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Platelet Membrane Skeleton Revealed by Quick-Freeze Deep-Etch

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Abstract

Actin polymerization is an essential component of platelet activation. Since actin appears to polymerize at its membrane-associated end, knowledge of the structural relationship of actin filaments to membrane is an important part of understanding that polymerization process. A membrane-associated actin-containing cytoskeleton has been described in human platelets biochemically and is composed, at least in part, by an association between glycoprotein Ib and the actin-binding protein originally isolated from macrophages. Many other actin-associated proteins with known sub-membranous localization in other systems have been found in platelets, including alpha-actinin, vinculin, and low levels of spectrin and the red cell protein Band 4.1. Because of the density of the platelet cytoplasm, the structure of the membrane-skeleton has not yet been visualized. We have used quick freeze-deep etch techniques to observe the sub-membranous cytoplasm and report visualization of a periodic, submembranous filament system not before seen in the platelet. This filamentous system was more easily observed in thrombin-stimulated platelets, but appeared to be present in resting, discoid cells as well. The filaments could also be readily observed when platelets are lysed after fixation, stained with tannic acid, and embedded for thin-sectioning. This membrane cytoskeleton was composed of 9 nm thick filaments lying 15 nm apart, and 15 nm from the membrane. The filaments appeared to lie in parallel and to encircle the cell. Similar filaments could be seen associated with intracytoplasmic membrane systems in activated cells.

The main function of the human blood platelet is to maintain hemostasis by plugging rents in vessel walls. To accomplish this, the freely circulating anucleate cell must recognize that a tear has occurred, and respond to it by sticking to the area of damage and to other platelets. This response involves a complicated set of events, which includes changes in the surface membrane, in cell shape, and in the polymerization state of actin. Many of these events take place on the plasma membrane. How actin and the membrane interact has been studied by a number of different investigators. In the resting discoid cell, glycoprotein Ib has been shown by immunoprecipitation to be associated with actin-binding protein, a 260 kD protein originally isolated from macrophages (Fox, 1985; Okita et al., 1985). Interaction between the actin-associated proteins, gelsolin and profilin, and phosphoinositides, metabolites of the membrane lipid, phosphatidylinositol, may be part of the mechanism mediating their interaction with actin (Janmey and Stossel, 1987; Lassing and Lindberg, 1985) which, under activating conditions, results in the elongation of actin filaments at the membrane cytoplasmic interface. During activation the platelet membrane becomes sticky, and adhesion complexes form which are similar to those in fibroblasts that attach the cell to extracellular matrix. These adhesions also require a structural association between the

membrane and actin filaments, and some appear to be mediated by a fibronectin-like receptor, glycoprotein Ilb/IIIa (Charo et al., 1986; Cieniewski et al., 1986; Gardner and Hynes, 1985; Isenberg et al., 1987), and adhesion plaque-associated cytoskeletal proteins, vinculin (Rosenfeld et al., 1985), talin (Beckerle et al., 1986), and alpha-actinin (Burn et al., 1985). Other proteins present in membrane-skeletons of other cells are also found in platelets, including spectrin and the red cell protein, Band 4.1 (Davies and Cohen, 1985). These proteins may also be involved in membrane-lipid distribution (Choe et al., 1985). Finally, actin is hypothesized to polymerize from its membrane-bound end (Tilney et al., 1981,1986; Tilney and DeRosier, 1986). Since actin polymerization is a significant, and perhaps essential, part of platelet activation, it is particularly important to gain a better knowledge of how actin-membrane associations occur.

Ultrastructural observation of the association between the cytoskeleton and the plasma membrane in the platelet has been difficult because it is too tiny to view with light microscopy, and it has a very dense cytoplasm in thin sections prepared for electron microscopy. However, a clear view of cytoskeletal membrane structure is crucial to an understanding of how the changes which occur during activation are mediated biochemically. To observe the cytoplasmic membrane interface, we have used the quick-freeze, deep-etch technique. This technique has the advantage of being applicable to living cells, and thus avoids fixation artefacts. Additionally, cells can be captured within seconds of stimulation and the changes observed. With deep-etching after fracturing, cytoplasmic filaments are readily visualized (Hirokawa and Heuser, 1981), along with membrane surfaces and fracture faces. Additionally, freeze substitution can be used to study these structures in thin sections of unfixed, quick-frozen cells.

MATERIALS AND METHODS

Platelet Suspensions

Whole blood was drawn through a 19 gauge needle from healthy volunteers taking no medications, into an anticoagulant solution calculated to give a final concentration of 0.38% sodium citrate and 0.1% EDTA. The red cells were removed by sedimentation at 150*g* for 15 min, and the platelet-rich plasma washed twice in acid-citrate-dextrose (ACD), sedimenting each time at 270*g* according to published protocols (Fox, 1985). The suspension was incubated at 37°C in Tyrode's buffer until the quick-freeze apparatus could be prepared (30 min–2 h). Platelets prepared in this way were dripped into fixative and prepared for TEM to check that they had returned to their resting discoid shape. No prostaglandins or other inhibitors were used to prevent activation, and the majority of platelets had returned to their discoid shape by TEM after 30 min in Tyrode's at 37°C.

Quick-Freezing

Ten microliter aliquots of platelet suspensions of about 500,000 cells/ml were dropped on a square of Whatman 52 filter paper glued to an aluminum quick-freeze support. The non-porous paper did not absorb the droplet, perhaps because of the dried glue. The support was turned upside down and mounted onto the specimen arm of a Polaron quick-freeze apparatus. When the copper block had reached temperatures below that of liquid nitrogen

(as attested by the presence of liquid gas forming on its surface), the specimen arm was released to plunge the specimen down upon the block, or the cell suspension was injected with thrombin, a second counted on a stop watch, and the specimen arm released to contact the block. Hence, the stimulation was 1 sec plus the time it took for the plunger to contact the block. This short stimulation time was chosen because we wished to capture the earliest morphological changes in response to thrombin, in the hopes of dissecting the sequence of events that produces the activated cell structure. In experiments with a longer stimulation time, the range of morphological changes was greater, with aggregation, degranulation, and other thrombin-mediated changes occurring. Thrombin (from John Fenton) injections were calculated to give a final concentration of 3 U/ml. A polymyxin B (Sigma, St. Louis, MO) stock solution was prepared and either injected with or without thrombin in the same manner to give a final concentration of 4 mM as described (Bearer and Friend, 1980). Frozen specimens were stored in liquid nitrogen until further processing.

Freeze-Fracture and Etching

Frozen platelets were mounted in a Balzer's 360 M freeze, fracture device, fractured at −115°C, and etched for 20 min at −110°C. After etching, replicas were made by rotary shadowing with the platinum gun at 24 angle and the carbon gun directly overhead. Replicas were washed in bleach and mounted on Formvar coated, carbon-stabilized, copper grids.

Freeze Substitution

Alternatively, frozen platelets were immersed in 5% osmium tetroxide in acetone at dry ice temperatures and incubated for 3 days. They were warmed qradually by passing 1 h at −20°C and 1 hr at 4°C. The loose cells were then stained with 1% uranyl acetate in 100% ethanol for 1 h at room temperature, and microfuged into pellets for embedding in Epon 812.

Glutaraldehyde Fixation

Washed platelets, platelet-rich plasma, or whole blood was fixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.2 for 1 h. Stimulated washed platelets were treated with 3 U of thrombin for 1 min before addition of fixative. Since aldehyde fixation is much slower of action than freezing, a longer stimulation was unavoidable, even should the fixative be added within 1–2 sec of the thrombin. Hence, a longer stimulation was chosen to obtain a more uniform degree of activation in all cells, such that the time of fixation would less markedly affect the time of activation. After postfixation in 1% Palade's osmium, they were en bloc stained in 1% tannic acid in 0.05 M sodium cacodylate, pH 7.0, and then in Kellenberger's uranyl acetate. Lysis was accomplished prior to fixation according to published techniques (Zucker-Frank-lin, 1976). Leaching was obtained by rapid dehydration in graded ethanol beginning at 70% followed by two prolonged changes in propylene oxide for 20 min at room temperature (RT). Dehydrated pellets were embedded in Epon. Silver gray sections were post-stained with Reynold's lead citrate and uranyl acetate.

OBSERVATIONS

Resting platelets (Fig. 1), fixed in gluteraldehyde, displayed a submembranous felt-work that was prominent, and possibly periodic (Fig. 1A) after the dense cytoplasm was leached

away by rapid dehydration. Removal of the cytoplasm by hypotonic lysis produced irregularly shaped cells with membrane-attached filaments (Fig. 1B). Quick-frozen resting platelets had smooth membranes and round granules (Fig. 1C). At low magnification, the majority had a discoid shape with only rare pseudopod projections. Sub-membranous filaments were not observed in fractured preparations, perhaps because of the dense cytoplasm, and a structure corresponding to the membrane-associated feltwork seen in thin-sections could not be recognized.

After stimulation, the membrane-associated periodic feltwork became universally identifiable in thin sections as punctate particles 9 nm in diameter (Fig. 2A, B). Grazing sections revealed these points to be filaments lying 15 nm apart and 15 nm within the plasma membrane. They occasionally extended to granules (Figs. 2A, 3E). Platelets quick-frozen 1–2 sec after thrombin stimulation had many of the features of activation, including projection of some pseudofilopodia, concentration of granules, and dilitation of the internal regions of the surface-connected canalicular system. Observation of the sub-membranous region revealed 9 nm filaments lying just beneath the plasma membrane and spaced about 15 nm apart (Fig. 3A–E). Although the feltwork was present in thin sections of unstimulated cells, filaments such as these were not observed in freeze-etch replicas. These filaments were thicker and more convoluted than the microfilaments, probably primarily composed of actin, commonly observed deeper into the cytoplasm.

Membrane lipid changes have been shown to occur in platelets upon activation (Chap et al., 1982; Lagarde et al., 1982; Morin, 1980; Schick, 1978; Spangenberg et al., 1984) and to be detectable with the antibiotic polymyxin B (Bearer and Friend, 1986; Lupu et al., 1986). Furthermore, lipid metabolites mediate the association of actin-binding proteins to actin (Janmey and Stossel, 1987; Lassing and Lindberg, 1985) and act as second messengers to induce the activity of protein kinase C, a major signal tranducer in the platelet (Spangenberg et al., 1984). To see if the membrane lipid changes occurred concomitant with increased visibility of the membrane-associated filaments, we stimulated platelets in the presence of polymyxin B (PXB), an antibiotic that perturbs membranes exposing anionic lipid in the outer leaflet (Bearer and Friend, 1986, 1980). The effect of PXB on membrane lysis of yeast has recently shown to be inhibited by mutations that increase the activity of protein kinases (Boguslawski and Polazzi, 1987), whose activity in platelets is induced by the metabolites of thrombin-stimulated catabolism of membrane lipids (Takai et al., 1986). While unstimulated cells had no response to PXB (Fig. 4A), the stimulated platelets quick-frozen within 1–2 sec of thrombin exposure had the expected membrane perturbations (Fig. 4B). Deep-etching revealed that the perturbed plasma membrane was not a result of ice crystal formation within the cytoplasm. Two adjacent cells (Fig. 4B) displayed either smooth membrane or membrane that was perturbed throughout, an additional circumstance unlikely to be caused by ice crystal formation. Furthermore, in freeze-subsituted quick-frozen platelets, there was no difference in the degree of ice crystal formation between untreated cells and those cells treated with thrombin and/or PXB.

It has been hypothesized that the surface-connected canalicular system (SCS) contributes to changes in the platelet plasma membrane which accompany activation. We therefore wished to see if morphological changes in the SCS commonly known to occur during activation had

begun within the 1—2 sec interval of our experiments. In thin sections of unfixed, quick-frozen resting cells, the SCS was a pentilaminar membrane structure without central lumen (Fig. 5A). The central lamina was darker and thicker than the usual membrane staining. At the confluence of the SCS with the plasma membrane was a narrow neck. After 1–2 sec of thrombin stimulation, the SCS opened in discrete areas within the cell while the portion adjoining the plasma membrane remained closed as a pentilaminar structure (Fig. 5B, C). This pentilaminar appearance of the SCS in the resting cell suggests that it may be less permeable to molecules in the media than the more open structure would be after stimulation. We have treated both resting and stimulated cells with a variety of electron-dense tracer molecules and found that the SCS in the resting cell is not accessible to uncharged molecules of more than 5 nm in diameter (E.L. Bearer, unpublished observation). This morphology does not rule out permeability to smaller molecules, or trafficking of intramembranous or membrane-bound macro-molecules which would allow selective entry to the system. No tight-junction-like configuration of the fractured membrane at the neck has been observed, but the system appears to be plugged by tannic-acid staining material in aldehyde-fixed, resting cells. In the fracture plane, tongue-shaped outlines of anastamosing SCS could be seen, perhaps analagous to similar structures observed in fractures of quick-frozen cells (Fig. 6A, B). Surface-connected pentilaminar membrane systems were also observed in blood leukocytes frozen in whole blood preparations. These observations implicate the pentilaminar membrane in providing additional surface membrane for cellular extensions.

Analysis of histograms of data from micrographs of these preparations show that virtually all cells which had been either fractured or sectioned through the sub-membranous cytoplasm had membrane-associated filaments. Half the cells in a given population had PXB membrane perturbations with stimulation. The SCS was internally distended in approximately two-thirds (Fig. 7).

DISCUSSION

The membrane participates in many of the events that accompany platelet activation. In this study, we prepared quick frozen-deep etched or freeze-substituted cells to observe the membrane and associated structures, such as the cortical cytoskeleton and the surface-connected canalicular system. We found that a membrane-associated cytoskeleton composed of 9 nm thick filaments, arrayed with a 15 nm periodicity, and at a distance of approximately 15 nm from the membrane, was visible, particularly clearly after 1–2 sec of throm-bin stimulation. Since these filaments were present in replicas of unfixed quick-frozen platelets as well as in traditional thin sections, they are unlikely to be an artefact of specimen preparation. The filaments were not identified in replicas of quick-frozen resting platelets, although an electron-dense fuzz was readily apparent in traditional aldehyde-fixed thin sections of resting platelets after the cytoplasm was leached away. This fuzz gives a suggestion of possible periodicity with dimensions similar to that observed in the more clearly delineated filaments present after activation. It remains a formal possibility that filaments do exist in the unstimulated cell but are obscured by the other components of the fuzz, and that the fracture plane does not pass at the right level to reveal them. It is also possible that the fuzz is the “nidus” for actin polymerization and filament formation, and

that it is the increased organization, length, and regular spacing that causes the prominence of the filaments in the stimulated cell. An electron-dense fuzz has been described at the tips of microvilli and stereocilia, another membrane-associated site where actin polymerization is thought to occur (Tilney et al., 1981, 1986; Tilney and DeRosier, 1986).

Although an array of oriented filaments has never before been described just beneath the cell membrane, the existence of such an array has been suggested by experiments in which the movement of colloidal gold particles has been observed on the surface of living macrophages (Sheetz et al., 1989) and neuronal growth cones (Forscher and Smith, 1988). These particles move in linear paths that are dependent on an intact actin network, since cytochalasin, which depolymerizes actin filaments, stops their movement. Since these experiments, like the quick-frozen platelets reported here, were performed on living cells in the absence of fixatives, it seems likely that this filament array has not been previously observed because it is not well preserved by standard fixations. The movement of these particles has been hypothesized to be mediated by an actin-based motor, possibly myosin I, which acts as a mechano-enzyme and can move beads along actin filaments. The electron-dense link between the filaments and the membrane observed in these tannic-acid stained thin sections might represent such a motor, since at this point in platelet activation the fibrinogen receptor, GPIIb/IIIa, moves from a diffuse to a focal distribution (Isenberg et al., 1987).

Such a periodic arrangement of membrane-associated filaments has also not been reportedly observed in platelets (Fox et al., 1985, 1987; Gonella and Nachmias, 1981; Jennings et al., 1981; Nachmias, 1980; Nachmias et al., 1977, 1979), but only one other study (Nakata and Hirokawa, 1987) has used quick-freezing as a means to observe the platelet cytoskeleton, and in that study the cells were fixed and treated with Triton to permeabilize the membrane and allow SI heavy meromyosin decoration of actin filaments. Hence, the membrane-associated cytoskeleton would potentially be lost. The most likely filamentous component of these filaments is actin, since it has been observed to be a major component of the platelet membrane. In addition, it polymerizes upon activation. However, the width of the filaments, 9 nm, suggests that if actin is present, it is thickened in some way by additional proteins, since cytoplasmic actin filaments have a 6—8 nm diameter. While a thick platinum shadow could increase the apparent width of the filaments in replicas, the filaments described here were 9 nm in thin sections, and the increase in width due to the platinum shadow was taken into account. The filaments could not be seen in freeze-subsitution specimens, probably because these preparations were not stained with tannic acid.

The other proteins potentially comprising the electron-dense fuzz or filaments include many other known actin-binding proteins. Recently, an immunocytochemical study of platelet “ghosts” revealed that macro-phage actin-binding protein, actin, and spectrin are associated with the plasma membrane (Fox et al., 1988). The harsh conditions necessary for antibody labelling in this study also did not appear to preserve intact filaments with the periodicity reported here. In my hands, these filaments did not label with anti-spectrin, anti-4.1, or anti-macrophage actin-binding protein antibodies (not shown). Hence, the filaments described here may be a unique structural molecule, or a composite of actin and other associated proteins. The width, 9 nm, is consistent with either spectrin or macrophage actin-binding

protein. In addition to these actin-binding proteins, the role of myosin, another filament-forming protein in high concentration in the platelet, during the early events of platelet activation, is only as yet poorly understood. We have identified many novel actin-binding proteins from human platelets by F-actin affinity chromatography (Bearer and Alberts, in preparation). Hence, the membrane-associated filaments could quite conceivably be an as yet undescribed cytoskeletal protein.

Our other observations include the activation-dependent susceptibility to PXB-induced membrane perturbations and concomitant changes in the SCS. We show that these perturbations are not present in resting cells treated with PXB, but occur in half the cells within 1–2 sec of thrombin activation. The artefacts of PXB treatment have been extensively reviewed elsewhere (Bearer and Friend, 1980, 1986; Karnovsky, 1982; Lupu et al., 1986). The fact that some membrane change occurs concurrently (within 2 sec of activation) with the increased visibility of cytoskeletal elements further supports the view that the membrane plays a role in mediating the cytoskeletal changes that accompany platelet activation. Other evidence for this mediating role include the effect of phosphoinositides, metabolites of the membrane lipid phosphatidylinositol, on the actin-binding proteins profilin (Lassing and Lindberg, 1985) and gelsolin (Janmey and Stossel, 1987), and changes in the phosphorylated state of the integral membrane glycoprotein GP Ib which mediates attachment of the membrane to macrophage actin-binding protein (Fox et al., 1988). Other investigators have shown that anionic lipids are exposed in the outer leaflet of the plasma membrane upon platelet activation (Chap et al., 1982; Lagarde et al., 1982; Morin, 1980; Schick, 1978; Spangenberg et al., 1984). Although the PXB effects may not be entirely due to that exposure, these observations suggest that membrane lipid reorganization also occurs very rapidly during platelet activation.

The closed appearance of the SCS has not been previously reported, probably due to the relatively slower fixation obtained by chemical fixatives, which could conceivably fix surface membrane ion channels prior to internal structures, and hence allow osmotic changes to occur, causing swelling or shrinking of organelles. Another freeze-substitution study showed that swelling of granules accompanies platelet activation (Morgenstern et al., 1987). This swelling has also not been observed in thin sections of platelets fixed in the traditional aldehydes. The internal dilitation of the SCS observed in stimulated cells may be a result of granule fusion to the SCS, shown to be one of the major pathways for platelet exocytosis (Stenberg et al., 1984). The changes in the configuration of the surface pore during stimulation have not been observed in aldehyde-fixed cells, but seem a logical mechanism for the rapid addition of membrane to the surface to provide for pseudopodial projections.

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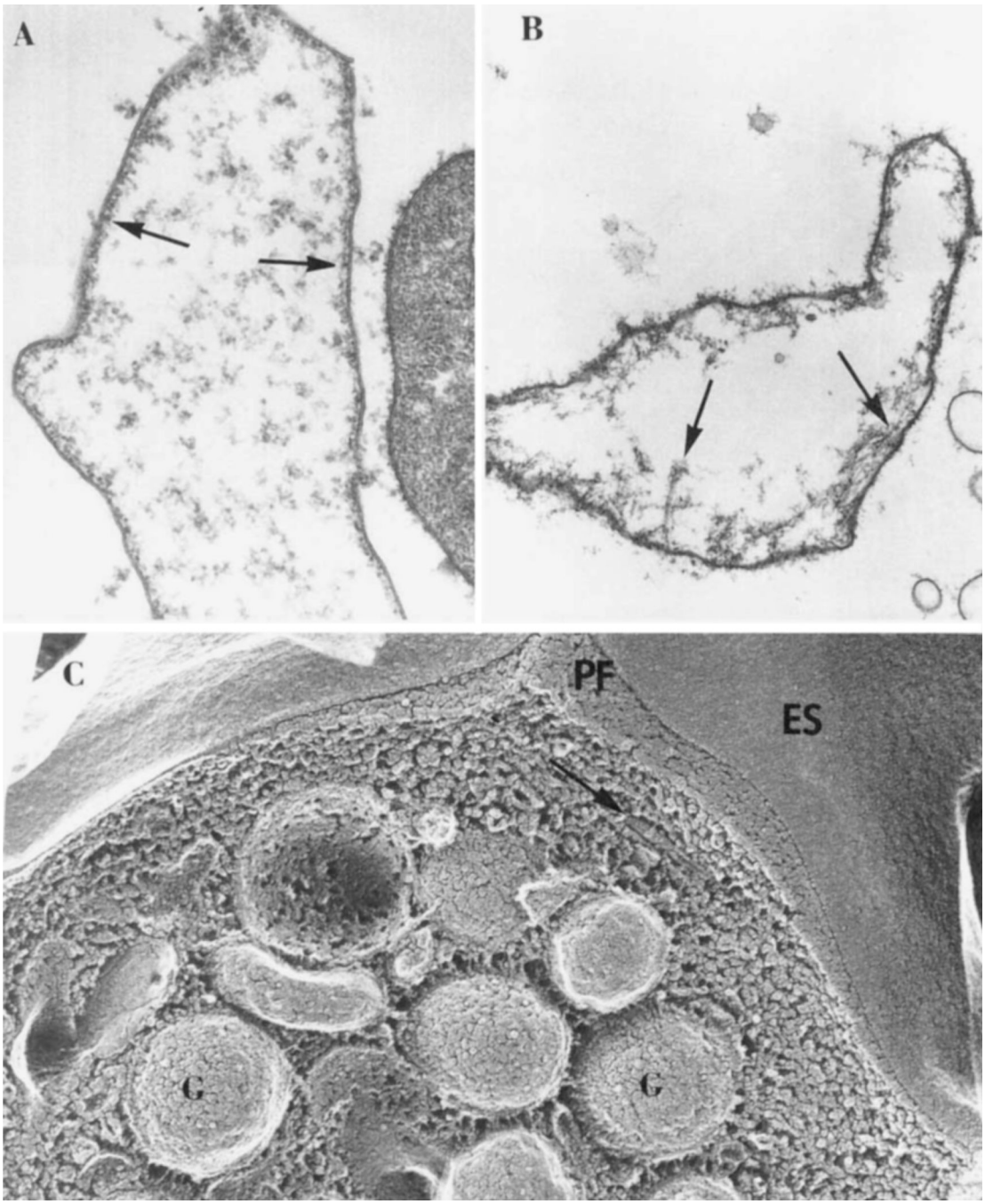

Fig. 1.

Resting platelets. **A:** A gluteraldehyde-fixed platelet leached of its cytoplasm after fixation displays a dense mesh (arrows) lining the cytoplasmic surface of the plasma membrane. **B:** A platelet, after hypotonic lysis, displays membrane attached filaments (arrows) extending into the empty cell interior. **C:** A quick-frozen, deep-etched resting platelet displays a smooth extracellular surface (ES), a peb-bled protoplasmic fracture face (PF), and dense cortical cytoplasm in which no filaments can be identified. A microtubule (arrow) is visible. Granules (G) are identified by their round shape. A, × 28,000. B, × 36,000. C, × 60,000.

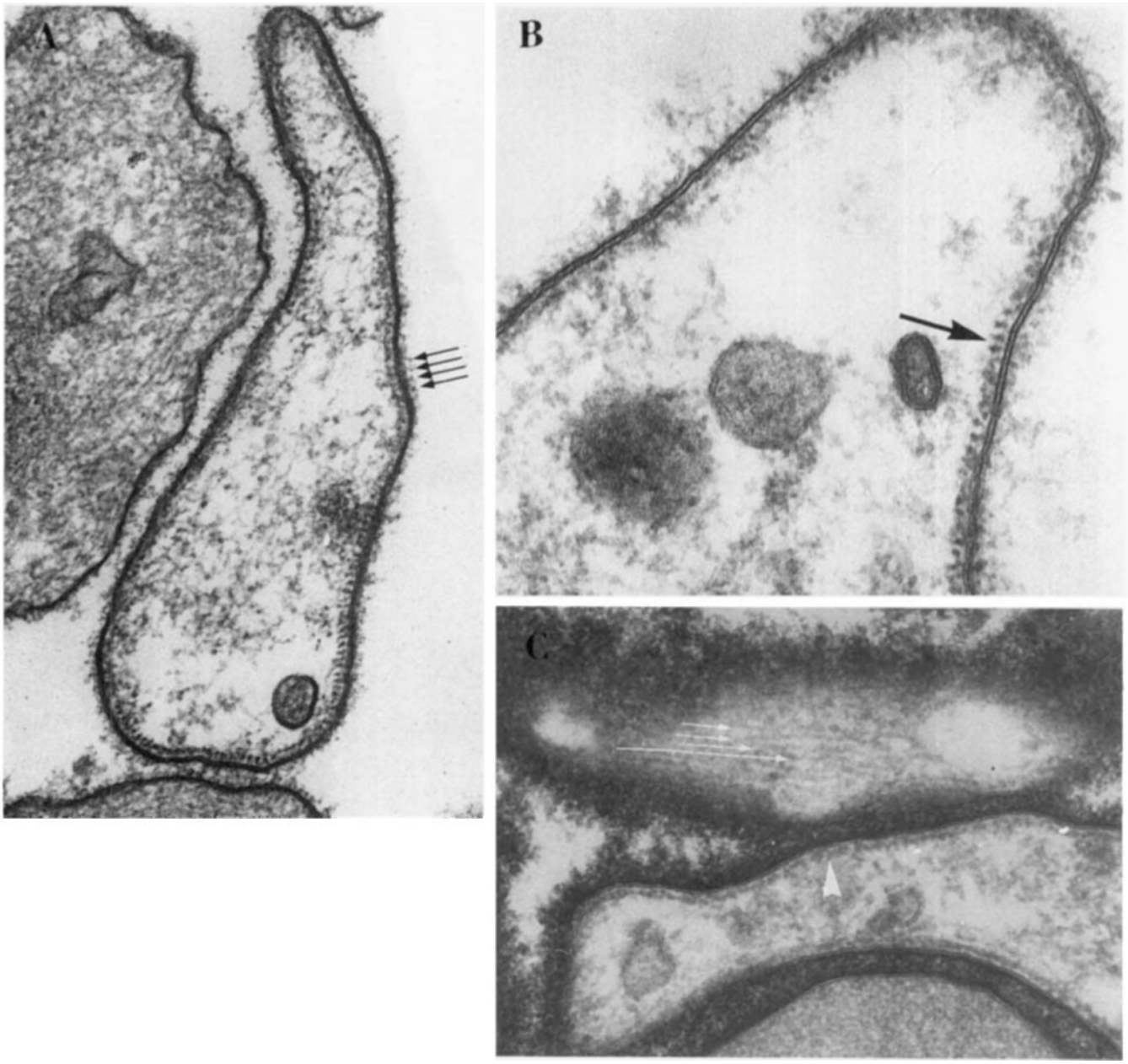

Fig. 2.

Thin sections of gluteraldehyde-fixed thrombin-stimulated platelets. **A:** A periodic array of electron-dense dots, 15 nm apart and about 15 nm from the membrane, is readily visible in this platelet fixed 1 min after thrombin stimulation and partially leached of its dense cytoplasm. A similar periodicity is discernible in the tannic-acid-stained extracellular coat (arrows). The dots are present around the full circumference of the cell, at both platelet-platelet contact points and at free surfaces. A residual granule and cytoplasmic filaments can also be seen. **B:** Higher magnification of another platelet treated as in A reveals connections between the dots (arrow) as well as with the membrane. Again, residual granules remain in the cytoplasm. **C:** Grazing section at the cytoplasmic-membrane interface reveals the “dots” to be most probably cross-sections of filaments (arrows). An adjacent platelet, in a different

plane of section, again reveals the membrane association (arrowhead). A, × 70,000. B, × 140,000. C, × 70,000.

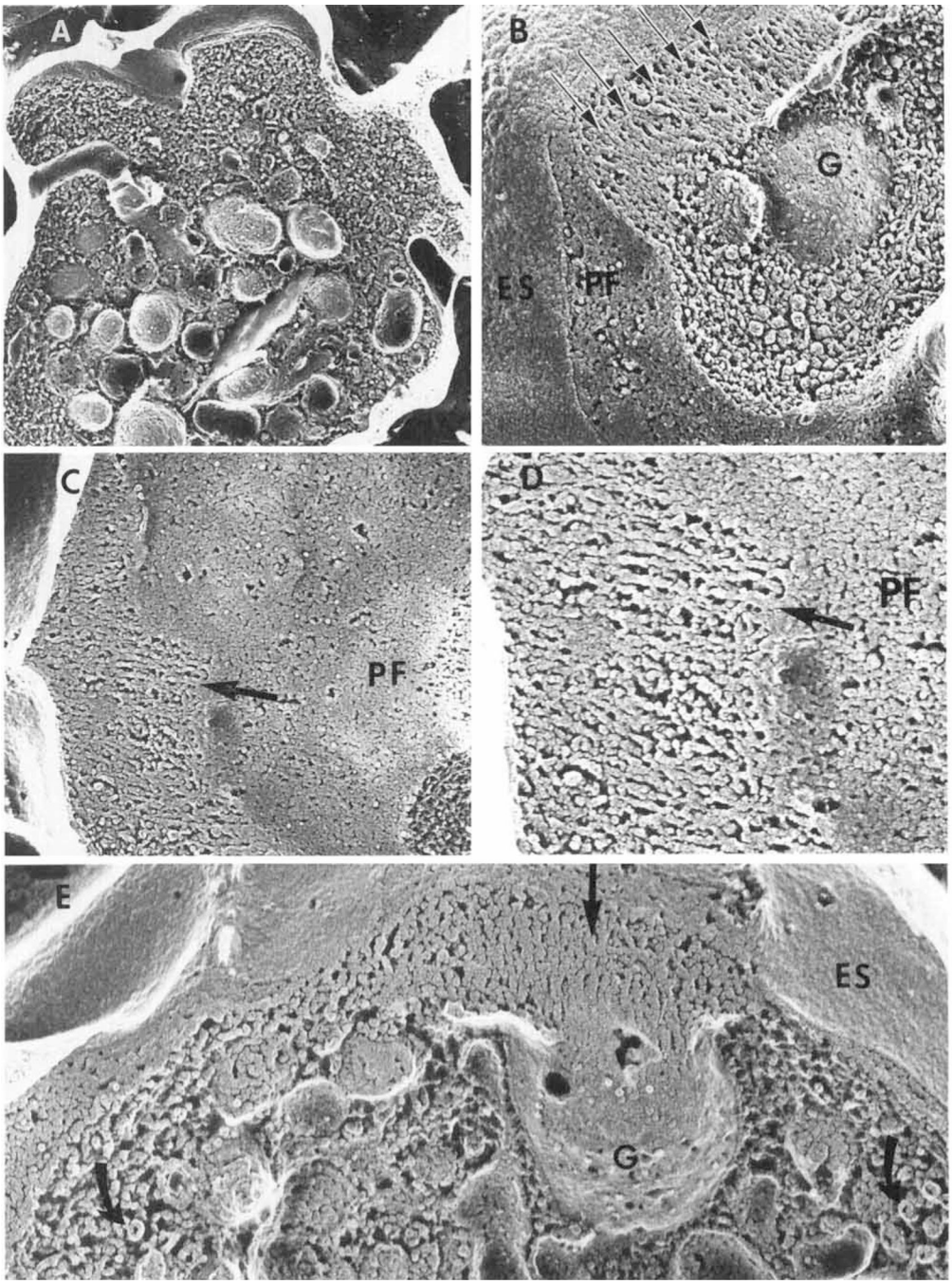

Fig. 3.

Quick-frozen, deep-etched replicas of stimulated platelets. **A:** After 1–2 sec of thrombin injection, washed platelets display aggregation of granules, changes in the canalicular system, and projection of pseudofilopodia. **B–D:** Grazing fractures through the cytoplasmic-membrane interface reveal 9 nm wide filaments, separated from each other by approximately 15 nm (arrows) and lying just within the membrane. Extracellular surface (ES); protoplasmic fracture face (PF). In B, a granule (G) is visible deep to the filaments in the cytoplasm. **E:** In rare fractures, continuity between filaments (arrow) and the

cytoplasmic surfaces of granule membranes were seen, as shown here. Cross-fractured microtubules are present encircling the periphery (curved arrows). A, × 16,000. B, × 100,000. C, × 100,000. D, × 150,000. E, × 80,000.

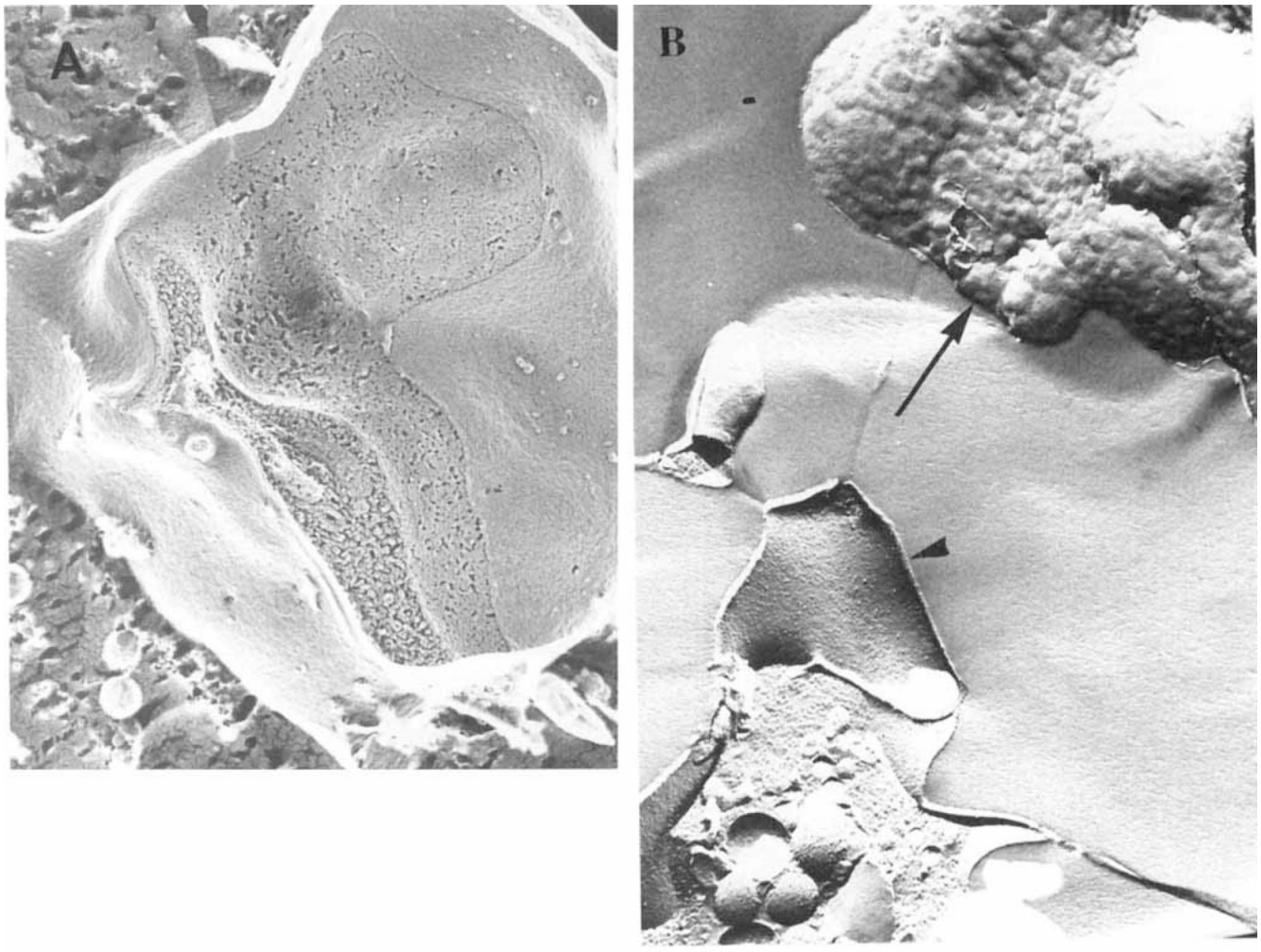

Fig. 4.

Polymyxin B treatment. **A:** Quick-frozen, fracture-etched platelets, whether stimulated or resting, had smooth, taut membrane surfaces. This unstimulated cell was treated with PXB, and no perturbation of the normally taut membrane is observed. **B:** When platelets are stimulated in the presence of PXB, surface membranes lose this taut appearance and become bubbly (arrow). Not all cells in a given population are affected—an adjacent cell has retained its taut, smooth membrane, showing that ice crystal formation is not likely to have caused the membrane perturbation seen in the adjacent cell, obviously at the same depth from the helium-cooled block. A, × 20,000. B, × 12,000.

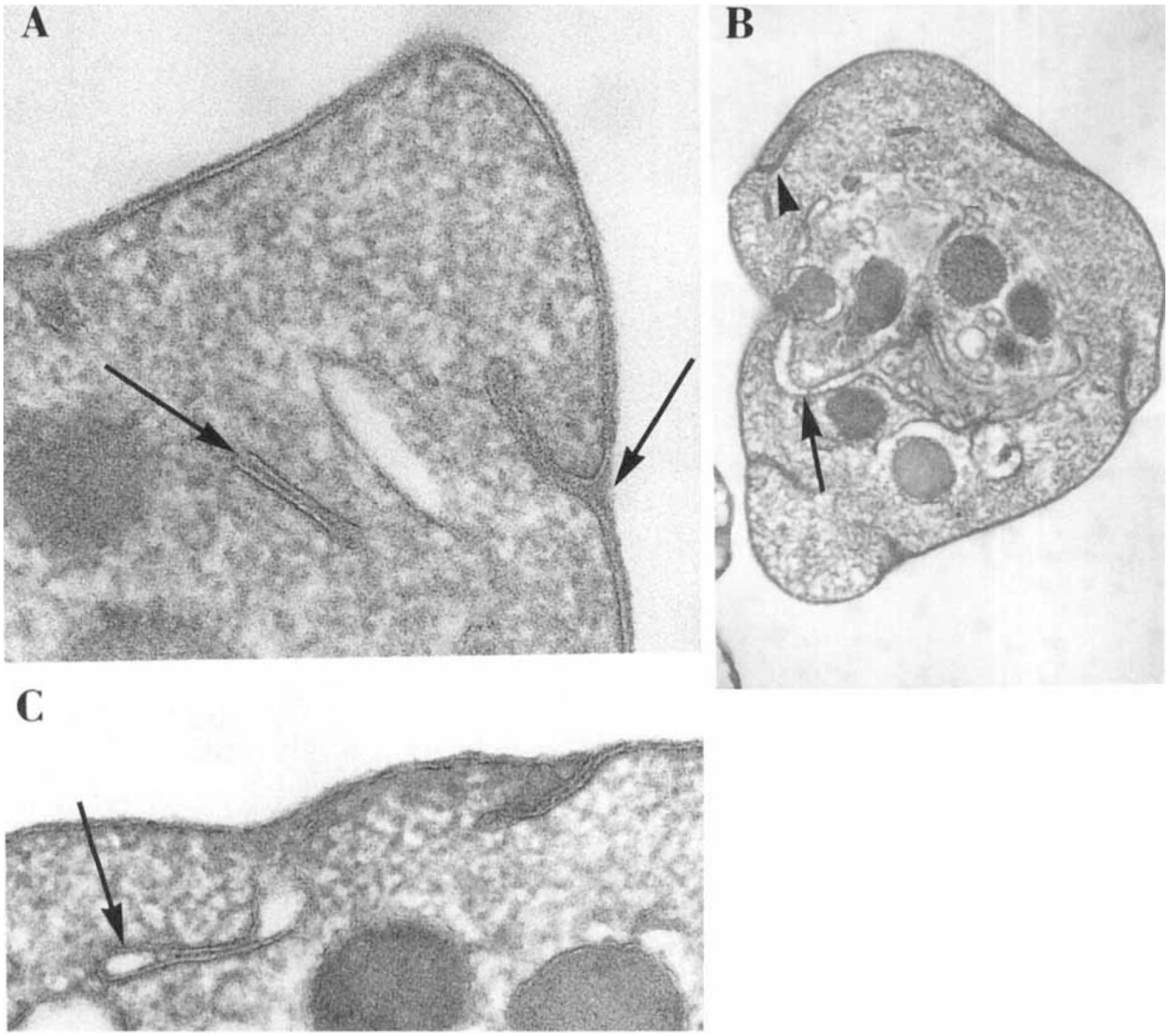

Fig. 5.

Thin sections of quick frozen-freeze substituted platelets. **A:** Unstimulated platelets have a discoid shape, with a pentilaminar surface-connected canalicular system (SCS) (arrows) both internally and at its junction with the plasma membrane. **B, C:** After 1–2 sec of thrombin stimulation prior to freezing, the inner portions of the SCS open (arrow in B and C), while the plasma membrane-SCS junction remains pentilaminar for some length into the cell's interior (arrow-head in B). Electron-dense material is sometimes seen at the mouth to the SCS. A, × 120,000. B, × 40,000. C, × 120,000.

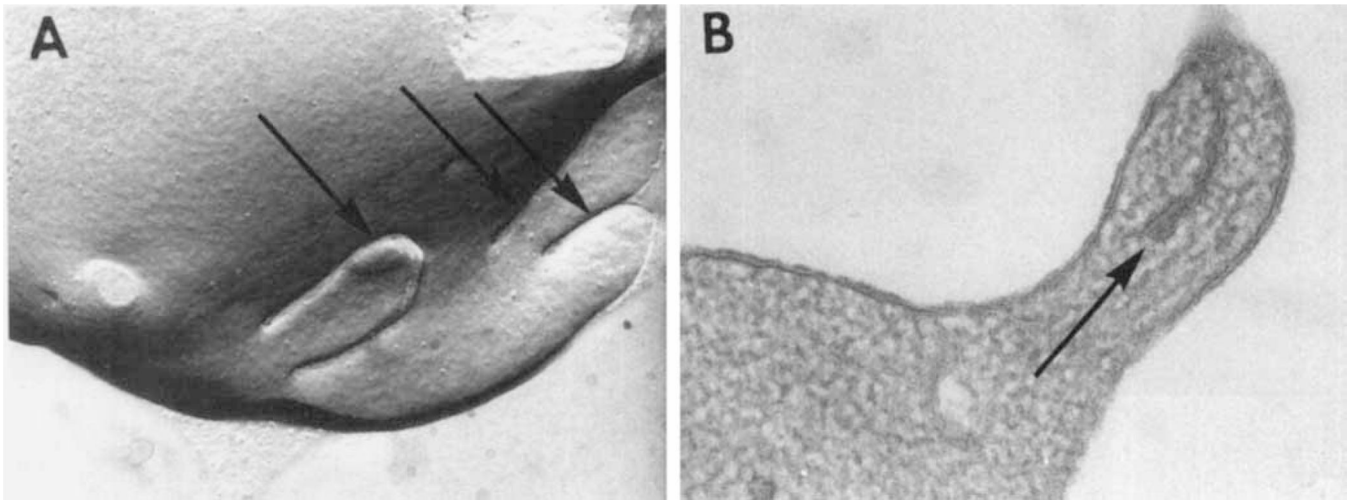

Fig. 6.

Surface-connected canalicular system in freeze-substituted, quick-frozen platelets. **A:** Quick-frozen, fractured platelets display serpiginous membrane furrows (arrows) and at the same time lose the pore-shaped openings into the SCS. **B:** Freeze-substituted platelets also display cytoplasmic pentilaminar membrane systems extending into early pseudofilopodia. A, × 20,000. B, × 70,000.

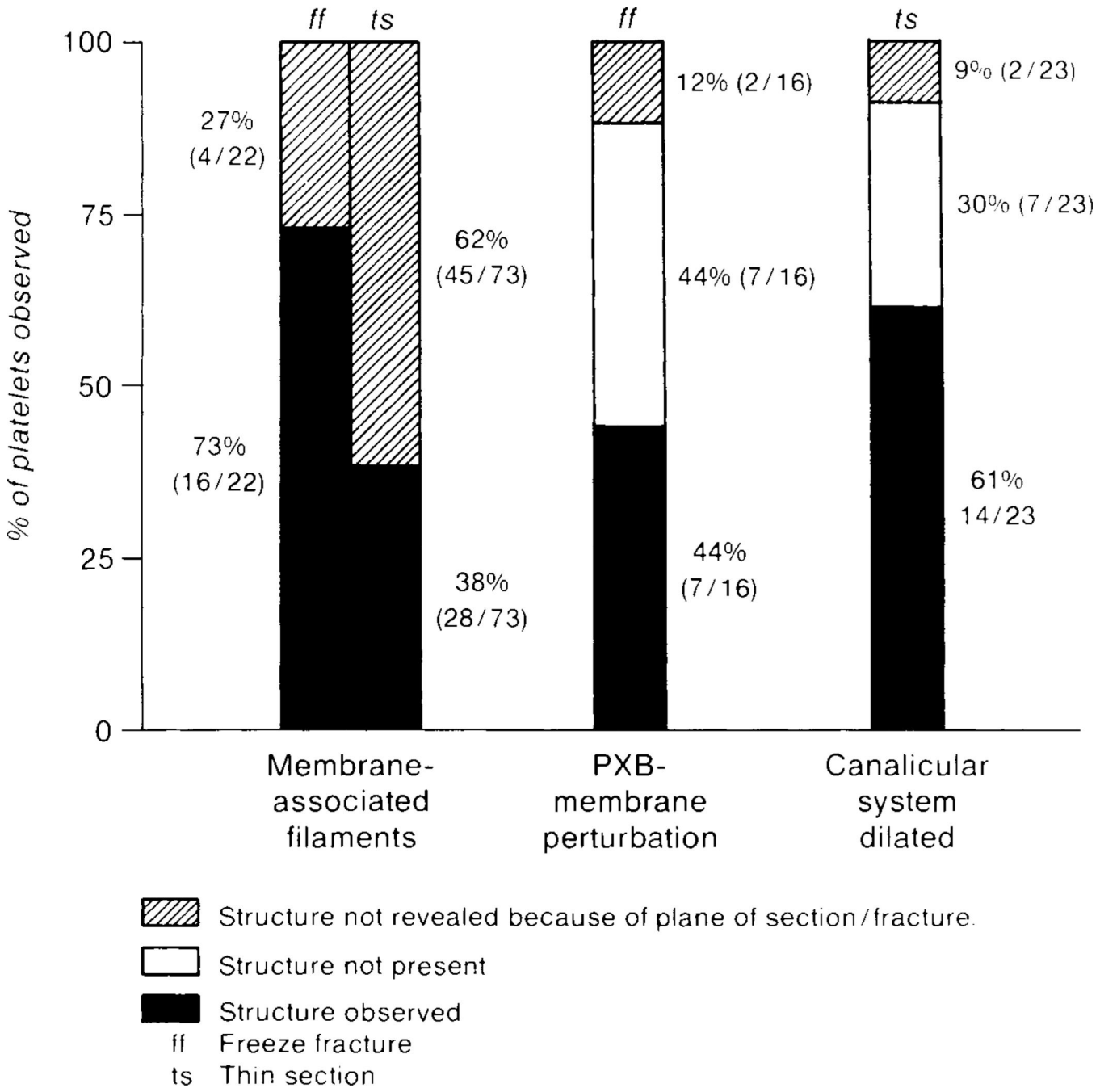

Fig. 7.

A histogram showing the percentage of stimulated cells in which a particular structure was identified. Note that all cells in which the sub-membranous cytoplasm could be observed had membrane-associated filaments. In contrast, only half the cells treated with PXB had membrane perturbations.