Role of the Membrane Skeleton in Preventing the Shedding of Procoagulant-rich Microvesicles from the Platelet Plasma Membrane

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Abstract. The platelet plasma membrane is lined by a membrane skeleton that appears to contain short actin filaments cross-linked by actin-binding protein. Actin-binding protein is in turn associated with specific plasma membrane glycoproteins. The aim of this study was to determine whether the membrane skeleton regulates properties of the plasma membrane. Platelets were incubated with agents that disrupted the association of the membrane skeleton with membrane glycoproteins. The consequences of this change on plasma membrane properties were examined. The agents that were used were ionophore A23187 and dibucaine. Both agents activated calpain (the Ca^{2+}-dependent protease), resulting in the hydrolysis of actin-binding protein and decreased association of actin with membrane glycoproteins. Disruption of actin–membrane interactions was accompanied by the shedding of proco-agulant-rich microvesicles from the plasma membrane. The shedding of microvesicles correlated with the hydrolysis of actin-binding protein and the disruption of actin–membrane interactions. When the calpain-induced disruption of actin–membrane interactions was inhibited, the shedding of microvesicles was inhibited. These data are consistent with the hypothesis that association of the membrane skeleton with the plasma membrane maintains the integrity of the plasma membrane, preventing the shedding of procoagulant-rich microvesicles from the membrane of unstimulated platelets. They raise the possibility that the procoagulant-rich microvesicles that are released under a variety of physiological and pathological conditions may result from the dissociation of the platelet membrane skeleton from its membrane attachment sites.

PLATELETS are rich in cytoskeletal proteins that regulate the contractile responses of these cells. For example, granule contents are secreted as the granules are centralized by a ring of contracting actin filaments and associated myosin, and fibrin clots are retracted when the interaction of myosin with actin filaments generates sufficient tension to retract the filopodia to which fibrin is bound (see Fox, 1987; Nachmias and Yoshida, 1988, for reviews).

The role of the cytoskeleton in the unstimulated platelet is less well understood. We have considered whether the cytoskeleton might play a role in regulating properties of the plasma membrane. Like the plasma membrane of other cells, the platelet plasma membrane consists primarily of a lipid bilayer with inserted proteins. It is not clear what stabilizes the membrane, preventing it from fragmenting, or what determines its contours, maintains the phospholipid asymmetry, or directs the lateral distribution or function of glycoproteins in the membrane. It is often suggested that submembranous actin filaments are responsible for regulating these properties of membranes. Although filaments in platelets, as in other cells, run beneath the plasma membrane, and although both lateral associations and end-on associations of the filaments with the membrane have been described (Nachmias, 1983; Karlsson et al., 1984; Boyles et al., 1985; Escolar et al.,

1986; Hartwig and Shevlin, 1986; Nakata and Hirokawa, 1987), it is hard to envisage how such filaments could impart the requisite flexibility to the membrane and allow the rapid changes in cell shape that can occur in response to stimulation. It seems that a separate structure that coats the lipid bilayer would be better suited for regulating these properties of the membrane and would allow their modulation by mechanisms other than those that regulate the contractile activities of the cytoplasmic actin filaments.

In fact, the only cell type in which these properties of the plasma membrane have been clearly shown to be regulated by the cytoskeleton is the human red blood cell. This cell does not contain cytoplasmic actin filaments, but rather a membrane skeleton composed of short actin filaments cross-linked by spectrin that is closely adherent to the plasma membrane (for reviews, see Bennett, 1985; Marchesi, 1985). It seems likely that other cells may contain a membrane skeleton in addition to the networks of actin filaments that fill their cytoplasm (Ben-Ze'ev et al., 1979; Apgar et al., 1985).

In recent years, we have provided evidence that platelets do indeed contain such a structure (Fox, 1985a; Fox and Boyles, 1988; Fox et al., 1988). Most of the actin filaments in platelets exist in a three-dimensional network that can be

visualized by thin-section microscopy, sedimented at low g forces from Triton X-100-lysed platelets, and depolymerized when Ca^{2+} is present in the lysis buffer (presumably as a result of the action of the Ca^{2+}-dependent protein, gelsolin). These filaments are not associated with any membrane glycoprotein that we can detect in unstimulated platelets (Fox, 1985a), but become associated with myosin (Fox and Phillips, 1982; Carroll et al., 1982) and the glycoprotein (GP)[1] IIb-IIIa complex (Phillips et al., 1980) after platelet activation. Presumably, these filaments are the ones that are involved in the contractile responses of cells. However, a small amount of the actin filaments in Triton lysates has very different properties. In contrast to the filaments involved in contractile functions, these filaments remain in the low-speed supernatant of platelets lysed with the conventional Triton X-100 lysis buffers; they can be sedimented only under high g forces (Fox, 1985a). They are resistant to the action of gelsolin (Fox, 1985a; Fox et al., 1988) and are selectively associated with specific membrane glycoproteins (Fox, 1985b). The filaments were identified as such because they bind phalloidin, but they are too short to be visualized by thin-section microscopy (Fox et al., 1988). They were shown to be a component of a structure that has an amorphous appearance and exists as a continuous layer that apparently follows the contours of the plasma membrane (Fox et al., 1988). We suggested that this structure represents a membrane skeleton similar to the one that coats the lipid bilayer of the red blood cell plasma membrane. Although this structure is probably associated with the cytoplasmic actin filaments in intact platelets (Fox et al., 1988), it is readily dissociated from these filaments in Triton X-100 lysates.

Our aim in the present study was to determine whether the membrane skeleton regulates properties of the plasma membrane. The approach that we used to address this question was to incubate platelets with agents that dissociate the membrane skeleton from the plasma membrane and to determine the consequences of this dissociation. Disruption of the actin–membrane interactions was accompanied by shedding of procoagulant-rich microvesicles from the plasma membrane. These results suggest that the membrane skeleton is important in maintaining the integrity of the membrane in the unstimulated platelet. They raise the possibility that the procoagulant-rich microvesicles that are shed from platelets aggregating in response to physiological agonists may result from activation-induced modification of the membrane skeleton.

Materials and Methods

Incubation of Platelet Suspensions

Venous blood was drawn from healthy adult donors. Platelets were isolated by centrifugation and were radiolabeled as described previously (Fox, 1985a). Surface glycoproteins were ^3H-labeled by the sodium metaperiodate/sodium [^3H]borohydride method, as described previously (Fox, 1985a). Platelets were resuspended at 37°C in a Tyrode's buffer containing 138 mM sodium chloride, 2.9 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose, 1.8 mM calcium chloride, and 0.49 mM magnesium chloride, pH 7.4, at a final concentration of 1×10^9 platelets/ml. Disruption of the actin–membrane interactions was achieved by incubating platelet suspensions at 37°C with 0.4–1.0 μM ionophore A23187 (Sigma

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1. *Abbreviation used in this paper*: GP, glycoprotein.

Chemical Co., St. Louis, MO) or 0.5 mM dibucaine-HCl (Sigma Chemical Co.). Ionophore A23187 was added in a final concentration of 0.2% (vol/vol) DMSO (J. T. Baker Chemical Co., Phillipsburg, NJ), while dibucaine was added in saline. In some experiments, as indicated, platelets were preincubated for 30 min at 37°C with 1 mg of leupeptin/ml or for 1–5 min with 10–100 μg of calpeptin/ml (Tsujinaka et al., 1988). Like leupeptin, calpeptin is an inhibitor of sulfhydryl proteases such as calpain. However, unlike leupeptin, calpeptin readily crosses the plasma membrane and does not inhibit the amidolytic activity of thrombin. Leupeptin and calpeptin were added to the platelet suspensions in a final concentration of 0.1–0.2% (vol/vol) DMSO. Leupeptin was obtained from Vega Biochemicals (Tucson, AZ), and calpeptin was the generous gift of Dr. Toshimasa Tsujinaka of Osaka University Medical School, Osaka, Japan.

Isolation of Subcellular Fractions

Platelets were lysed by the addition of an equal volume of buffer containing 2% (vol/vol) Triton X-100 (Sigma Chemical Co.), 10 mM EGTA, 100 mM Tris-HCl, pH 7.4. Triton X-100-insoluble actin filaments and Triton X-100-soluble fractions were separated by centrifugation of platelet lysates at 100,000 g for 2.5 h. Both cytoplasmic actin filaments and the filaments of the membrane skeleton sediment under these conditions (Fox, 1985a).

Isolation and Analysis of Platelet Microvesicles

Platelets were incubated with agents that disrupted the membrane skeleton. Intact platelets were then sedimented by centrifugation of the platelet suspension at 15,600 g for 1 min at ambient temperatures. The microvesicles were isolated from the 15,600-g supernatant by sedimentation at 100,000 g for 2.5 h, or by filtration of the supernatant through polycarbonate filters (0.2 μm; Millipore Corp., Bedford, MA).

For visualization of microvesicles, 200 mM EGTA (pH 9.0) was added to the platelet suspensions to a final concentration of 5 mM. Intact platelets were immediately removed by centrifugation, and microvesicles were isolated from the resulting supernatant on polycarbonate filters. The filters were fixed with 0.2 M glutaraldehyde, 60 mM cacodylate buffer, pH 7.4. Samples were then processed for EM as described previously (Fox et al., 1988).

Analytical Procedures

Samples were analyzed on SDS-polyacrylamide gels in the presence of reducing agent by the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue, and stained proteins quantitated by densitometry. The membrane glycoproteins of ^3H-labeled platelets were detected by fluorography of gels treated with EN^{3}HANCE (New England Nuclear, Boston, MA).

Polyclonal antibodies against actin-binding protein and talin were raised in rabbits, affinity purified, and characterized as described previously (Fox et al., 1985). Polyclonal antibodies against GP IIb-IIIa were provided by Dr. David Phillips of COR Therapeutics, Inc. (South San Francisco, CA). Normal rabbit IgG was obtained from Sigma Chemical Co. Immunoblotting was performed as described by Towbin et al. (1979).

Protein concentrations were estimated by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Procoagulant activity was assayed as described by Bevers et al. (1982). This assay was not affected by the concentrations of calpeptin that were present.

Results

Loss of Actin–Membrane Interactions in Platelets in which Calpain Was Activated by Dibucaine or Ionophore A23187

The GP Ib–IX complex is the major site of attachment of the membrane skeleton to the plasma membrane (Fox, 1985a). This complex consists of two disulfide-linked subunits, GP Ib$_\alpha$ ($M_r = 145,000$) and GP Ib$_\beta$ ($M_r = 24,000$), that are complexed in a 1:1 ratio with another membrane glycoprotein, GP IX ($M_r = 22,000$). Previously (Fox, 1985b), we have shown that actin–membrane interactions are dissociated in platelets in which calpain is activated with ionophore

A. Immunoblot

minus calpeptin   plus calpeptin

ABP—
200K—
100K—
91K—

0 .5 1 5 10 15 0 .5 1 5 10 15

B. Fluorogram

minus calpeptin   plus calpeptin

$GPIb_\alpha$—

$GPIb_\beta$—
GP IX —

0 .5 1 5 10 15 0 .5 1 5 10 15
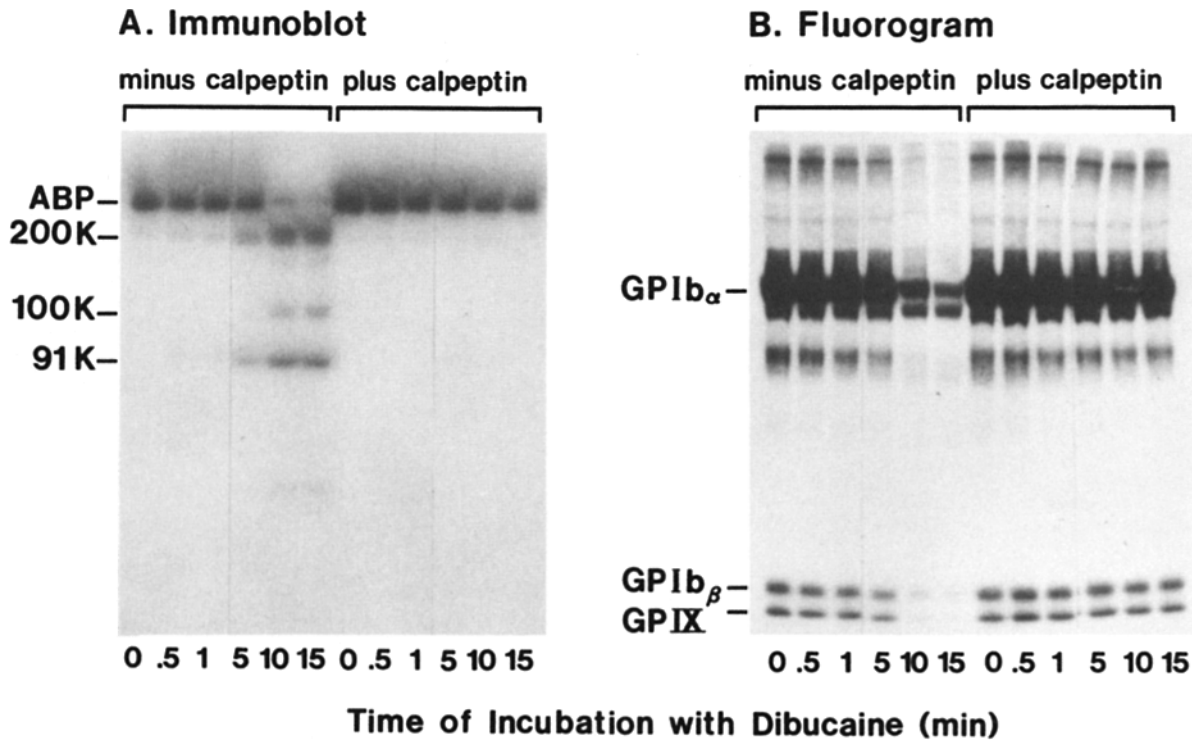
Time of Incubation with Dibucaine (min)

Figure 1. SDS-polyacrylamide gels showing calpain-induced hydrolysis of actin-binding protein and decreased association of the GP Ib–IX complex with actin filaments in platelets incubated with dibucaine. Suspensions of ^{3}H-labeled platelets were incubated for 5 min in the presence or absence of 50 μg of calpeptin/ml. 0.5 mM dibucaine was then added. At the indicated intervals, aliquots were solubilized in an SDS-containing buffer and proteins were separated on SDS-polyacrylamide gels containing 7.5% acrylamide. Hydrolysis of actin-binding protein was detected on immunoblots using actin-binding protein antibodies (*A*). Additional aliquots were removed from the incubation and solubilized in a Triton X-100-containing buffer; the Triton X-100-insoluble actin filaments (*B*) and Triton X-100-soluble fractions (data not shown) were isolated, solubilized in an SDS-containing buffer, and analyzed on SDS-polyacrylamide gels containing a 5–15% exponential gradient of acrylamide. ABP, actin-binding protein; 200K, 100K, and 91K are hydrolytic fragments of M_r = 200,000, 100,000, and 91,000, respectively, that are generated when actin-binding protein is hydrolyzed within platelets by calpain (Fox et al., 1985). Samples containing total platelet suspensions originated from 3 × 10^7 platelets; those containing Triton X-100-insoluble actin filaments originated from 6 × 10^7 platelets.

A23187, as shown by decreased recovery of GP Ib$_\alpha$ along with the insoluble actin filaments that are sedimented after lysis of platelet suspensions with Triton X-100. Another agent that activates calpain, in this case without activating the platelets, is dibucaine (Nachmias et al., 1979). Fig. 1 shows that hydrolysis of actin-binding protein in dibucaine-treated platelets (Fig. 1 *A*) was accompanied by dissociation of the GP Ib-IX complex from the membrane skeleton, as indicated by decreased sedimentation of the three components of the GP Ib-IX complex (GP Ib$_\alpha$, GP Ib$_\beta$, and GP IX) with the Triton X-100-insoluble actin filaments (Fig. 1 *B*) and increased recovery of the three glycoproteins in the Triton X-100-soluble fraction (data not shown).

In platelets aggregating in response to the physiological agonist thrombin, the amount of actin-binding protein that is hydrolyzed is small (estimated from immunoblots to be less than 10% of the total) (Fox et al., 1983). To determine whether hydrolysis of this small amount of actin-binding protein was sufficient to cause dissociation of the membrane skeleton from plasma membrane glycoproteins, we incubated platelets with dibucaine under conditions in which the extent of hydrolysis of actin-binding protein was comparable to that which occurred in aggregating platelets. Since the sensitivity of different platelet suspensions to dibucaine ap-

peared to vary considerably (in some suspensions actin-binding protein was completely hydrolyzed within minutes of addition of 0.5 mM dibucaine, whereas other platelets required 30 min before hydrolysis was detectable), time-course experiments were performed. In the experiment shown in Fig. 1, for example, incubation of the platelets with dibucaine for 5 min was sufficient to induce hydrolysis of amounts of actin-binding protein that were comparable to those hydrolyzed in

Table I. Disruption of Actin–Membrane Interactions in Platelets Exposed to Dibucaine

Addition to platelet suspension	GP Ib$_\alpha$		Filamentous actin*
	Triton X-100-insoluble	Triton X-100-soluble	
	% total platelet GP Ib$_\alpha$		*% of total*
None	60.0 ± 4.9	40.1 ± 1.7	67.8 ± 6.1
Dibucaine	14.7 ± 2.7	78.2 ± 6.8	58.6 ± 3.6

Suspensions of platelets that had been surface-labeled by the sodium metaperiodate/sodium [^{3}H]borohydride method were incubated for 5 min with no addition or with 0.5 mM dibucaine. Values given are the mean ± SE from three donors.
* Sedimented actin expressed as a percentage of the total platelet actin.

A. Immunoblot

minus leupeptin | plus leupeptin

ABP_
200K⁻

100K_
91K⁻

0 2.5′ 5′ 7.5′ 10′ 0 2.5′ 5′ 7.5′ 10′

B. Fluorogram

minus leupeptin | plus leupeptin

GPI a_
GPIb$_\alpha$-

GPIIb$_\alpha$-
GPIIIa⁻

GPIV_
GPV⁻

GP Ib$_\beta$⤸
GPIX⤹

0 0 15′ 15′
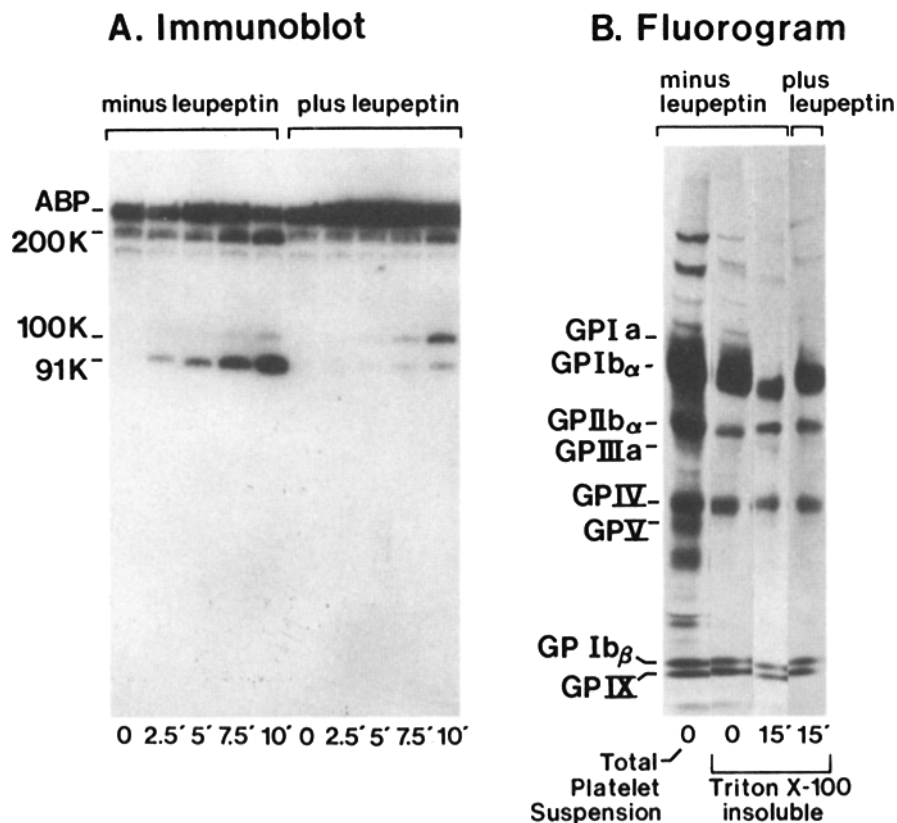
Total⤴
Platelet
Suspension

Triton X-100
insoluble

Figure 2. SDS-polyacrylamide gels showing the effect of leupeptin on the dibucaine-induced hydrolysis of actin-binding protein and release of membrane glycoproteins from the membrane skeleton. Platelets were surface-labeled by the sodium metaperiodate/sodium [³H]-borohydride method and suspended at 1×10^9 platelets/ml in a Tyrode's buffer. Suspensions were incubated in the presence or absence of 1 mg of leupeptin/ml for 30 min at 37°C. Dibucaine was then added to a final concentration of 0.5 mM. (A) At intervals, aliquots of platelet suspensions were solubilized in an SDS-containing buffer and electrophoresed on SDS-polyacrylamide gels containing 7.5% acrylamide, and the polypeptides were transferred to nitrocellulose paper. Actin-binding protein (ABP) and its products of hydrolysis by calpain (200K, 100K, and 91K) were detected with antibodies against actin-binding protein. (B) Suspensions were solubilized directly into an SDS-containing buffer (total platelet suspension) or were lysed by addition of a Triton X-100-containing buffer. Triton X-100-insoluble filaments were isolated by centrifugation of the lysates at 100,000 g for 2.5 h and solubilized in an SDS-containing buffer. Samples were electrophoresed through SDS-polyacrylamide gels containing a 5-15% exponential gradient of acrylamide. ³H-labeled glycoproteins were detected by fluorography.

aggregating cells (i.e., <10% of the total). Quantitation of the data presented in Fig. 1 B, shows that hydrolysis of these small amounts of actin-binding protein was sufficient to cause considerable release of the GP Ib–IX complex from the membrane skeleton. Thus, using decreased sedimentation of GP Ib$_\beta$ with Triton-insoluble actin filaments as a marker for release of the complex from the membrane skeleton, 27% of the complex was released after 1 min, and 44% after 5 min of incubation. Using GP IX as a marker, 21% of the complex was released after 1 min, and 42% after 5 min. The decreased sedimentation of this glycoprotein complex occurred without any significant decrease in the amount of sedimentable actin filaments (Table I). Thus, the glycoproteins were presumably released from the membrane skeleton because the linkage protein, actin-binding protein, was hydrolyzed.

To confirm that there was a causal relationship between the dibucaine-induced hydrolysis of actin-binding protein and dissociation of glycoproteins from the membrane skeleton, platelets were preincubated with calpain inhibitors. One inhibitor was calpeptin, a recently synthesized inhibitor of calpain that readily crosses membranes (Tsujinaka et al., 1988). Calpain initially hydrolyzes actin-binding protein into two fragments of $M_r = 200,000$ and 100,000 and then hydrolyzes the $M_r = 100,000$ fragment such that a $M_r = 91,000$ polypeptide is generated (Fox et al., 1985). Calpeptin totally inhibited both of these actions of calpain (Fig. 1 A). Inhibi-

tion of the dibucaine-induced hydrolysis of actin-binding protein was accompanied by an inhibition of the dibucaine-induced release of the plasma membrane glycoproteins from the membrane skeleton (Fig. 1 B). The ionophore A23187-induced hydrolysis of actin-binding protein and dissociation of the GP Ib–IX complex from the membrane skeleton were also totally inhibited by calpeptin (data not shown). The second calpain inhibitor that was used was leupeptin. Although leupeptin does not readily cross cell membranes, we found that by preincubating the platelets with 1 mg of leupeptin/ml for 30 min, sufficient leupeptin entered the platelets to produce a partial inhibition of the subsequent dibucaine-induced hydrolysis of actin-binding protein (Fig. 2 A). A similar level of inhibition was reported recently by other workers (Tsujinaka et al., 1988); these workers also found that preincubation of the platelets with high concentrations of leupeptin was needed to effect entry of the inhibitor into platelets. Leupeptin also caused partial inhibition of disruption of actin-membrane interactions (Fig. 2 B, compare fourth lane with third lane).

Disruption of the Actin–Membrane Interactions Is Accompanied by Shedding of Microvesicles from the Plasma Membrane

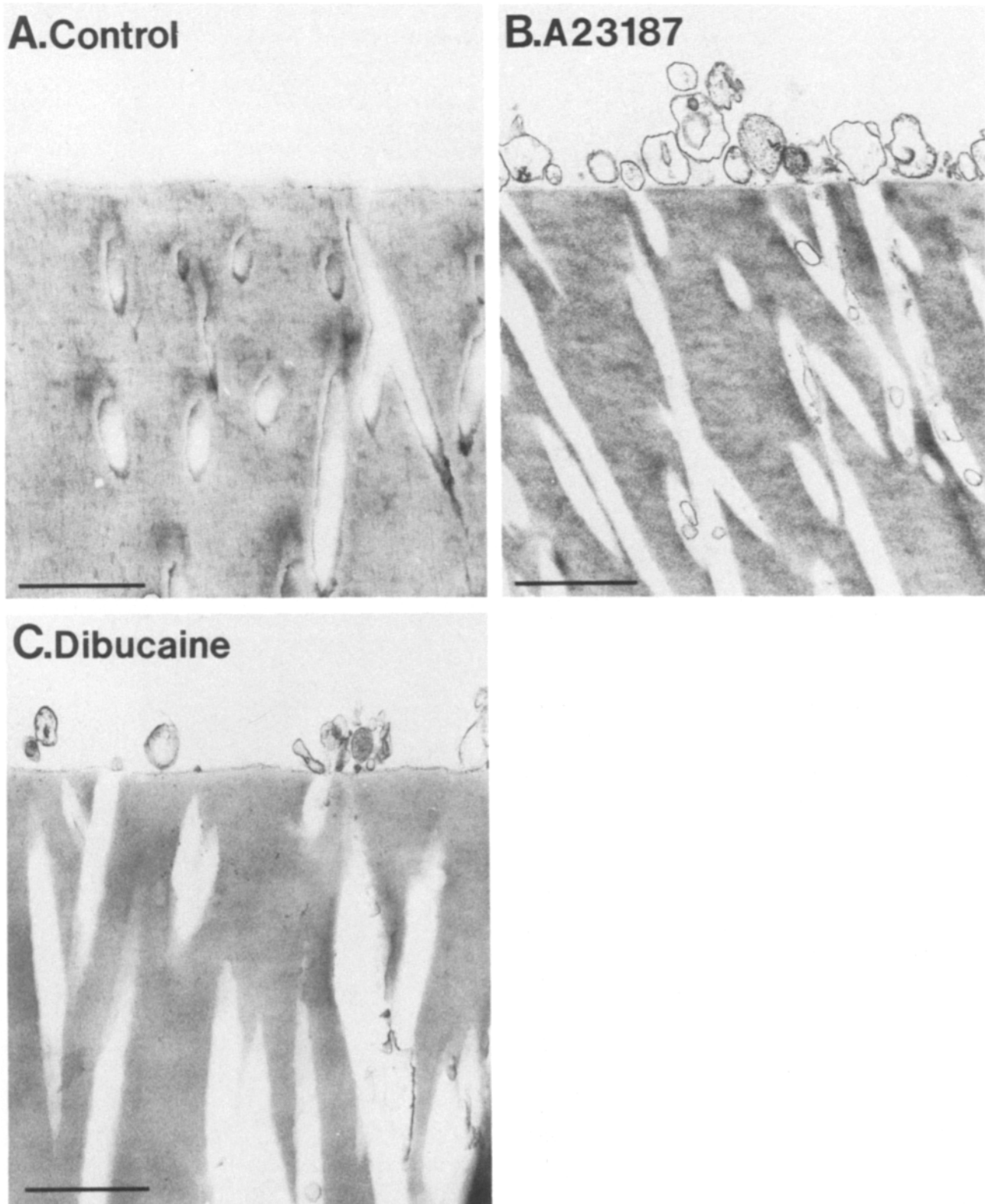By analogy to the red blood cell (Lux, 1979; Mohandas et

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Figure 3. Electron micrographs of microvesicles that were released during incubation of platelet suspensions with agents that disrupted actin–membrane interactions. Platelet suspensions were incubated alone (*A*) or in the presence of 0.4 μM ionophore A23187 for 5 min (*B*) or 0.5 mM dibucaine for 5 min (*C*). Intact platelets were sedimented by centrifugation of the suspensions at 15,600 g for 1 min. Microvesicles were isolated from the resulting supernatant by filtering the supernatant through polycarbonate filters that had a 0.2-μm pore size. The polycarbonate filters were fixed and then examined by EM. Microvesicles were present in suspensions of platelets in which the actin–membrane interactions had been disrupted. These vesicles ranged in size from ~800 nm in diameter to vesicles that were small enough to enter the pores of the filters. Many of the vesicles were empty, whereas others contained an amorphous material. Bar, 1 μm.

GPIa⌐
GPIb_α-
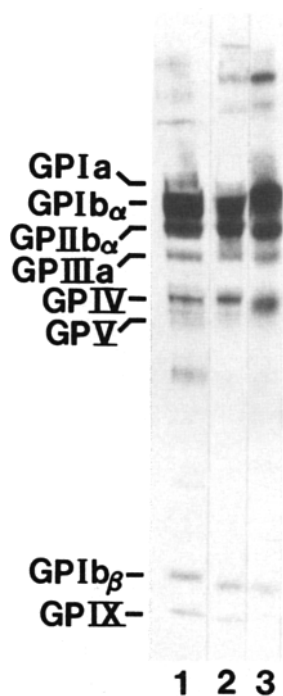GPIIb_α⌐
GPIIIa⌐
GPIV-
GPV⌐

GPIb_β-
GPIX-

1 2 3

Figure 4. SDS-polyacrylamide gels showing the surface glycoprotein composition of the microvesicles shed from the plasma membrane during exposure of platelets to agents that disrupted the membrane skeleton. Platelets in which the surface membrane glycoproteins had been ³H-labeled were solubilized directly with an SDS-containing buffer (lane *1*) or were incubated with 0.5 mM dibucaine for 10 min (lane *2*) or 1 μM ionophore A23187 for 10 min (lane *3*). Suspensions were centrifuged at 15,600 g for 1 min to sediment intact platelets. The material remaining in the supernatant was centrifuged at 100,000 g for 2.5 h to obtain the microvesicles. Microvesicles were electrophoresed through SDS-gels containing a 5–15% exponential gradient of acrylamide. ³H-labeled membrane glycoproteins were detected by fluorography. Lane *1* contained 3 × 10⁷ platelets from a total platelet suspension; lane 2 contained the microvesicles isolated from 24 × 10⁷ of the platelets shown in lane *1*. Lane *3* contained microvesicles isolated from 6 × 10⁷ platelets in a separate experiment.

intact platelets were ³H-labeled by the sodium metaperiodate/sodium [³H]borohydride technique and platelets were incubated with agents that decreased association of the membrane skeleton with the plasma membrane. At the end of the incubation, intact platelets were removed by low-speed centrifugation. The microvesicles were isolated by high-speed centrifugation of the platelet-free supernatant. Glycoproteins present on the microvesicles (Fig. 4) were identified (on two-dimensional nonreduced-reduced SDS gels [data not shown]) as GP Ia, the three components of the GP Ib–IX complex, GP IIb-IIIa and GP IV. At least one glycoprotein (GP V) was absent. Based on these observations, we chose to assay the amount of microvesicles by the amount of GP Ib_α, GP IIIa, GP Ib_β, or GP IX shed from the platelet.

As an initial test of the idea that microvesicles were shed because actin–membrane interactions were disrupted, the time courses of actin-binding protein hydrolysis, release of the GP Ib–IX complex from the membrane skeleton, and the shedding of microvesicles were compared. As shown in Fig. 5, the time courses of the three events were quite similar. Interestingly, the shedding of microvesicles appeared to lag behind the hydrolysis of actin-binding protein and release of GP Ib–IX from the membrane skeleton. Only a small amount of GP Ib–IX was present in the microvesicles compared with the amount that was released from the membrane skeleton. These results are consistent with the hypothesis that several actin–membrane interactions must be disrupted before a region of membrane is destabilized sufficiently to bleb off.

al., 1983), we predicted that the membrane skeleton might contribute to maintaining the stability of the plasma membrane; thus, one consequence of disruption of the actin–membrane interactions might be the shedding of microvesicles from the plasma membrane. To test this hypothesis, platelets were incubated with the agents that decreased the association of the membrane skeleton with the plasma membrane. Incubations were terminated by sedimentation of intact platelets at 15,600 g. The resulting platelet-free supernatant was passed through filters, and the filters (Fig. 3, *A–C*) were examined morphologically to determine whether any microvesicles were present. Few microvesicles were recovered from suspensions of control platelets (Fig. 3 *A*). However, microvesicles were present in suspensions of platelets in which the membrane skeleton had been disrupted by either ionophore A23187 (Fig. 3 *B*) or dibucaine (Fig. 3 *C*). The vesicles varied considerably in size, from ∼800 nm in diameter to vesicles as small as 40 nm that were visualized in the 200-nm pores of the filters. Many of the vesicles appeared empty. Others contained an amorphous material whose appearance resembled the membrane skeleton that we have described previously (Fox et al., 1988). Presumably, the vesicles were shed from regions of the plasma membrane that had lost their association with the membrane skeleton; the vesicles that contained amorphous material may have formed by fusion of two separate regions that had lost actin–membrane interactions and by their detachment along with the intervening region of membrane, which remained associated with pieces of the submembranous skeleton.

To be able to quantitate the formation of microvesicles, plasma membrane glycoproteins present on the microvesicles were identified. The surface-membrane glycoproteins of
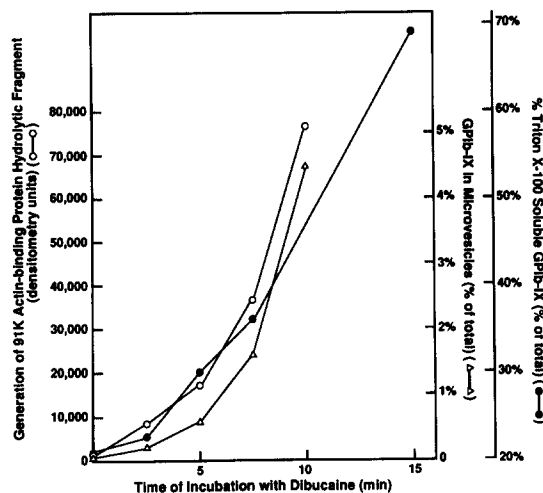

Figure 5. Time course of dibucaine-induced hydrolysis of actin-binding protein, release of the GP Ib–IX complex from the membrane skeleton, and the shedding of microvesicles from the plasma membrane. Platelets in which the surface-membrane glycoproteins had been ³H-labeled were incubated with 0.5 mM dibucaine for 0–15 min. The extent of hydrolysis of actin-binding protein (*ABP*) was assessed by quantitation of the amount of the $M_r = 91,000$ hydrolytic fragment by densitometry of immunoblots. The release of the GP Ib–IX complex from the membrane skeleton was assessed by the amount of GP Ib_β and GP IX that were soluble in Triton X-100, as quantitated by densitometry of autoradiograms. The shedding of microvesicles from the plasma membrane was assessed by the release of GP Ib_β and GP IX from the platelet surface, as quantitated by densitometry of autoradiograms. (●), Triton X-100-soluble GP Ib_β plus GP IX; (○) 91-kD hydrolytic fragment of actin-binding protein; (△) GP Ib_α plus GP IX in the microvesicles.
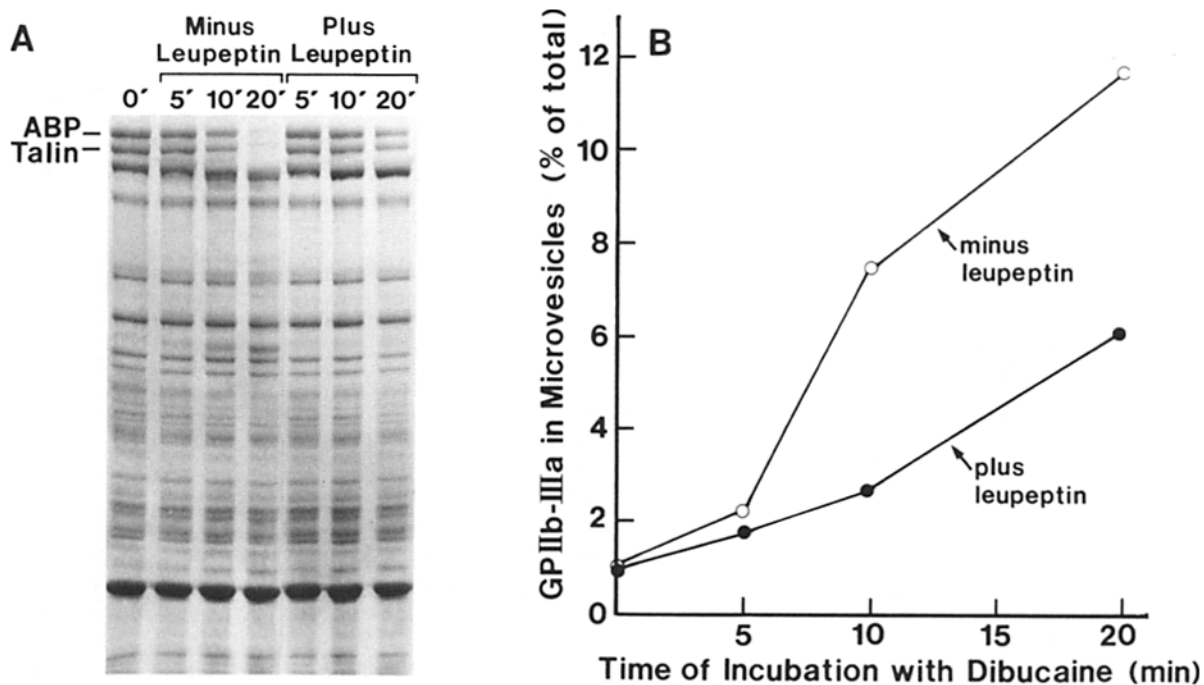
Figure 6. Effect of leupeptin on the dibucaine-induced hydrolysis of actin-binding protein and shedding of microvesicles from the plasma membrane. Suspensions of unlabeled platelets were incubated for 30 min in the presence or absence of 1 mg of leupeptin/ml. Dibucaine was then added to a final concentration of 0.5 mM. (*A*) At intervals, aliquots of platelet suspensions were solubilized in an SDS-containing buffer and electrophoresed on SDS-polyacrylamide gels containing 7.5% acrylamide. The polypeptides were detected with Coomassie brilliant blue. (*B*) At intervals, intact platelets were removed from suspension by centrifugation at 15,600 *g* for 1 min. The resulting platelet-free supernatant was dotted onto nitrocellulose paper, which was incubated with antibodies against the GP IIb–IIIa complex and then with ^{125}I-labeled protein A. The amount of GP IIb–IIIa was quantitated by densitometry of autoradiograms and expressed as a percentage of the total amount of GP IIb–IIIa in the platelet suspension.

As a further test of the idea that microvesicles were shed as a consequence of dissociation of the membrane skeleton from the plasma membrane, platelets were preincubated with inhibitors of calpain prior to the addition of dibucaine or ionophore A23187. These inhibitors decreased the shedding of microvesicles from the plasma membrane. Thus, leupeptin, which partially inhibited dibucaine-induced hydrolysis of actin-binding protein (Fig. 6 *A*), partially inhibited the shedding of microvesicles (Fig. 6 *B*). Calpeptin, which totally inhibited the disruption of actin–membrane interactions (Fig. 1 *B*) almost totally inhibited the dibucaine-induced (Fig. 7 *A*) and the ionophore A23187-induced (Fig. 7 *B*) shedding of microvesicles from the plasma membrane.

Characterization of the Composition and Functional Activity of the Microvesicles

The protein composition of the microvesicles was examined on SDS-polyacrylamide gels. Ionophore A23187 induced secretion of the contents of the α-granules; thus, several of the major proteins present in the low-speed supernatant from ionophore-treated platelets were α-granule proteins thrombospondin, fibrinogen, β-thromboglobulin, and platelet factor 4 (Fig. 8 *A*, lane 2). Some of these proteins remained in the supernatant (Fig. 8 *A*, lane 3) when microvesicles were sedimented by high-speed centrifugation, but considerable (although variable) amounts of the secreted proteins sedimented at high *g* forces with the microvesicles (Fig. 8 *A*, lane 4). Several other proteins also sedimented. One of these was

identified as actin (by two-dimensional isoelectric focusing [data not shown]); others were identified by immunoblotting as the M_r = 200,000 fragments generated when actin-binding protein (Fig. 8 *B*) and talin (Fig. 8 *C*) are hydrolyzed by calpain. In many experiments variable amounts of the intact proteins were also present. The invariable presence of hydrolytic fragments is consistent with the microvesicles being shed from regions of the platelet in which calpain was active. The composition of the particulate material shed from dibucaine-treated platelets was quite similar to that from ionophore A23187-treated platelets except that the α-granule proteins were not present (data not shown). Another difference was that considerable amounts of myosin were present, perhaps because in dibucaine-treated platelets (in which secretion does not occur) myosin remained near the membrane, while in ionophore A23187-treated platelets it became centralized.

Microvesicles Shed from Platelets Contain Procoagulant Activity

Both of the agents that dissociated the membrane skeleton from its membrane attachment site (dibucaine (Fig. 9 *A*) and ionophore A23187 [data not shown]) induced the generation of procoagulant activity in suspensions of platelets. Procoagulant activity was present in the platelet-free supernatant (Fig. 9 *A*). This platelet-free activity appeared to be associated with the microvesicles: it was enriched in the microvesicles that were sedimented from the platelet-free supernatant (Ta-
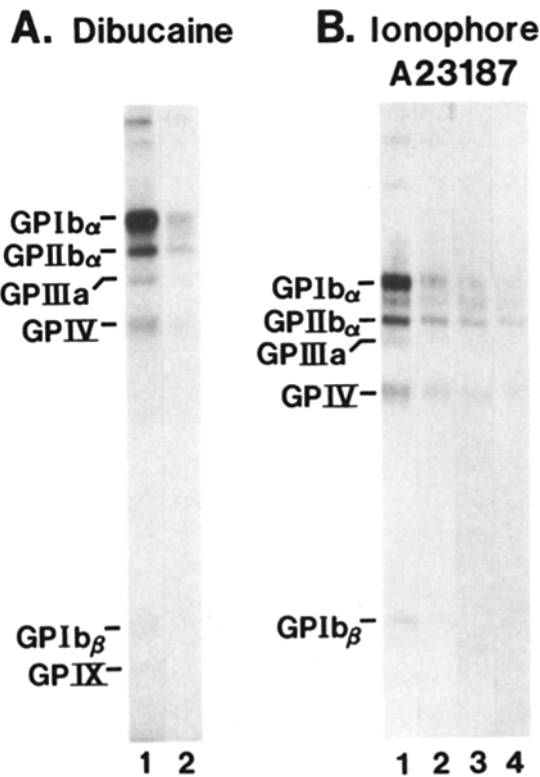
A. Dibucaine   B. Ionophore A23187

GPIbα⁻
GPIIbα⁻
GPIIIa⁻
GPIV⁻

GPIbα⁻
GPIIbα⁻
GPIIIa⁻

GPIV⁻

GPIbβ⁻
GPIX⁻

GPIbβ⁻

1 2   1 2 3 4

Figure 7. Fluorograms of SDS-polyacrylamide gels showing the effect of calpeptin on the shedding of microvesicles. Suspensions of ^{3}H-labeled platelets were preincubated for 5 min in the presence of calpeptin: 0 μg/ml (lanes *1*), 10 μg/ml (lanes *2*), 50 μg/ml (lane *3*), or 100 μg/ml (lane *4*). Suspensions were then incubated with 0.5 mM dibucaine for 5 min (*A*) or with 1 μM ionophore A23187 for 5 min (*B*). Incubations were terminated by removal of intact platelets. Microvesicles that had been shed from 6×10^8 platelets were isolated from the resulting supernatant by centrifugation. Samples 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.

ble II), and it was nearly absent in the supernatant from platelets in which disruption of actin–membrane interactions and shedding of microvesicles had been prevented by inclusion of calpeptin (Fig. 9 *B*).

Discussion

Previously, we have suggested that platelets contain a membrane skeleton that is distinct from the cytoplasmic component of the cytoskeleton and lines the plasma membrane in much the same way as the skeleton of the red blood cell lines its membrane (Fox et al., 1988; Fox and Boyles, 1988). To determine whether the platelet membrane skeleton, like that of the red blood cell, stabilizes the plasma membrane, platelets were incubated with ionophore A23187 and dibucaine, agents that activated calpain and thus disrupted the association between the membrane skeleton and plasma membrane glycoproteins. Incubation of platelets with these agents caused the shedding of procoagulant-rich microvesicles from the plasma membrane. These microvesicles contained calpain-generated hydrolytic fragments of actin-binding protein

and talin. Shedding of the microvesicles correlated with the extent of disruption of the membrane skeleton. When disruption of the membrane skeleton was inhibited by inhibitors of calpain, the shedding of the microvesicles was inhibited. These results are consistent with the hypothesis that microvesicles form as a consequence of the calpain-induced disruption of actin–membrane interactions. We suggest, therefore, (*a*) that association of the membrane skeleton with membrane glycoproteins stabilizes the plasma membrane of unstimulated platelets, preventing the shedding of procoagulant-rich microvesicles from the plasma membrane, and (*b*) that one consequence of the activation of calpain in platelets can be the shedding of procoagulant-rich microvesicles.

The reason that the microvesicles contain procoagulant activity is not clear. Zwaal and co-workers have noted a correlation between the extent of hydrolysis of actin-binding protein within the platelet and the externalization of negatively charged phospholipids (Comfurius et al., 1985). They suggested that the platelet cytoskeleton was responsible for maintaining the asymmetry of the phospholipids, and that hydrolysis of the cytoskeleton during platelet activation allowed negatively charged phospholipids to "flip" to the outer bilayer, where they could provide new binding sites for the prothrombinase complex. Although we have not determined whether expression of the procoagulant activity detected in the present study results from externalization of phospholipids, our data are consistent with the idea that procoagulant activity can be generated when actin–membrane interactions are disrupted. Thus, the agents that disrupted the membrane skeleton also led to increased procoagulant activity, some of which was shed with the microvesicles. One possible explanation for the presence of procoagulant activity in microvesicles could therefore be that dissociation of the membrane skeleton from the plasma membrane results in increased expression of binding sites for the prothrombinase complex on regions of the membrane that are subsequently shed. However, other possibilities exist; for example, procoagulant activity may occur as a consequence of negatively charged lipid becoming exposed during the physical process of microvesicle formation. Further studies will be needed to evaluate these possibilities.

The present studies provide evidence that microvesicles are shed when calpain is active in platelets and are consistent with the hypothesis that this results from the calpain-mediated disruption of the membrane skeleton. Calpain is activated when platelets aggregate in response to a physiological agonist. Although the agonist-induced activation of calpain was first reported in 1983 (Fox et al., 1983), the possibility that calpain plays a role in stimulus–response coupling in platelets has not been widely accepted. The main reason for this may be that only small amounts of substrates are hydrolyzed (typically, <10% of the total actin-binding protein in platelets is hydrolyzed). The present study shows that hydrolysis of these small amounts of substrate is sufficient to cause release of 40–50% of the GP Ib–IX complex from the membrane skeleton and to elicit the generation of procoagulant-rich microvesicles. Recently, Ezzell et al. (1988) estimated that only ∼20% of the total actin-binding protein in platelets is associated with the GP Ib–IX complex. Thus, for hydrolysis of <10% of the total actin-binding protein to result in release of 40–50% of the GP Ib–IX complex from the membrane skeleton, one would have to propose that the mem-
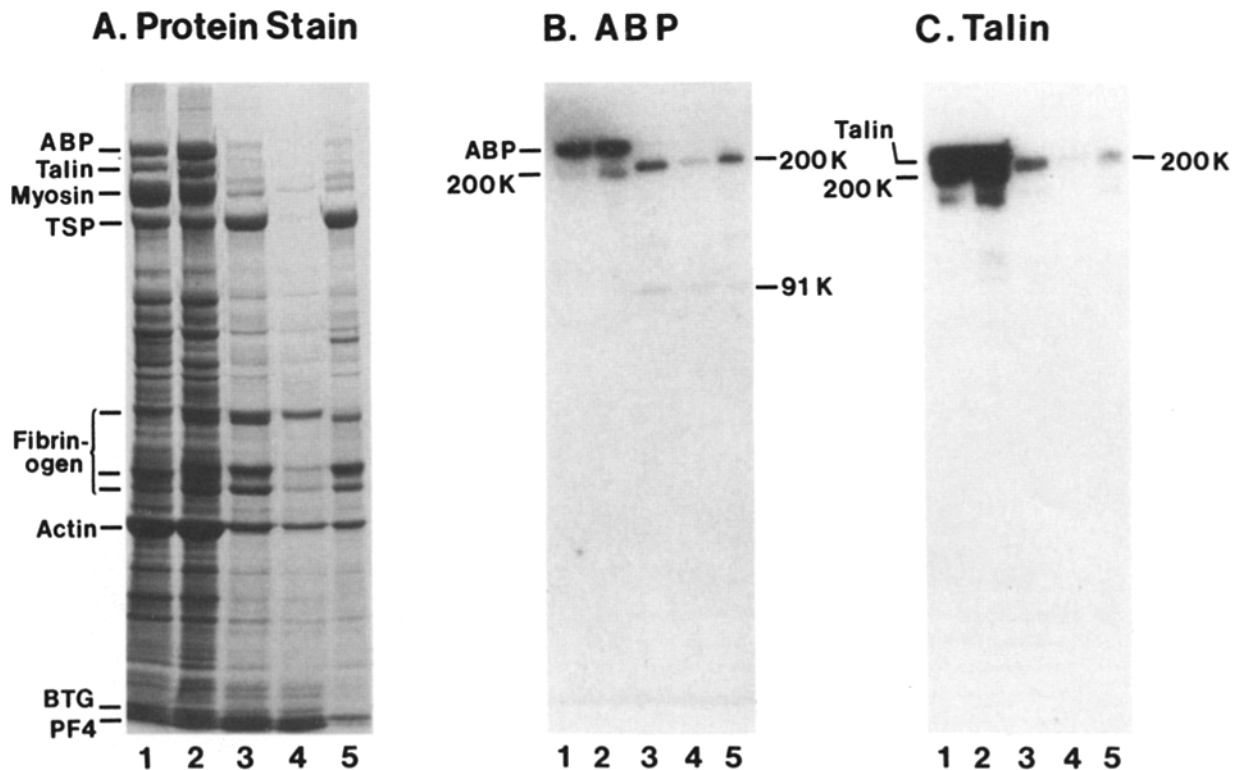
A. Protein Stain　　B. ABP　　C. Talin

Figure 8. SDS-polyacrylamide gels showing the protein composition of the microvesicles shed from the plasma membrane during exposure of platelets to agents that disrupted the membrane skeleton. Platelet suspensions were stirred in the presence of 1 μM ionophore A23187 for 10 min. Control suspensions (lanes *1*) or suspensions that had been stirred in the presence of ionophore (lanes *2*) were solubilized directly into an SDS-containing buffer or were centrifuged at 15,600 *g* for 1 min to sediment intact platelets. The material remaining in the supernatant from ionophore-treated platelets was either solubilized in SDS (lanes *3*) or centrifuged at 100,000 *g* for 2.5 h to obtain the 100,000-*g* supernatant (lanes *4*) and the sedimented microvesicles (lanes *5*). All samples were electrophoresed through SDS-gels containing a 5–15% exponential gradient of acrylamide. Lanes *1* and *2* contained protein from 3×10^7 platelets; the remaining lanes contained protein originating from 6×10^7 platelets. In *A*, proteins were detected with Coomassie brilliant blue. In *B* and *C*, actin-binding protein and talin were detected on immunoblots. ABP, actin-binding protein; TSP, thrombospondin; BTG, β-thromboglobulin; PF4, platelet factor 4. The $M_r = 200,000$ products of hydrolysis of actin-binding protein and talin are both shown as 200K.

brane-bound pool of actin-binding protein was preferentially hydrolyzed by calpain. This possibility is not unreasonable, given the considerable body of literature suggesting that calpain can be activated by binding to the plasma membrane (for review, see Suzuki et al. [1988]). There are many reports that procoagulant-rich microvesicles are shed from platelets in a variety of physiological and pathological conditions (Bode et al., 1985; Crawford, 1971; Dombrose et al., 1981; George et al., 1982; 1986*a,b*; Sandberg et al., 1982; 1985; Sims et al., 1989; Solberg et al., 1987; Wiedmer et al., 1986; Wolf, 1967). The present study raises the possibility that these vesicles may form as a consequence of the calpain-induced disruption of actin–membrane interactions. Studies are under way to test this hypothesis.

The only cell type in which a membrane skeleton has been well characterized is the red blood cell. In this cell, the membrane skeleton also stabilizes the plasma membrane. The existence of a variety of hereditary defects has provided clear evidence that defective association of the skeleton with the plasma membrane leads to the shedding of microvesicles from the plasma membrane (Lux, 1979). As in the platelet, there is also evidence that the membrane skeleton prevents the expression of procoagulant activity on the surface of the red blood cell by maintaining the asymmetry of negatively

charged phospholipids (Franck et al., 1985). The red blood cell membrane skeleton consists of a network of short actin filaments, cross-linked by spectrin. It is so closely adherent to the plasma membrane that it is hard to visualize in the presence of an intact plasma membrane (Hainfeld and Steck, 1977; Liu et al., 1987; Shen et al., 1986). However, because the red blood cell does not contain cytoplasmic actin filaments, the membrane skeleton is the only structure that remains after solubilization of the membranes with detergent. Thus, the red blood cell membrane skeleton has been readily amenable to study and has been shown to regulate many of the properties of the plasma membrane.

In other cell types, the major structure remaining after solubilization of the plasma membrane with detergents is a dense network of cytoplasmic actin filaments. Thus, that a membrane skeleton may also exist in other cell types has often been overlooked. Recently, however, the observation that proteins such as spectrin, 4.1, or ankyrin are present in a sub-membranous location has been taken as an indication that a membrane skeleton may be present in other cells (Bennett, 1985; Marchesi, 1985). Further, the finding that a detergent-insoluble shell containing uncharacterized proteins remains after extraction of various cell types under conditions in which cytoplasmic actin filaments have been de-
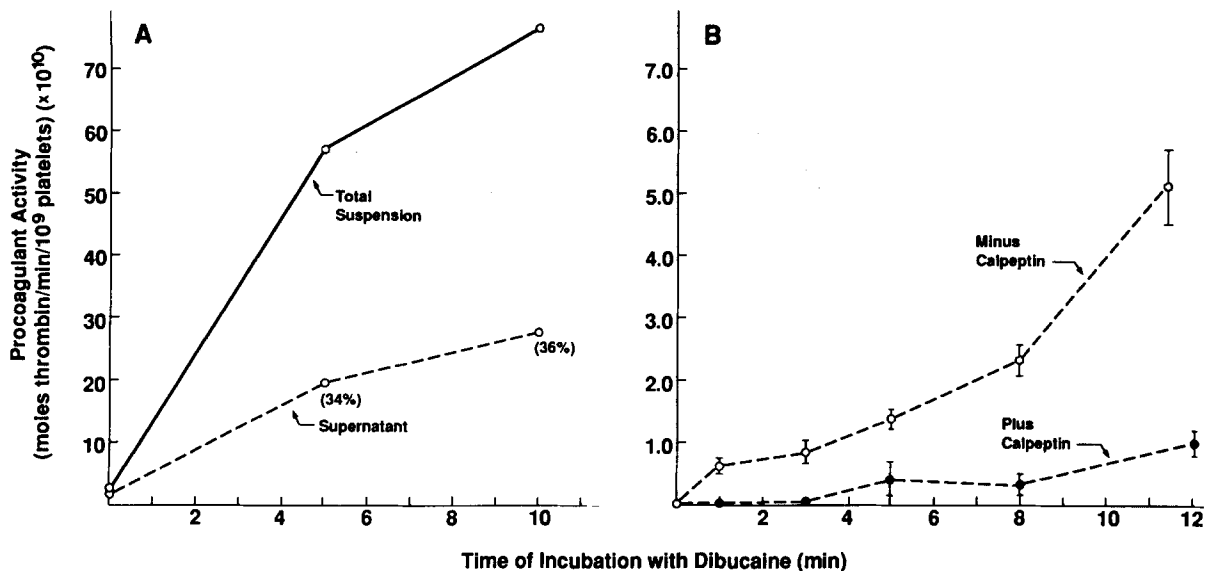
Figure 9. Generation of procoagulant activity on dibucaine-treated platelets. (A) Platelet suspensions were incubated with 0.5 mM dibucaine. At intervals, aliquots of suspension were removed and the procoagulant activity in the total suspension (*solid line*) or in the supernatant remaining after the intact platelets had been removed by sedimentation (*broken line*) was assayed. The numbers in parentheses show the prothrombinase activity in the supernatant expressed as a percentage of that in the total platelet suspension. (B) Platelets were preincubated in the presence (●) or absence (○) of 50 μg of calpeptin/ml for 5 min before dibucaine addition. The procoagulant activity present in the platelet-free supernatant was assayed at the intervals indicated.

polymerized (Ben-Ze'ev et al., 1979; Apgar et al., 1985; 1986) has led to the suggestion that a peripheral layer of proteins may function as a membrane skeleton in cells other than the erythrocyte. Thus, the findings on the role of the membrane skeleton in platelets may have relevance to other cell types. Microvesicles that are shed physiologically, for example, as a mechanism of modifying the cell surface during development (Johnstone et al., 1987), or of enhancing cell-substrate attachment (Schubert et al., 1983), or the procoagulant-rich microvesicles that are shed from tumor cells (Van De Water et al., 1985) could result from modulation of components of a membrane skeleton. The present evidence that calpain-induced changes to the membrane skeleton can regulate the stability of the plasma membrane therefore suggests a general mechanism by which membrane-associated properties of cells might be regulated.

Table II. Enrichment of Dibucaine-induced Procoagulant Activity in the Platelet Microvesicles That Fragmented from the Plasma Membrane

Fraction	Specific procoagulant activity ($\times 10^{10}$)
	mol of thrombin/min/mg of protein
Low-speed supernatant	25.7
High-speed supernatant	19.3
High-speed pellet (microvesicles)	111.8

Platelet suspensions were incubated for 15 min in the presence of 0.5 mM dibucaine. Intact platelets were removed by centrifugation of the suspension at 15,600 g for 1 min. The resulting supernatant was centrifuged at 100,000 g for 2.5 h to sediment the microvesicles.

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