Membrane Structure of Nonactivated and Activated Human Blood Platelets as Revealed by Freeze-Fracture: Evidence for Particle Redistribution during Platelet Contraction

B. VAN DEURS and O. BEHNKE
Institute of Anatomy, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark

ABSTRACT The distribution of intramembrane particles of nonactivated and activated human blood platelets was studied by freeze-fracture under various experimental conditions to see whether morphological evidence for a structural coupling between the platelet actomyosin system and the fibrin network in a retracting clot could be established.

Membrane particles were evenly distributed in nonactivated platelets; the total number (E + P faces) was ~ 1,500/μm^2 of membrane, and there were two to three times more particles present on the E face than on the P face. Transformation of discoid platelets to "spiny spheres" by cooling did not change the particle distribution. Platelet activation and aggregation by serum or ADP caused no change in membrane particle density or distribution. Particle distribution was not changed in Ca^{2+}-activated platelets fixed immediately before fibrin formation, but after fibrin formation and during clot retraction, particles were sometimes most frequent on the P face and tended to form distinct clusters, and aggregates of E face pits were observed.

Blood platelets contain contractile proteins that are distinct as filaments in platelets in retracting clots. We suggest that the redistribution of particles seen in activated platelets during clot retraction reflects the establishment of mechanical transmembrane links between the platelet actomyosin system and the fibrin net. The P-face particle clusters may represent sites of force transmission between actin filaments bonded to the inside of the membrane and the fibrin network at the outside. Thus, whereas membrane particles may not be directly involved in the attachment of actin filaments to membranes, the transmission of the force of the contractile system to an exterior substrate apparently involves the intramembrane particles.

The attachment of actin filaments to cell membranes and the possible interaction with intramembrane proteins have been subjects of considerable interest for several years (see references 12, 17, 25, and 26). For example, evidence has been presented that in sperm acrosomes and intestinal microvilli, actin filament attachment occurs in particle-free domains of the membrane as revealed by freeze-fracture (25).

The blood platelet would appear a priori to be a promising system for studies of possible interaction between actin filaments and membranes. The demonstration of an actomyosin like protein in human blood platelets (5) was one of the first reports on the existence of contractile proteins in mammalian nonmuscle cells. Since then, the properties of platelet contractile proteins have been extensively studied (for review, see references 7 and 8).

Although the disk-shaped, nonactivated platelet contains much actin and myosin (13, 15, 20), only few filamentous structures are visible in the electron microscope (4). When platelets are activated, however, actin filaments appear abundantly in the platelet cytoplasm (2, 4, 18) together with myosinlike tactoids (4, 19, 30). Interaction between platelet actin and myosin is considered responsible for platelet contractile

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activity as it manifests itself in the consolidation of a hemostatic plug or in the retraction of a clot.

The mechanism by which platelets pull the fibrin net together in a retracting clot is a matter of dispute (2, 7, 21), but whatever the mechanism is, for it to be operational the contractile force of the platelet actomyosin system must somehow be transmitted across the platelet membrane to the fibrin network. This requirement implies that the platelet membrane is an intermediary, with components of the contractile system attached to the membrane's inside and fibrin fibers to its outside. Actin has been demonstrated in platelet plasma membrane fractions (24), and platelets contain α-actinin (11), which suggests a membrane-actin interaction most likely via α-actinin (10, 14).

The experiments reported here were performed to analyze the platelet membrane structure under different experimental conditions and to see whether platelet activation and the establishment of the implied coupling between the platelet contractile system and the fibrin net was reflected in membrane structure as revealed by freeze-fracture (27). We found that intramembrane particles were evenly distributed, being most frequent on the E face in the nonactivated platelet, whereas in a retracting clot, particles were sometimes more abundant on the membrane's P face and tended to aggregate into clusters. We discuss the significance of these observations in terms of relative strengths of the bonding to either side of the membrane of the integral membrane proteins, and in terms of membrane-contractile filament interaction.

MATERIALS AND METHODS

Preparation of Platelets

Blood samples from healthy humans of both sexes were collected by venipuncture of the antecubital vein. The blood was drawn into a plastic syringe containing 1 ml of 3.8% sodium citrate, prewarmed to 37°C, to 9 ml of blood. In some experiments with nonactivated platelets, 0.1% EDTA was added to the sodium citrate. The samples were transferred to plastic vials and centrifuged at 37°C for 10–15 min at 150 *g*, and the supernatant platelet-rich plasma (PRP) was then subjected immediately to the procedures described in this section.

Experimental Procedures

NONACTIVATED PLATELETS: Platelets in PRP were processed directly as described below.

COOLING AND CALCIUM-ACTIVATION OF PLATELETS: 1-ml samples of PRP were maintained at 4°C for 1 h, and then processed as described below.

Platelets were activated by adding 50 μl of a 0.5 M $CaCl_2$ solution to 1 ml of PRP. Platelet morphology was watched closely in a drop of the Ca^{++} activated PRP in a dark-field microscope as described previously (3). Fixative was added immediately to the sample when the platelet disk-shape was lost and the platelet spines had formed, and before fibrin fibers had appeared. The shape-change occurs within a couple of minutes and is nearly synchronous in the whole population. The activated platelets were subsequently processed as described below.

ACTIVATION OF PLATELETS BY SERUM AND BY ADP: Platelets were activated by adding 50–100 μl of serum freshly extruded from a retracting clot. Platelet shape-change occurred within seconds after the addition, and platelets were fixed immediately before fibrin formation was visible. The specimens were processed as described below.

Shape-change and aggregation of platelets were induced by adding 50 μl of a stock solution of 1 mg ADP/ml (Sigma Chemical Co., St. Louis, Mo.) to 1 ml of PRP. Immediately after shape-change had occurred and aggregation had taken place, the aggregates were fixed and processed as described below.

PLATELETS IN A RETRACTING CLOT: PRP was recalcified as previously described and maintained at 37°C. Clots formed within 5–6 min, and were allowed to retract for 30–45 min. Small pieces of the clots were then fixed for 24 h by immersion in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and then processed for freeze-fracture.

Fixation of Platelets in Suspension

4 ml of 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 24°C was added to 1 ml of PRP either immediately after its preparation or after experimentation. After 5 min, platelets were sedimented by centrifugation (at 22°C), the supernatant plasma was discarded, and the sedimented platelets were fixed for an additional hour in fresh 2% glutaraldehyde. The fixed platelets were then sedimented into pellets by centrifugation for 5 min in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, Calif.).

Freeze-Fracture

After the fixed platelet pellets and the retracted clots were cut into small cubes (~0.5 mm), the material was cryoprotected by immersion for 60 min at 4°C in 15% glycerol in 0.1 M sodium cacodylate buffer, pH 7.2, followed by 30% glycerol in the same buffer for at least 60 min. Specimens were mounted on gold disks and frozen in a mixture of liquid and solid nitrogen (a nitrogen slush) (28).

In some experiments, unfixed, pelleted platelets and retracted clots were either frozen directly (without fixation or cryoprotection = fresh-frozen material) or were cryoprotected in glycerol before being frozen in the nitrogen slush.

Fracturing and replication were performed in a Balzers 301 freeze-etch apparatus (Nordic Balzers AB, Sweden) with electron beam evaporation equipment. Specimen stage temperature was −104°C. Immediately after fracturing, platinum was evaporated under an angle of 45° for 8–10 s (~25 Å as measured with a quartz crystal thin-film monitor). Carbon was thereafter evaporated for ~12 s (~300Å) to stabilize the replica.

Replicas were rinsed for some hours in hypochlorite or Sputofluol (Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.) followed by distilled water, and were finally collected on Formvar-coated grids.

Quantitation of Intramembrane Particles

The density of intramembrane particles (IMP) was determined on prints (X 100,000) of platelets selected in the electron microscope at a low magnification, at which particles are not visible but where identification of E and P faces is possible (see Results). Particles were counted by use of a 2 × 3-cm mesh on areas of exposed platelet membranes that were considered to be approximately plane and that did not include openings of the surface-connected system of the platelet or any other irregularities. The number of IMPs per square micrometer (mean ± SD) of the fractured platelet membrane was calculated (Table I).

RESULTS

Nonactivated Platelets

Replicas of fractured platelets fixed in suspension in citrated or citrate-EDTA PRP (Fig. 1) very often exposed the platelet surface membrane, although cross-fractures revealing components of the platelet cytoplasm such as the surface-connected membrane system (SCS) and platelet granules (cf. Fig. 7) were also frequent.

E and P faces of the fractured platelet membranes were determined (*a*) by evaluating the curvature of the exposed membrane, and (*b*) by determining whether the SCS-openings to the surface appeared as depressions (P face) or elevations (E face) (Figs. 1 and 2).

IMP were judged to be evenly distributed in the membrane.[1] They appeared about two and one-half times more frequently on the E face than on the P face (Fig. 2). We found in total ~1,500 particles/μm^2 of platelet membrane (E + P face; Table I). These values for particle density and distribution (Table I) are very close to those previously reported (6).

This P/E face ratio of particles was found also in unfixed

[1] There are three ways to indicate the distribution of particles in a replica, using area frequency distribution analysis: random, regular, and clustered. Even distribution is here defined either as random or regular, and clustered distribution will, thus, refer to a directly visible "uneven" particle pattern.

TABLE I

Density and E- and P-Face Distribution of IMP in Freeze-Fractured Human Platelets after Various Experimental Procedures

	Experiment	$\bar{X}$ ± SD*			
		IMP_P	IMP_E	IMP_{P+E}‡	$K_P = IMP_P/IMP_E$§
1	Nonactivated (citrated)	463 ± 136 (48)	1,045 ± 213 (66)	1,508	0, 44
2	Nonactivated (citrated), unfixed‖	305 ± 127 (36)	850 ± 204 (36)	1,155	0, 36
3	Nonactivated (citrate-EDTA)	478 ± 145 (30)	1,123 ± 163 (36)	1,601	0, 43
4	Cooling (4°C)	388 ± 139 (30)	1,128 ± 203 (24)	1,516	0, 34
5	Ca^{2+} activation (before fibrin formation)	465 ± 120 (42)	925 ± 181 (42)	1,390	0, 50
6	Serum activation	412 ± 102 (30)	1,098 ± 183 (36)	1,510	0, 38
7	ADP activation	402 ± 120 (30)	1,008 ± 207 (48)	1,410	0, 40
8	Ca^{2+} activation (clot retraction)	670 ± 303 (38)	842 ± 247 (38)	1,512	0, 80

* IMP density per square micrometer P face (IMP_P) and E face (IMP_E); mean ± SD, numbers in parenthesis = number of measurements.
‡ Total number of IMP (P + E) per square micrometer platelet membrane.
§ K_P, Particle partition coefficient.
‖ Except for experiment *2*, glutaraldehyde fixation were used in all experiments shown here.

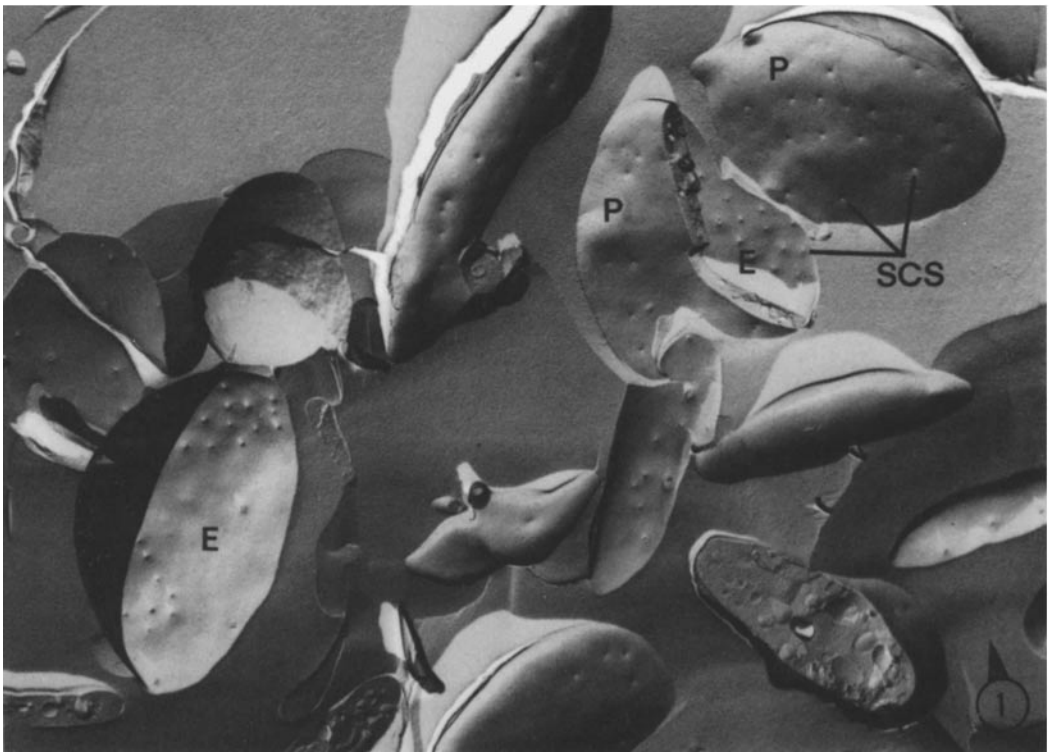


FIGURE 1 Survey micrograph of nonactivated, fractured platelets. The platelets are disk-shaped, and on the exposed membranes the openings of the surface-connected membrane system (*SCS*) are seen. *E* and *P* on this and the following micrographs refer to E face and P face, respectively, of the fracture. The E and P faces can be determined by the curvature of the membrane and by the appearance of the SCS-openings (elevations on E face, and depressions on P face). The arrowhead on the circle surrounding the figure number on this and the following micrographs indicates the direction of platinum shadowing. × 12,600.

platelets cryoprotected in glycerol although the total amount of particles was somewhat smaller (Table I). Redistribution of particles induced by glycerol (16) was not observed. Replicas prepared from fresh-frozen platelets (= no fixation, no cryoprotection) in most cases exhibit pronounced freezing damage, and structures revealed in the fractures were often difficult to identify. Attempts to count particles on this material were, therefore, suspended.

Effects of Cooling

Cooling induces a rapid depolymerization of the platelet marginal bundle of microtubules (see reference 1), and the platelets assume the shape of irregular "spiny spheres" (Fig. 3). Platelets cooled for 1 h showed a distribution frequency of particles indistinguishable from that described for nonactivated platelets (Table I).

Effects of Platelet Activation by Calcium, Serum, and ADP

Replicas of the plasma membrane of calcium-activated platelets before fibrin had formed (Figs. 4 and 5) showed a distri-

FIGURE 2 Fractured membranes of nonactivated platelets seen at higher magnification demonstrate that particles are more frequent on the E face than on the P face. × 96,000.

bution and frequency of intramembrane particles as described in nonactivated platelets (see Table I).

Platelets were activated and aggregated by the addition of a small amount (50–100 μl) of serum freshly extruded from a retracted clot. Replicas of membranes of aggregated platelets (Fig. 6) showed no appreciable change in particle distribution and frequency.

Addition of ADP to citrated PRP induces a platelet shape-change followed by platelet aggregation (Fig. 7). Replicas of fractured platelet aggregates showed the same distribution and frequency of particles as described above (Table I).

Platelets in a Retracting Clot

Clotting was induced in citrated PRP by recalcification, and clots were allowed to retract at 37°C for 30–45 min. Replicas of clots fixed during retraction showed platelet aggregates

consisting of irregularly-shaped platelets with many pseudopods (Fig. 8). These aggregates appeared more or less densely packed with single platelets or platelet pseudopods occurring in between the aggregates (Figs. 9–13). Openings of the SCS were infrequently seen.

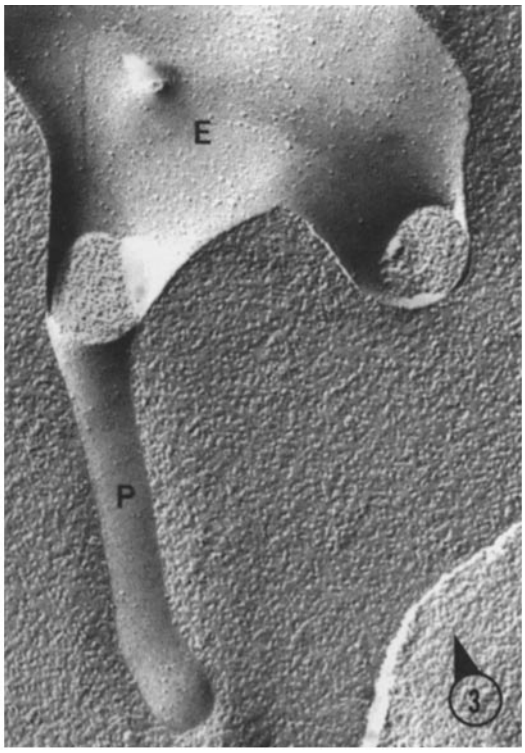
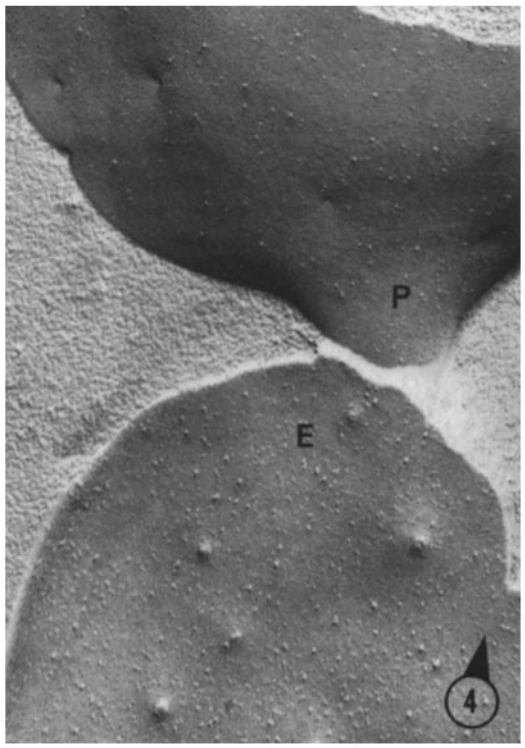
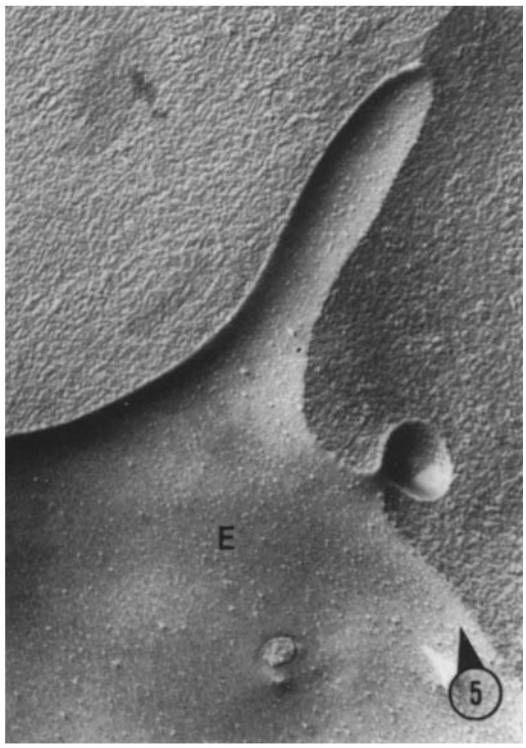


FIGURE 3 Fractured membrane from a cooled, irregularly shaped (spiny-sphere) platelet. Particles are predominant on the E face. × 57,600.

A remarkable feature of cleaved platelet membranes in the retracted clots was the irregularity of particle distribution and frequency. Within the platelet aggregates, most of the exposed membranes exhibited evenly distributed particles which were most frequent on the E face (Fig. 8 and Table I), a situation similar to that seen, for example, after ADP or serum activation. However, E faces with rather few particles and P faces with many particles were encountered (features which are indicated by the mean values and K_p value in Table I), and in some cases P faces distinctly exhibited an abundance of particles (Fig. 9). Furthermore, in many cases particles on the P face were aggregated to form patches surrounded by more or less particle-free areas (Figs. 10–12). On E faces, patches with densely packed pits were occasionally seen; these patches presumably correspond to P-face particle aggregates (Fig. 13). The aggregated particles were more irregular in shape and less distinct than nonaggregated particles. Because of the distribution and the appearance of the P-face particles under these circumstances, systematic counts of particles per square unit could not be performed, and we are, therefore, uncertain on which fracture face of the membrane the particles are more frequent in membranes with aggregated particles. Evaluation of numerous micrographs convincingly showed that the distribution of particles on E and P faces varied, and aggregates of P-face particles may, therefore, not necessarily indicate that there are more P-face particles than E-face particles per square micrometer fractured platelet membrane. Regionally, however, this was obviously the case.

The presence of clustered P-face particles was confirmed in unfixed, cryoprotected material, and in fresh-frozen (noncryoprotected) material (Figs. 14 and 15).





FIGURES 4 and 5 Platelet membranes after Ca^{2+} activation and before fibrin formation. There are more particles on the E face than on the P face. × 57,600.

DISCUSSION

This study shows that (*a*) the plasma membranes of nonactivated human blood platelets have about two and one-half times more particles (assumed to represent integral membrane proteins) associated with the external side (E face) of the lipid bilayer than with the protoplasmic side (P face) and that (*b*) the membranes of activated platelets in a retracting clot sometimes have their particles associated predominantly with the protoplasmic side where they tend to form aggregates. These properties of the platelet membrane in different functional states will be discussed in terms of a particle partition coefficient and membrane-contractile system interaction.

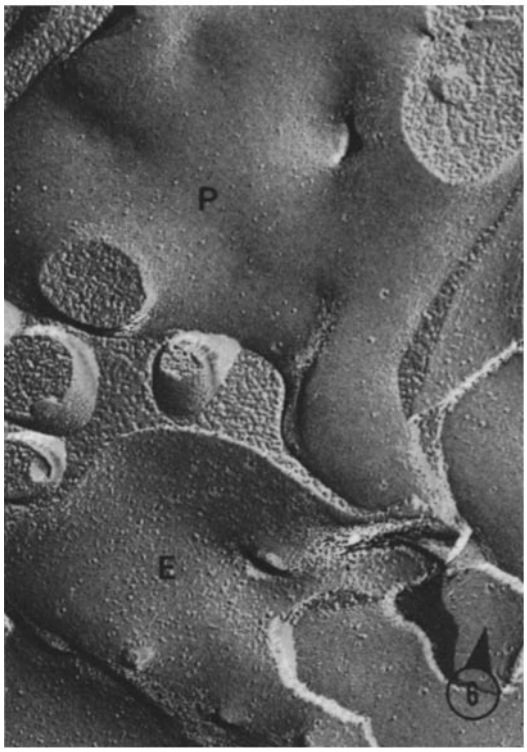


FIGURE 6 Fractured membranes of a serum-induced platelet aggregate. There are more particles on the E face than on the P face. × 57,600.

The fracture of a frozen biological membrane (in the membrane's plane) is assumed to follow "a plane of minimal resistance" and to leave covalent bonds unbroken. In principle, the fracture can pass either superficially or deeply, relative to an integral protein,[2] and provided the relative strengths of bonding of the integral proteins to either side of the membrane are similar, the fracture by a stochastic process may result in the same amount of particles being present on the P and E faces. This concept has been expressed in terms of the particle partition coefficient, $K_p = C_P/C_E$, where C_P and C_E represent the concentrations of particles on the P face and the E face, respectively (IMP_P and IMP_E in Table I; [23]). Thus, in a fractured membrane with equal bond strengths on either side of the integral proteins, $K_p = 1$.

In most studies of the distribution of particles between E and P face reported so far, a $K_p > 1$ has been found, typically by a factor of ~3. This distribution, then, seems to be true for glutaraldehyde-fixed mammalian membranes, and, thus, the membrane of the fixed, nonactivated platelet in which we found $K_p < 1$ (Table I) seems to be a unique case.

Because glutaraldehyde is a potent cross-linking agent (29), fixation of membranes with this aldehyde before cleavage may change the in vivo K_p value significantly by introducing strong bonds between the integral proteins of a membrane and molecules on the membranes' inner or outer aspect, resulting in an artifactual predominant localization of particles on either face.

A glutaraldehyde-induced shift in a K_p value has, for example, been documented in the case of tight-junction proteins (considered a class of "globular" integral proteins; 29), and for nonjunctional proteins in endothelial cell membranes (9). In either case a $K_p < 1$ was found in unfixed material, whereas a $K_p > 1$ was found after glutaraldehyde fixation. In most mammalian membranes, however, $K_p > 1$, i.e., membrane particles are present predominantly on the P face irrespective of the procedure used in the preparation of the tissue (22).

In the nonactivated platelet membrane $K_p < 1$, whether the platelets had been fixed in glutaraldehyde or not. Thus, the unique value of the platelet membrane does not appear to be a fixation artifact and it suggests that the integral proteins of

[2] If an integral membrane protein penetrates the hydrophobic part of the lipid bilayer as a straight polypeptide chain, it is likely that it would simply be cross-fractured and, thus, be invisible in a replica.

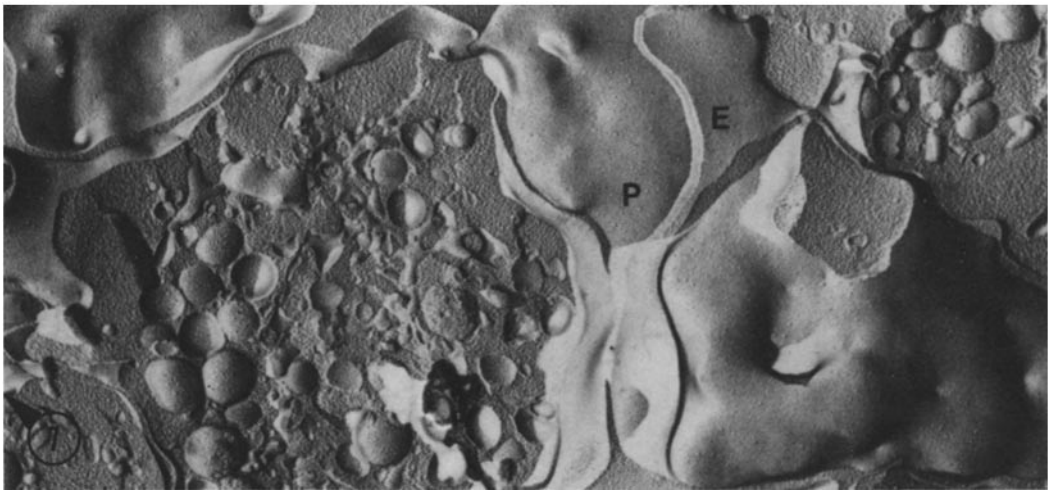


FIGURE 7 A cross-fractured platelet and fractured membranes of an ADP-induced platelet aggregate. The highest particle density is seen on the E face. × 24,000.

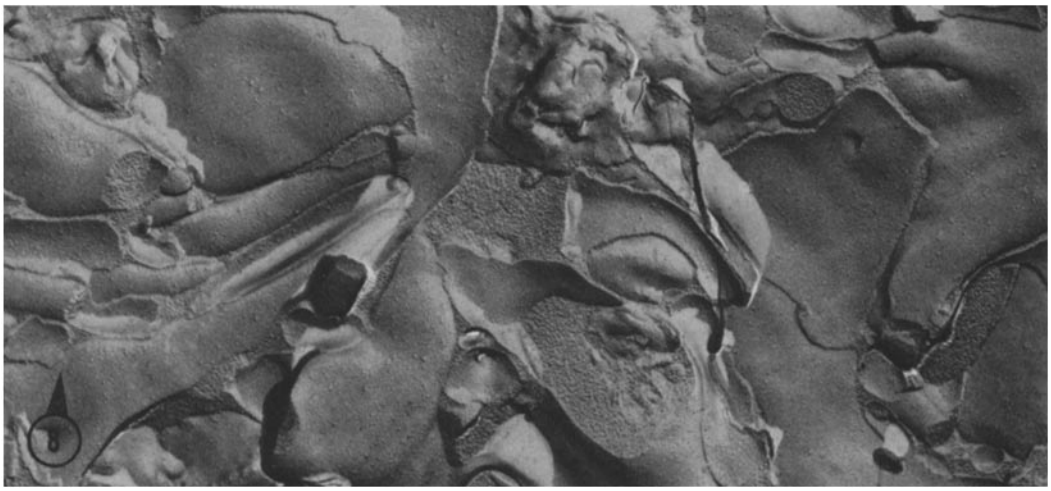


FIGURE 8 Survey micrograph of membranes of aggregated, Ca^{2+}-activated platelets in a retracting clot (after fibrin formation). × 28,500.

FIGURE 9 Platelet in a retracting clot. In this case most particles are present on the P face, but the particles are not clustered. × 57,600.

this membrane are more strongly bonded to the external than to the internal side of the membrane. We cannot as yet explain the firm association of the major part of integral proteins of the platelet membrane with the external leaflet, but we tentatively suppose that cross-links between exterior, hydrophilic chains of integral glycoprotein molecules and of the carbohydrate moiety of glycolipids are responsible for the unique platelet K_p (Fig. 16).

Replicas of cleaved membranes of aggregated platelets present in retracting clots revealed a heterogenous picture of particle distribution and association. IMP were sometimes predominant on the P face, where they could be evenly distributed or clustered. This observation indicates that in some platelets in a retracting clot a major part of the integral proteins of the platelet membrane lose their relatively firm association with the exterior membrane surface and instead become more firmly bound to the interior surface, as revealed by particle aggregates on the P face. Moreover, on the E face, distinct pits that normally are invisible appear in clusters presumably corresponding to the P-face particle aggregates. We suggest that interaction established between the platelet membrane and contractile proteins may explain this change in integral membrane protein topography on the following two premises: (*a*) The platelet is a potential smooth muscle cell; it contains actin, myosin, tropomyosin, and α-actinin, and activation induces a cascade of events, notable among which is polymerization of platelet actin (2). (*b*) Clot retraction is believed to be effected predominantly by contraction of platelets pulling the fibrin net together. As mentioned above, to be operational in this respect the platelet contractile system must be physically linked via the platelet membrane to the fibrin fibers. It is likely that α-actinin, which seems to be associated with the platelet membrane (11), establishes the link of the cytoplasmic side to platelet actin. Bridges forming focal contacts between fibrin fibers and the outside of the platelet membrane (2) may establish the link to the external side, creating a focal site of force transmission.

We believe that at the presumed sites of force transmission the integral membrane proteins may be more firmly bound to the actin side (P-face) than to the fibrin side (E face; Fig. 16). On the basis of morphological observations, it has been argued (2) that clots may retract because platelets move relative to the fibrin net, thereby pulling the fibrin fibers toward the platelet aggregates. The suggested mechanism implies labile bonds between platelets and fibrin, permitting cycles of attachment and detachment of platelets to fibrin, and perhaps also of actin to the platelet membrane. This may explain why only some

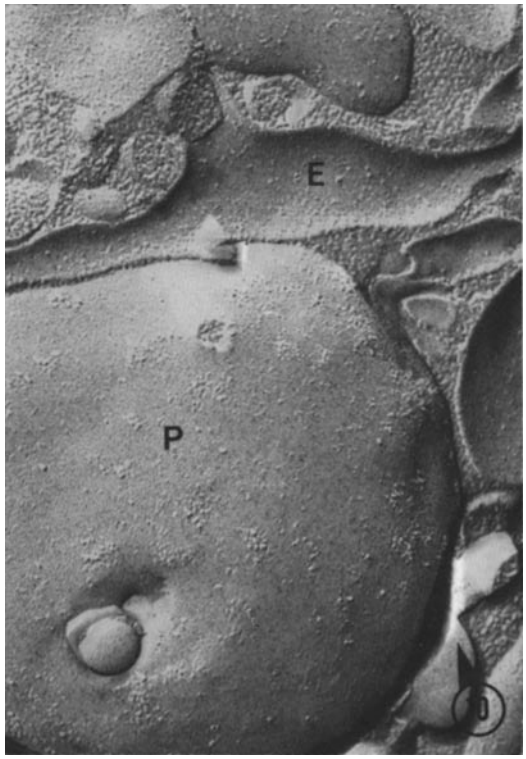


FIGURE 10 Platelets aggregated in a retracting clot. One of the exposed membranes clearly exhibits particle clusters on the P face. × 57,600.

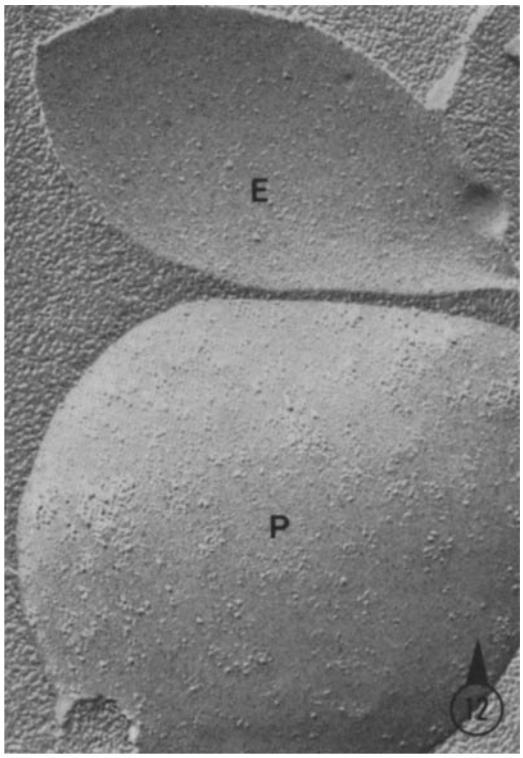


FIGURE 12 In this micrograph of platelets in a retracting clot, both E and P faces are seen, the latter exhibiting many clustered particles. × 57,600.

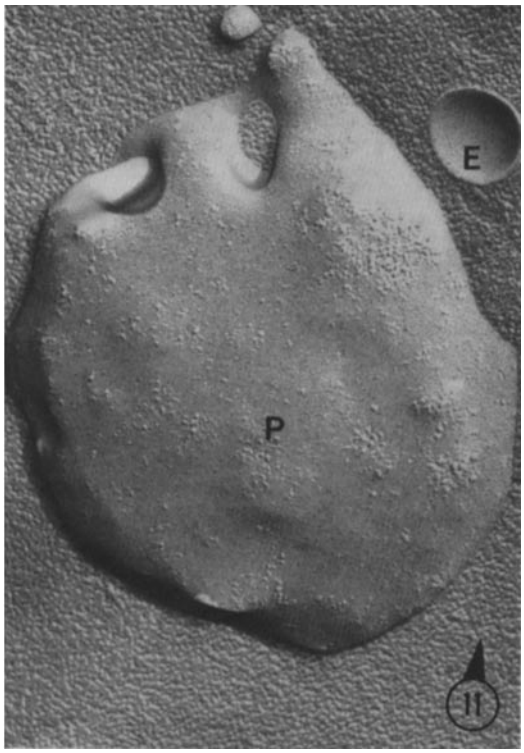


FIGURE 11 Another example of distinct P-face particle clusters. × 57,600.

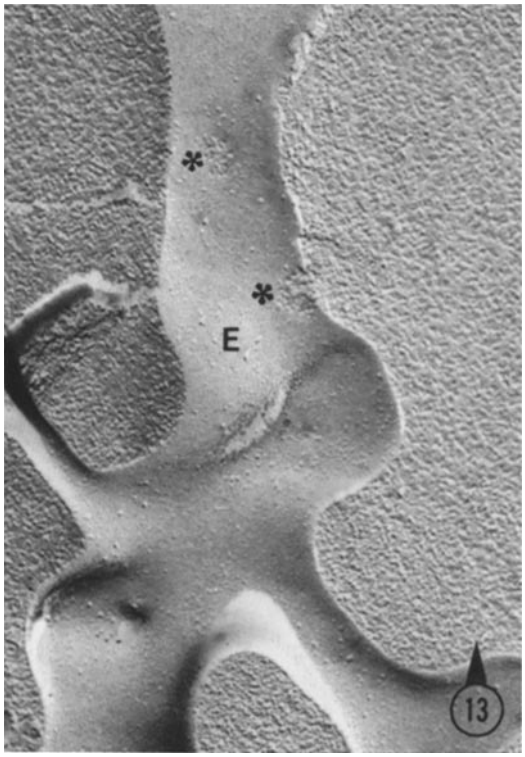
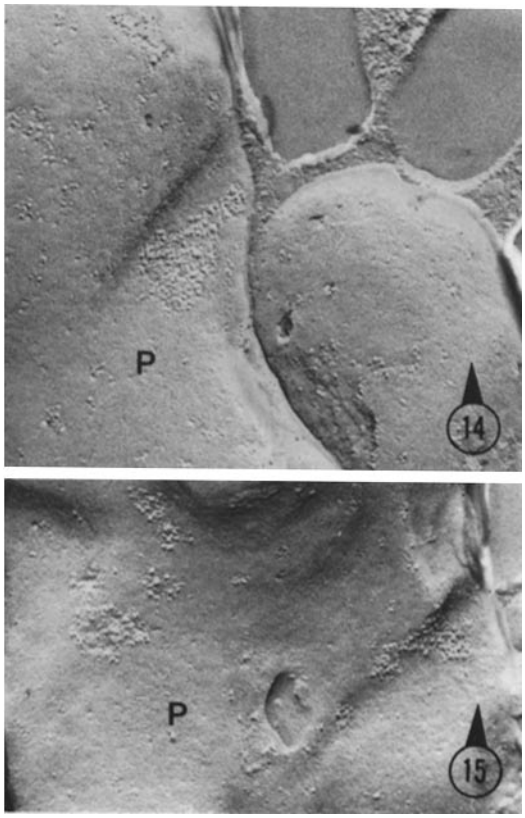


FIGURE 13 E face of a platelet membrane in a retracting clot. (*), Aggregates of pits. × 57,600.

platelet membranes in a retracting clot exhibited P-face particle clusters.

In the discussion of whether actin filaments attached to the inner membrane surface cause intramembrane particle aggregation, observations on other systems of actin filament - membrane interaction, which seem to be in conflict with our interpretation, are of interest. In sperm cells of the horseshore crab and mussel, actin filaments are attached to the acrosome



FIGURES 14 and 15 P faces of rapidly frozen platelet membranes in a retracting clot. Particle clusters are evident. × 57,600.

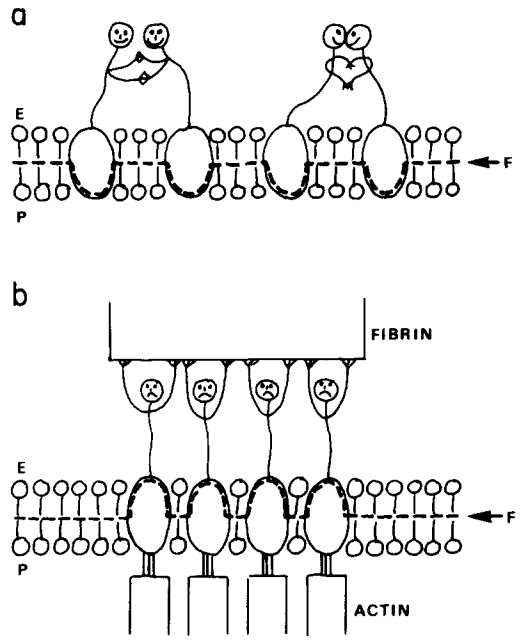


FIGURE 16 A schematic presentation of our interpretation of the present freeze-fracture observations on membranes of nonactivated platelets (*a*) and of platelets in retracting clots (*b*). In (*a*) relatively strong bonds between the external segments of the evenly distributed integral glycoproteins are indicated, explaining the fracture plane (dashed line labeled *F*). In (*b*) bonds have been established between the actin filaments on the inside of the membrane and the integral proteins. This causes aggregations of the membrane proteins and may explain the fracture plane provided the bonds to the external fibrin network are weaker. For details, see Discussion.

membrane, and in intestinal cells, actin filaments anchor at the tip of microvilli (25). In these cases, the sites of filament attachment have been shown by freeze-fracture to be devoid of IMP (25). However, although the contractile filaments in these systems adhere to the membrane's inside, perhaps via α-actinin, they have no obvious need for interaction with transmembrane proteins to establish transmission of force between themselves and an exterior substrate. Thus, membrane particles may not be directly involved in the attachment of actin filaments to membranes (25), but when the contractile force of the actomyosin system is transmitted to an exterior substrate (such as the fibrin network in the case of platelets in a retracting clot), involvement of IMP seems plausible. It should be noted that polymerization of platelet actin in itself does not induce a change in intramembrane topography because the membranes of ADP- or serum-activated platelets showed a particle distribution indistinguishable from that of nonactivated platelets.

In conclusion, our observations show that the platelet membrane exhibits certain unique properties and suggest that a modulation of membrane topography occurs when platelets exert a contractile function, and that the modulation is caused by an interaction of the integral membrane proteins and the platelet contractile system when they act in concert to exert force upon an exterior substrate.

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