecyl sulfate (SDS)-containing buffer in the presence of reducing agent (16). For analysis of procoagulant activity, microvesicles were sedimented at 100,000 × g and resuspended in a Tyrode’s buffer at a final concentration of 50–200 μg of protein/ml.

Analytical Procedures—Samples were analyzed on SDS-polyacrylamide gels in the presence of reducing agent by the method of Laemmli (17). Gels were stained with Coomassie Brilliant Blue. The membrane glycoproteins detected on immunoblots of platelets were [H]-labeled, and these radiolabeled glycoproteins were detected by fluorography of gels treated with ENHANCE (Du Pont-New England Nuclear). Coomassie Brilliant Blue-stained proteins were quantitated by densitometry of SDS gels, and [H]-labeled glycoproteins were quantitated by densitometry of the bands of interest on exposed fluorograms.

Polycynal antibodies against actin-binding protein and against talin were raised in rabbits, affinity-purified, and characterized as described (16). Affinity-purified rabbit polyclonal antibodies against glycoprotein (GP) Ibα, were provided by Dr. Lawrence Fitzgerald of Cor Therapeutics, South San Francisco, CA. Normal rabbit immunoglobulin G was obtained from Sigma. Immunoblotting was performed as described by Towbin et al. (18). Protein concentrations were estimated by the Bio-Rad protein assay.

Procoagulant activity was assayed by a modification of the method of Bever et al. (19). Platelets were incubated with various agents at a concentration of 0.1 × 10^10 to 1.0 × 10^10 platelets/ml. Platelets were then diluted to a final concentration of 0.02–0.25 × 10^10 platelets/ml in a Tyrode’s solution that contained 0.6 mM factor X, (Sigma), 1.1 mM MgCl₂, 0.15 mM CaCl₂, 1.3 μM prothrombin (Sigma). Incubations were stirred for 1–15 min. The amount of thrombin formed was determined by removing 20–50 μl of the incubation and placing it into 1 ml of the chromogenic substrate S2238 (150 μM). Thrombin was determined by a curve that was obtained using purified thrombin. When activation of platelets was induced with thrombin, control assays that contained the same concentration of thrombin (in the absence of platelets) were included. In some experiments, as indicated in the text, intact platelets were removed from suspension by centrifugation at 15,600 × g as described above, and the procoagulant activity remaining in the microvesicle-containing supernatant was assayed. Calpeptin, MDL, and EST had no effect on the assay for procoagulant activity at the concentrations used.

RESULTS

Evidence That Activation of Calpain by Physiological Agonists Causes the Shedding of Microvesicles—Experiments were performed to determine whether activation of calpain in platelets aggregating in response to physiological agonists correlated positively with the shedding of microvesicles. Calpain activation was detected by the hydrolysis of actin-binding protein. The shedding of microvesicles was detected by the presence of plasma membrane GP IIb, in the particulate fraction shed from platelets. As reported previously (1), there was little hydrolysis of actin-binding protein in platelets stirred in the absence of an agonist. Few microvesicles were shed from these platelets (Table I). When platelets were stirred in the presence of thrombin, calpain was activated and caused the hydrolysis of actin-binding protein (data not shown and Ref. 1). The thrombin-induced activation of calpain was accompanied by the increased shedding of microvesicles (Table I). Collagen, another agonist that causes activation of calpain (1), also caused the shedding of microvesicles, as did a combination of the two agonists (Table I). If platelets are incubated with an agonist but the suspension is not stirred, there is little activation of calpain (1, 2, 5). Few microvesicles were shed under these conditions (Table I).

Previously, we have shown that the calpain-induced hydrolysis of actin-binding protein is not detectable until after suspensions of stirred platelets have aggregated (1). Thus, in test further the possibility that the formation of microvesicles is related to activation of calpain, experiments were performed to determine the time at which microvesicles were shed from the platelet membrane. Microvesicle formation was detected by the presence of radiolabeled membrane glycoproteins in particulate material shed from platelets. When platelets were stirred in the presence of collagen, aggregation was under way by 30 s to 1 min after addition of the agonist (Fig. 1A). As indicated by the hydrolysis of actin-binding protein, activation of calpain became detectable only after this time (Fig. 1B). The major shedding of microvesicles also occurred after this time (Fig. 1C). If platelets are incubated with prostaaglandin E₂ prior to addition of an agonist, the intracellular changes that induce platelet responses (for example, the elevated Ca²⁺

<table>
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<tr>
<th>Agonist</th>
<th>GP Ibα in microvesicles</th>
<th>% total platelet GP Ibα</th>
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<td>None (stirred)</td>
<td>0.9</td>
<td></td>
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<tr>
<td>Thrombin, stirred</td>
<td>11.2</td>
<td></td>
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<td>Collagen, stirred</td>
<td>6.6</td>
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<tr>
<td>Collagen and thrombin, stirred</td>
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<tr>
<td>Thrombin, not stirred</td>
<td>3.7</td>
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1 The abbreviations used are: EGTA, ethylenebis(oxethyleneniterol)tetraacetic acid; SDS, sodium dodecyl sulfate; GP, glycoprotein.
Role of Calpain in Microvesicle Shedding

FIG. 1. Temporal correlation among platelet aggregation, activation of calpain, and shedding of microvesicles. Suspensions of platelets in which the surface glycoproteins had been \(^{3}H\)-labeled were preincubated for 5 min in the absence or presence of 5 \(\mu\)M prostaglandin E\(_{1}\) (PGE\(_{1}\)). Suspensions were stirred subsequently in the presence of 20 \(\mu\)g of collagen/ml for the indicated times. Platelet aggregation was detected by an increased light transmittance (panel A). Calpain activation was detected by terminating incubations with an SDS-containing buffer and analyzing the hydrolysis of actin-binding protein (ABP) on immunoblots (panel B). For the detection of microvesicle formation (panel C), incubations were terminated by the addition of EGTA and the removal of intact platelets by centrifugation for 2.5 h at 100,000 \(\times\) g; the sedimented microvesicles were solubilized in an SDS-containing buffer, and electrophoresed through SDS-polyacrylamide gels containing a 5-15\% exponential gradient of acrylamide; \(^{3}H\)-labeled glycoproteins were detected by fluorography. Each lane contained the microvesicles shed from 3 \(\times\) 10\(^{7}\) platelets.

As a direct test of the possibility that microvesicle formation resulted from activation of calpain, platelets were incubated with inhibitors of calpain before the addition of agonist. Calpeptin, at concentrations of 10-20 \(\mu\)g/ml, almost completely prevented hydrolysis of actin-binding protein in aggregating platelets (15). Higher concentrations of calpeptin were required to inhibit the calpain-induced hydrolysis of talin (15). Incubation of platelets with calpeptin before the addition of collagen, thrombin (data not shown), or a combination of the two agonists (Fig. 2) inhibited the shedding of the procoagulant-rich microvesicles from the platelets. Calpeptin almost completely inhibited microvesicle formation at the lower concentrations, i.e. those that were sufficient to inhibit hydrolysis of actin-binding protein. Higher concentrations (200 \(\mu\)g/ml) that also inhibited the hydrolysis of talin (15) had no further inhibitory effect on the generation of microvesicles (Fig. 2). Quantitation of the data shown in Fig. 2 revealed that a concentration of 200 \(\mu\)g/ml of calpeptin/ml inhibited the shedding of 70\% of the microvesicles. MDL and EST, two other membrane-penetrating inhibitors of calpain (13, 14), also inhibited the hydrolysis of actin-binding protein (Fig. 3A) and the shedding of microvesicles (Fig. 3B).

Evidence That Microvesicles Are Shed from Aggregating Platelets Because Actin-Membrane Interactions Are Disrupted—Previously, we provided evidence that the shedding of microvesicles could be induced by disrupting actin-membrane interactions.
brane interactions (9). Because the linkage protein between actin and membrane glycoproteins (actin-binding protein) (21, 22) is hydrolyzed by calpain in aggregating platelets (3), it appeared possible that the calpain-induced shedding of microvesicles induced by physiological agonists resulted from the calpain-induced disruption of actin-membrane interactions. Because of technical problems associated with efficiently solubilizing aggregated platelets, it has not been possible to compare the protein composition of the membrane skeleton of unstimulated platelets with that from platelets in which calpain has been activated by exposure of platelets to a physiological agonist. Therefore, to assess the possibility that the microvesicles shed from platelets incubated with physiological agonists resulted from disruption of the membrane skeleton, we determined whether these microvesicles were similar in composition and function to those generated by agents that disrupt the membrane skeleton (9). Some of the proteins were actin (data not shown), actin-binding protein and its M₉ = 200,000 calpain-generated hydrolytic fragment (Fig. 4A), and talin and its M₉ = 200,000 calpain-generated fragment (Fig. 4B). The identity of the latter two proteins and their hydrolytic fragments was demonstrated on immunoblots. Several other Coomassie Blue-staining proteins were selectively absent from the microvesicles generated by the agonists (see Ref. 9). As seen in Fig. 1, analysis of microvesicles shed from platelets in which the membrane glycoproteins had been radiolabeled showed that the microvesicles contained GP Ib, GP IIb, GP IIIa, and GP IV, but did not contain other H-labeled glycoproteins such as GP V. Again, this composition is the same as that of the microvesicles shed from platelets in which actin-membrane interactions had been disrupted (9).

In Fig. 4, we electrophoresed equal microgram quantities of protein from intact platelets (lanes 1), the platelet-free supernatant (lanes 2), and the supernatant (lanes 3) and sedimented microvesicles (lanes 4) resulting from high speed centrifugation of the platelet-free supernatant. By comparison of lanes 4 with lanes 1, it can be seen that both GP Ibα and GP IIbα were enriched in the microvesicle fraction compared with intact platelets. This is consistent with the microvesicles being depleted selectively of other platelet proteins. Interestingly, however, considerable amounts of platelet GP Ib were also released from platelets (Fig. 4C, lane 2) in a soluble form,
remaining in the high speed supernatant when the microvesicles were sedimented. In contrast, essentially all of the GP Ib, (Fig. 4D) and GP Ibβ and GP IX (data not shown) that was shed from platelets was present in the microvesicles.

**Evidence That the Calpain-induced Generation of Procoagulant Activity May Be Associated with the Calpain-induced Shedding of Microvesicles**—The microvesicles that are shed when actin–membrane interactions are disrupted contain procoagulant activity on their surface (9). It is well known that procoagulant activity is generated on the surface of platelets when they are activated (see Ref. 23 for review). Fig. 5 shows that much of the collagen-induced procoagulant activity was present in the microvesicle-containing supernatant that remained when intact platelets were sedimented. The platelet-free procoagulant activity was enriched in the microvesicles that subsequently were sedimented from the platelet-free supernatant at higher centrifugal forces (Table II). The procoagulant activity was also enriched in the microvesicles compared with the total platelet suspension (data not shown).

We have shown that there are at least two mechanisms by which procoagulant activity is generated on the surface of platelets and that one of these results from an action of calpain (15). To determine whether the calpain-induced shedding of microvesicles was associated with the calpain dependent mechanism of procoagulant generation, we compared the effect of calpeptin on the procoagulant activity in a total platelet suspension with that shed in the form of microvesicles. As reported (15), calpeptin at concentrations that totally inhibited the calpain-induced hydrolysis of actin-binding protein only partially inhibited the collagen-induced generation of procoagulant activity in a platelet suspension (Table III). However, these concentrations of calpeptin almost completely inhibited the generation of that component of procoagulant activity that was shed from the platelet (Table III).

**DISCUSSION**

Although calpain is present in all eukaryotic cells, the function of this protease has remained obscure. The platelet was the first cell in which activation of calpain in response to a physiological agonist was demonstrated (1). However, because only small amounts of calpain substrates are hydrolyzed, some investigators have questioned the likelihood that activation of calpain could lead to any physiological event (24). The present study indicates that activation of calpain is responsible for the shedding of many of the procoagulant-containing microvesicles from the membrane of aggregating platelets.

There have been many reports that platelet-derived membrane vesicles, often referred to as platelet dust, are present in plasma (25–27). More recently, George and co-workers (28) have shown that these microvesicles are present in higher concentrations in the circulation of patients who have undergone cardiac surgery. The formation of microvesicles in platelets...

**FIG. 5.** Collagen-induced procoagulant activity is present in the microvesicle-containing supernatant. Platelet suspensions (1.3 × 10⁹ platelets/ml) were stirred at 37 °C in the presence of 20 μg of collagen/ml. At intervals, the suspensions were diluted to 5.6 × 10⁹ platelets/ml by the addition of factor V, factor X, and prothrombin, and the procoagulant activity (solid line) was measured as described under “Materials and Methods.” At the same intervals, duplicate incubations were centrifuged at 15,600 × g for 1 min, and the prothrombin activity that remained in the microvesicle-containing supernatant was determined (broken line). The supernatants in parentheses show the prothrombinase activity in the supernatant expressed as a percentage of that in the total platelet suspensions.
let concentrates during their storage for blood bank use has been reported (29, 30), and agonists (31–34) and the complement proteins C5b-9 (35) have been shown to induce microvesicle formation in suspensions of washed platelets. Interestingly, the microvesicles detected by others (like the ones generated when calpain is activated) have often been reported to contain procoagulant activity. However, the fact that the shedding of procoagulant-containing microvesicles is a physiological response of platelets to agonists has not been accepted widely, perhaps because the mechanism of formation of the microvesicles has remained obscure. It has often been argued, for example, that microvesicles are shed simply as a consequence of physical agitation of platelet suspensions. The present study shows that microvesicles are not formed by agitation of unstimulated platelet suspensions; rather, they require the presence of a physiological agonist for their formation. Further, the inhibition of microvesicle formation in the presence of prostaglandin E₁, provides a direct demonstration that the formation of microvesicles is a component of the signal-response pathway. Finally, the finding that their formation correlates with the activation of calpain and is inhibited markedly (although not totally) by inhibitors of calpain suggests that many of the microvesicles that form in a suspension of stirred platelets do so as a result of the agonist-induced activation of calpain in platelets.

By analogy to the red blood cell (36), it appears possible that microvesicle shedding could result from decreased association of the membrane skeleton with the cytoplasmic surface of the plasma membrane. In a previous study, we used the nonphysiological agents dibucaine and ionophore A23187 to provide evidence that activation of calpain leads to dissociation of the membrane skeleton from its major site of attachment to the membrane (9). Disruption of the actin-membrane interaction correlated with the shedding of procoagulant-containing microvesicles (9). The association of the membrane skeleton with the plasma membrane is also disrupted when platelets are incubated with N-ethylmaleimide (37). The disruption of actin-membrane interactions by this agent is also accompanied by the shedding of procoagulant-containing microvesicles. Several lines of evidence suggest that the microvesicles shed when calpain is activated by physiological agonists may also result from disruption of actin-membrane interactions. First, calpeptin inhibited the shedding of microvesicles at concentrations that were sufficient to inhibit the calpain-induced hydrolysis of actin-binding protein (a component of the membrane skeleton that links this structure to the plasma membrane) (21, 22), but not talin. Second, the composition of the microvesicles was similar to that of the microvesicles generated when the membrane skeleton was disrupted. Third, like the microvesicles shed when actin-membrane interactions are disrupted, those shed from aggregating platelets had procoagulant activity on their surface.

Other investigators have concluded that shedding of procoagulant-containing microvesicles is not caused by activation of calpain (10, 11, 38). Perhaps one reason for the conflicting conclusions has been the assumption that there is only one mechanism involved in the shedding of microvesicles. The present study suggests that this may not be the case. Collagen and thrombin caused some microvesicles to be shed under conditions in which calpain was not activated (e.g. thrombin in the absence of stirring), but once calpain was activated, there was a large increase in the amount of microvesicles that were shed. In the former case, shedding of the vesicles may have been caused by the calpain-independent mechanism by which C5b-9 proteins induce the shedding of microvesicles (11). In the latter case, as the present study suggests, the shedding of microvesicles could result from the calpain-induced hydrolysis of the membrane skeleton. It is conceivable that the calpain-independent shedding could result from a different reorganization of the membrane skeleton. One could envisage a mechanism, for example, in which the binding of Ca²⁺/calmodulin to spectrin (which is a component of the platelet membrane skeleton) (4) affects organization of the membrane skeleton (39).

A second reason for the differing conclusions about the role of calpain in the generation of microvesicles (10, 11, 38) may be the assumption that the generation of procoagulant activity is always associated with the shedding of microvesicles. Our recent study indicates, however, that there are at least two mechanisms by which procoagulant activity is generated (9). The major component of the procoagulant activity is generated under conditions in which calpain is not activated (and few microvesicles are shed). This procoagulant activity presumably results from an event such as activation of prebound factor V (24) or the Ca²⁺-induced activation of aminotranslocase (38). Because few microvesicles are shed under these conditions (at least when thrombin or collagen is the agonist), most of this procoagulant activity presumably remains on the surface of the platelet.

Once calpain is activated, however, there is a small increase in the generation of procoagulant activity (15) and a large increase in the amount of microvesicles that are shed. The calpain-induced component of the procoagulant activity is present primarily on the microvesicles that are shed from the plasma membrane. The presence of this procoagulant activity on the microvesicles could be a result of externalization of the negatively charged phospholipids (which provide the binding sites for the prothrombinase complex) during the physical process of microvesicle shedding. Alternatively, the membrane skeleton normally could participate in maintaining the asymmetric distribution of the negatively charged phospholipids, or activation of calpain could inhibit directly the activity of aminotranslocase, thus preventing the re-internalization of the phospholipids. In either of the latter two cases, activation of calpain would result in a loss of phospholipid asymmetry in the same areas of the membrane as were shed subsequently from the platelet. Future studies will be needed to distinguish between these potential mechanisms.

A third reason for disagreement over the mechanism involved in microvesicle shedding may result from the fact that different methods have been used to detect microvesicles. In our studies, we have isolated the microvesicles by differential centrifugation. The present study shows that while some GP Ibₐ is shed from the surface of aggregating platelets because it is a component of the microvesicles, much more is lost in the form of a soluble protein that we recently have shown to be glycopallidin. In contrast, essentially all of the GP Ibₐ, GP Ibₜ, and GP IX that was lost from the platelet was present in microvesicles. This is interesting because the shedding of microvesicles has often been assayed by flow cytometry using antibodies directed against GP Ibₐ to identify platelet-derived material (10). The use of GP Ibₐ, GP IX, or GP Ibₜ antibodies might provide a more reliable method for quantitating the percentage of plasma membrane that is shed as microvesicles. Further, the microvesicles range in size from approximately 800 nm in diameter down to approximately 40 nm (9). This raises the possibility that analysis of microvesicle formation by flow cytometry may underestimate the formation of microvesicles, unless the gating is such that particles as small as 40 nm can be detected.

In the past, the function of the microvesicles shed from
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Calpain was largely ignored. However, the present demonstration that these are formed as a consequence of post-aggregation stages of platelet activation and the fact that they are present in the circulation (25–28) suggest that their production represents an important consequence of the later stages of platelet activation. This is significant because it suggests that the presence of microvesicles in the circulation may provide a marker for determining whether platelet thrombi are forming. Because the microvesicles contain procoagulant activity, one of their major functions could be to accelerate coagulation. Therefore, it will be of interest to determine whether the microvesicles contain more procoagulant activity per unit of membrane than areas of the membrane that remain associated with the platelet. Although the present study provides evidence that the procoagulant activity is enriched in the microvesicles per unit of protein, it does not comment on the potential enrichment per unit of membrane area. Even if the microvesicles are not enriched in procoagulant activity per unit of membrane, it appears likely that the shedding of microvesicles may provide a mechanism for effectively increasing the accessibility of procoagulant membrane glycoproteins that can bind to components of the extracellular matrix, microvesicles that break away from the aggregate presumably could bind to the vessel wall and thus stabilize the clot by anchoring the developing fibrin network to sites other than those to which the initial layer of adherent platelets is attached.

We conclude from the present study that activation of calpain in aggregating platelets is responsible for the shedding of procoagulant-rich microvesicles from these cells. Calpain may therefore be critical in enabling platelets to perform their hemostatic function in the circulation effectively. It appears likely that the microvesicles form because calpain activation induces local disruption of the association of the platelet membrane skeleton with the plasma membrane. Because the plasma membrane of other cells probably is lined by a membrane skeleton (36, 40), our findings on the role of calpain in regulating properties of the platelet plasma membrane may have relevance to other cell types. Many of the proteins that are assumed to mediate actin-membrane interactions (for example, actin-binding protein (3), spectrin (4, 41), talin (5), protein 4.1 (42), α-actinin (43), and vinculin (44)) are present in other cells and are substrates for calpain. Activation of calpain has been implicated in the regulation of the activity of the glutamate receptor in hippocampus synaptic plasma membranes (45), and leupeptin has been shown to inhibit the production of microvesicles that is induced in hepatocytes when intracellular Ca2+ concentrations increase (46). Hence, it is possible that, as in the platelet, calpain-induced changes in a membrane skeleton might regulate actin-membrane interactions and, thereby, properties of the plasma membrane in these cell types.

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