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MICROMORPHOLOGIC CONSEQUENCES FOLLOWING PERTURBATION OF ERYTHROCYTE MEMBRANES BY TRYPSIN, PHOSPHOLIPASE A, LYSOLECITHIN, SODIUM DODECYL SULFATE AND SAPONIN

A CORRELATED FREEZE-ETCHING AND BIOCHEMICAL STUDY

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SUMMARY

1. Treatment of erythrocyte membranes with phospholipase A and low levels of saponin induces alternation in the fracture plane of freeze–etch preparations in such a way, that exposed membrane faces are covered with an array of plaques made up of adherent membrane material.

2. Increasing levels of saponin, lysolecithin and sodium dodecyl sulfate lower the number of membranes fracturing tangentially, presumably because the altered balance between polar and apolar forces in the membranes allows the fracture plane, normally guided by hydrophobic regions in the membrane, to follow directions other than along the membrane plane.

3. Trypsin also generates new polar groups at its site of action, but does not influence the plane of fracture.

4. Our data suggest that alternations of the fracture plane occur preferentially in lipid domains.

INTRODUCTION

Biophysical and biochemical approaches to the characterization of biomembranes have long been supplemented by their direct electron-microscopic visualization. Moreover, the freeze-etching technique, in which cells are physically "fixed" by low temperature, now allows one to probe structurally into the apolar cores of membranes and, by deep etching, to directly view polar external faces[1,2]. The molecular character and organization of membrane components appears to be largely preserved by the latter method and studies on lipid–water systems indicate that no changes of lipid phase occur during the rapid cooling employed[3]. However, it is still uncertain what a freeze-etch image represents at the molecular level and we are therefore correlating our biochemical and biophysical investigations of erythrocyte membranes,

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with their ultrastructural appearance by freeze-etch electronmicroscopy. In this report we describe the effects of trypsin, phospholipase A, lysolecithin, saponin and sodium dodecyl sulfate on erythrocyte membranes.

MATERIALS AND METHODS

Crystalline trypsin was obtained from Sigma Chemical Co. (St. Louis, Mo.). Phospholipase A was purified from *Naja naja* venom (Sigma Chemical Co.) as in ref. 4 and used as stated in the text. Our stock solutions containing 0.6 mg protein/ml 0.1 M Tris–HCl, pH 7.4, were stored at —75°. Crystalline bovine serum albumin was obtained from Behringwerke, Marburg, Germany and pure oleic acid from Carl Roth OHG, Karlsruhe, Germany. To saturate bovine serum albumin with oleate, we dissolved 7 mg of the protein in 3 ml 0.1 M Tris–HCl, pH 7.4, overlaid this with 1 mM oleate in heptane (1 ml), mixed at room temperature for 24 h and then isolated the albumin solution after phase separation[5]. The lysolecithin used, was obtained from Sigma Chemical Co. and showed only a single component, with the same mobility as authentic lysolecithin upon thin-layer chromatography on carboxymethylcellulose silica gel, using chloroform–methanol–water (65:40:10, v/v/v) as solvent[6], a technique used also for our other phospholipid assays. Lysolecithin was dispersed in 0.1 M Tris–HCl, pH 7.4, by 2 min sonification (Branson Sonifier Model S125, 2A, 20000 Hz)[6]. Pure sodium dodecyl sulfate was obtained from E. Merck, AG, Darmstadt. Saponin (Riedel de Haen, Hannover) was used without further purification.

Erythrocyte membranes were prepared according to the method of DODGE *et al.*[7], but incorporating the modifications described in refs. 8 and 9. We used 0.1 M Tris–HCl, pH 7.4 as buffer, unless stated otherwise and in our control studies added this buffer instead of the reagents. After all incubations, membranes were washed twice with 0.1 M Tris–HCl, pH 7.4 and each time sedimented at 16000 $\times$ g_{av}. (Sorvall RC2B centrifuge, rotor SS34) or 75000 $\times$ g_{av}. (Spinco L265B centrifuge, rotor 50Ti). Sediments were frozen immediately, without cryoprotective agents, in Freon 22 (Schick and Co., Stuttgart). Electrophoretic molecular sieving of control and treated membranes in 5.8% polyacrylamide, containing 1% sodium dodecyl sulfate was as in ref. 9. Protein was quantified as in refs. 8 and 9 and protein staining of polyacrylamide gels was as in ref. 9.

Freeze-etching was performed according to the method of MOOR[10,11] using the Balzers' apparatus type 360M (Balzers AG, Liechtenstein). The etching time and temperature were 60 sec and —100°.

After deposition of platinum–carbon and carbon films on the desired surfaces, the replicas were removed with 40% chromic acid, washed with distilled water and viewed on formvar-coated copper grids in a Siemens Elmiskop 1A. Dimensions were calibrated by use of standard carbon grating replicas. Particle counts were performed under a dissection microscope on calibrated positives with a calibrated grating placed over flat areas of membrane.

RESULTS

The effects of trypsinization

Treatment of erythrocyte ghosts with low levels of trypsin, (*i.e.* up to 0.01 mg enzyme per mg membrane protein in 5 ml 5 mM phosphate buffer, pH 8.0, at 37°

for 60 min) produces no detectable micromorphologic alterations, even though gel electrophoresis in sodium dodecyl sulfate indicates considerable cleavage of the major membrane peptides (Fig. 1b), in accord with the data of STECK *et al.*[12].

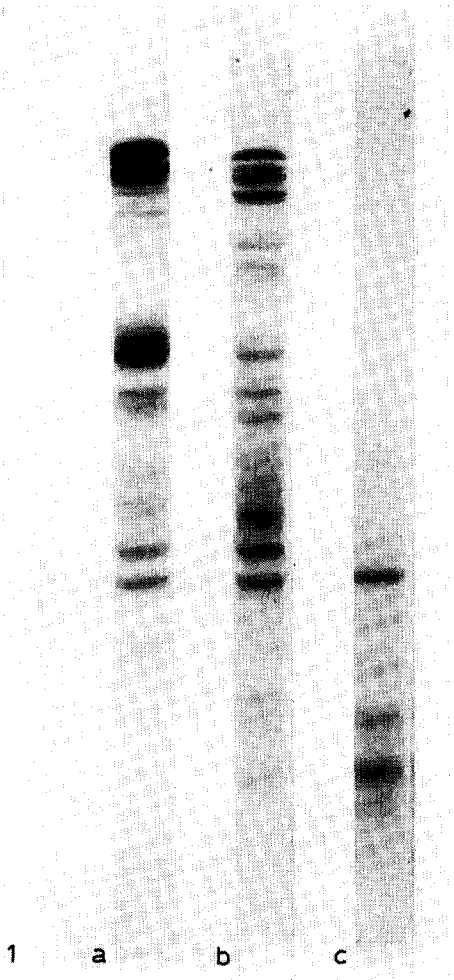

Fig. 1. Electrophoreses of trypsinized membranes in polyacrylamide, containing 1% sodium dodecyl sulfate and 0.04 M dithiothreitol. a. Control membranes. Incubated with buffer only. b. Membranes treated with 0.001 mg trypsin per mg membrane protein show considerable cleavage of the major peptides. c. Treatment with 0.1 mg trypsin per mg membrane protein effects massive breakdown of all membrane peptides.

Exposure of intact erythrocyte ghosts and "right-side-out" vesicles to high trypsin levels (0.1 mg trypsin per mg membrane protein) at 37° for 60 min decreases the number of membrane-associated particles not significantly but rearranges them into large clusters, separated by regions of smooth surface (Fig. 4), as also noted in refs. 13 and 14. The etched outer membrane surfaces appear affected allowing visualization of the underlying particle clusters (Fig. 4, arrow), similar to the surface irregularities described on etched surfaces by PINTO DA SILVA *et al.*[15]. Importantly, gel

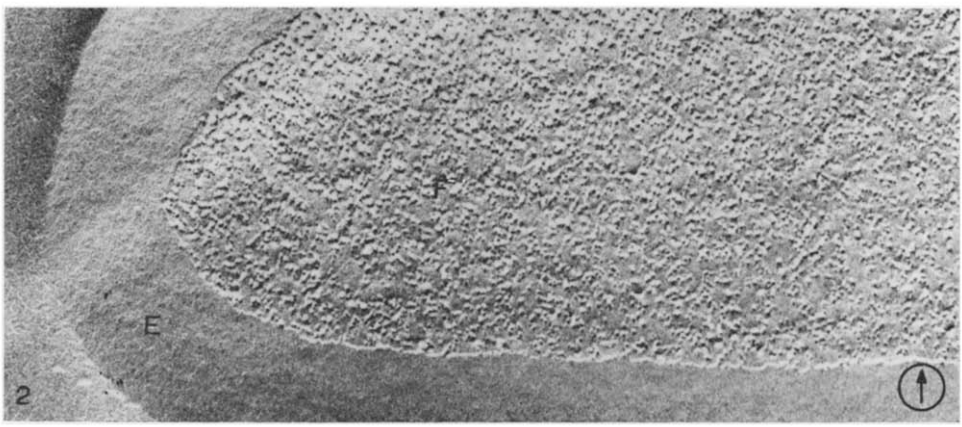

Fig. 2. Control membranes, kept for 1 h in 0.1 M Tris, pH 7.4, 2 mM $CaCl_2$ at 37°, and then pelleted at 16000 × *g*. The normal fracture plane (F) and the etched-out membrane surface (E) have the usual appearance. 80000 ×. Encircled arrow indicates direction of shadowing.

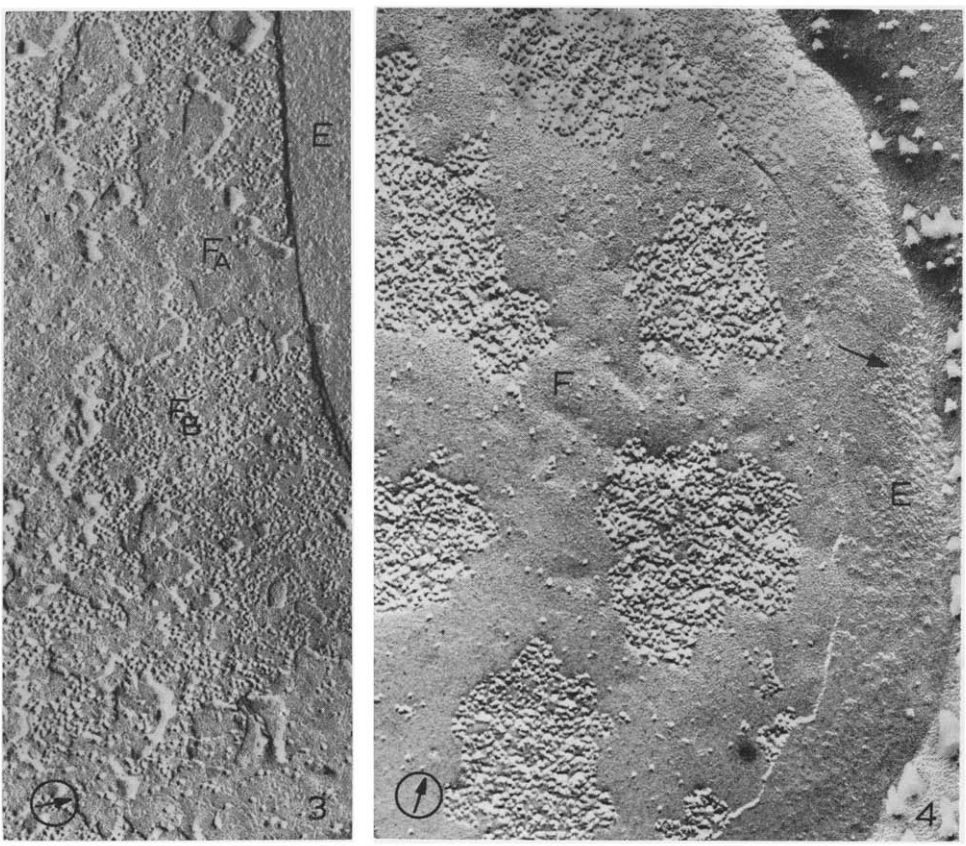

Fig. 3. As Fig. 2, but membranes pelleted at 75000 × *g*. Occasional plaques composed of parts of adjacent membranes are exposed (F_A, F_B). 80000 ×. E, etched-out membrane surface; encircled arrow indicates direction of shadowing.

Fig. 4. Membranes after massive trypsinization. The membrane-associated particles have rearranged in clusters, separated by smooth areas. Underlying clusters of particles have become prominent on the etched surface. There are no plaques. 80000 ×. Abbreviations, see Fig. 2.

electrophoresis in sodium dodecyl sulfate shows that these conditions effect the massive cleavage of membrane peptides (Fig. 1c), already reported in ref. 12. In contrast, equivalent trypsination of inside-out vesicles does not evoke such morphologic and electrophoretic changes, in accord with other data, showing that the internal surfaces of isolated erythrocyte membranes are resistant to the action of proteases[12].

The effects of phospholipase A

When erythrocyte ghosts are treated with 5 μg phospholipase-A per 100 μg membrane protein in 1 ml 0.1 M Tris–HCl, pH 7.4, 2 mM $CaCl_2$, at 37°, 50–70 % of the membrane phosphatides are cleaved to lysophosphatides and fatty acids according to estimates by thin-layer chromatography[6]; however, the products of enzyme action remain associated with the membranes[5,16,17]. The firm pellet, obtained by centrifugation at 16000–20000 $\times$ $g_{av.}$ for 20 min, after 15 min enzyme treatment showed 95 % of the membrane faces to be covered with a rather regular array of plaques, made up of adherent membrane and 80–100 Å thick (Fig. 5); we cannot give more exact layer thicknesses, because of local variations in the shadowing angle. However, the background membrane faces and the adherent layers exhibit the usual membrane-associated particles and their size and distribution are not measurably affected.

A slightly similar micromorphology has been previously reported after tight packing of normal ghosts[18]. Accordingly we centrifuged untreated ghosts at 75000 $\times$ $g_{av.}$ for 30 min and found that such packing does indeed produce regions with adherent membrane plaques in about 50 % of fractured ghosts; however, these occur much less frequently, occupy smaller membrane domains, are more variable in size than after phospholipase A treatment (Fig. 3). The etched surface of membranes is also affected by phospholipase A treatment.

Membrane-bound fatty acids and lysophosphatides are not directly responsible for the peculiar micromorphology seen after phospholipase A since neither their extraction with defatted bovine serum albumin[5] nor treatment with oleate-saturated albumin had any micromorphological consequences in control or phospholipase A treated ghosts. It is also unlikely that membranes become adherent through calcium bridges between newly formed fatty acids in apposed membranes, since repeated washing of the membranes with 0.1 M Tris–HCl, pH 7.4, 5 mM EDTA, did not abolish the plaques.

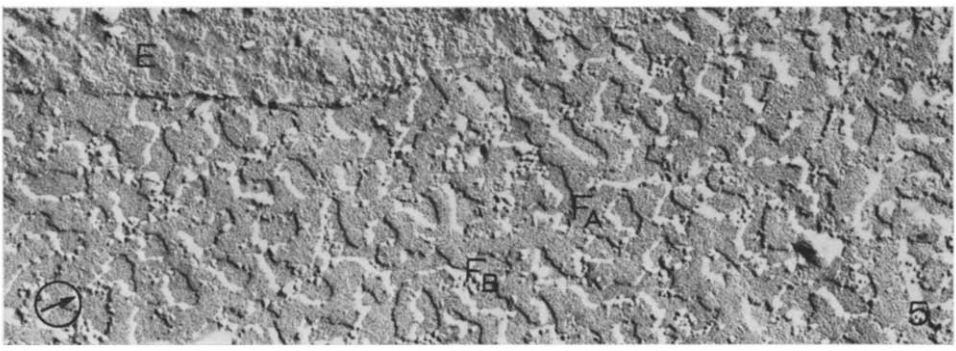

Fig. 5. Membranes treated with phospholipase A for 15 min. The fracture plane alternates frequently and regularly between adjacent membrane faces (F_A to F_B, and *vice-versa*), creating plaques of approximately equivalent surface areas and derived from adjacent membranes. 80000 $\times$.

After phospholipase A treatment for an intermediate period, 45 min, the membranes develop large (1000–3000 Å), flat mounds and/or craters (Fig. 6) which disappear when the incubation is extended to 90 min. These could represent the beginnings of vesiculation, but we have never actually observed budding off, nor do we obtain the change in light-scattering, which occurs during vesiculation.

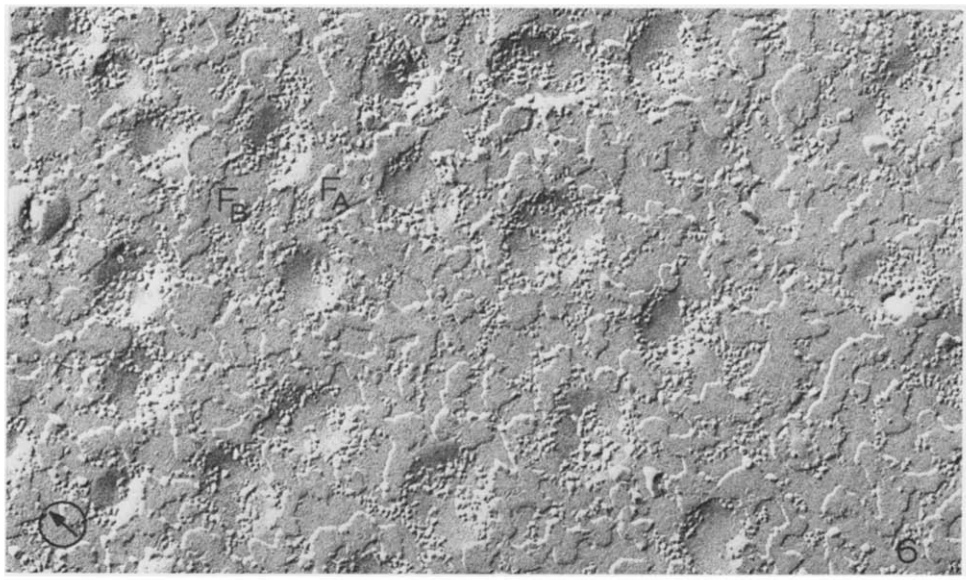

Fig. 6. Appearance after treatment with phospholipase A for 45 min. Flat elevations and depressions are now prominent, but are no longer visible after 90 min. 80000 ×.

The effects of added lysolecithin

Treatment of the membranes to 40 μg lysolecithin per 100 μg membrane protein at room temperature, produces no micromorphologic alterations even after prolonged exposure. At twice this level of lysolecithin light scattering of the ghost suspension decreases abruptly and fractures tangential to the membrane plane become infrequent. At 120 μg lysolecithin per 100 μg membrane protein, light scattering is about 1/2 of the control value and only transverse fractures are seen. No plaques were found at any of the lysolecithin levels tested.

The effects of sodium dodecyl sulfate

At low levels of sodium dodecyl sulfate (1 mg sodium dodecyl sulfate per mg membrane protein in 5 mM phosphate buffer, pH 8.0), where certain membrane-enzymes are activated and conformational changes are slight[19,20], as well as at 4 mg detergent per mg protein, where the peptides unfold and the enzymes become inactive[19,20], no micromorphologic changes occur besides some vesiculation. However, at high detergent levels (1 %), where the membrane peptides dissociate from their lipids into units of mol. wt. <300000 (refs. 9, 12), the membranes disrupt, tangential fractures are seldom and no particles are observed. Plaques were not seen even at low detergent levels.

The effects of saponin

Treatment of erythrocyte membranes with 0.02 % saponin in 0.1 M Tris–HCl, pH 7.4, for 1 h at 37° does not change the gross micromorphology of the ghosts, nor the size and distribution of the membrane-associated particles. However, low saponin levels produce plaques similar to those following phospholipase A-treatment (Fig. 7) and small holes on the etched surface become visible. As the saponin concentration is raised, the fracture-planes follow the membrane contours less and less, so that at 0.1 % saponin, all membranes are fractured transversely, giving the same appearance as with high levels of lysolecithin.

Fig. 7. Membranes exposed to 0.02 % saponin. The appearance is as after phospholipase A, with adhesion of membrane surfaces resulting in an alternation of the fracture plane ($F_{A,B,C}$). See text for further comment. 80000 ×.

DISCUSSION

The low temperatures used in freeze-etching drastically weaken the hydrophobic forces, which normally stabilize macromolecular assemblies[22] and it is probably that the fracture plane in freeze-etching is guided by such regions of low molecular cohesion[23–25]; the phenomena we detect must be viewed in this context. We reason that phospholipase A, as well as restricted levels of saponin introduce polar groups within hydrophobic lipid domains, and also at membrane surfaces, but do not grossly disrupt membrane structure. However, of the physical forces influencing contact between membrane surfaces (*e.g.* ref. 26), a major factor preventing adhesion is the electrostatic repulsion between surfaces of like charge; the activities of the involved charges decrease with temperature fostering adhesion between membrane surfaces, especially at the low ionic strengths employed here, and allowing newly formed polar regions to deflect the hydrophobically-guided fracture plane. We therefore interpret

the plaques to arise from an alternation of the fracture plane between membranes which have become closely adherent. As might be anticipated from their bulky polar groups, lysolecithin and sodium dodecyl sulphate do not produce this phenomenon. Control studies with phospholipase C (*Clostridium welchii*; 50 μg enzyme per mg protein; 0.1 M Tris–HCl, 2 mM $CaCl_2$; 37°; 30 min), yield results quite unlike those seen after phospholipase A, although about 80 % of the membrane phosphatides are split. Rather than plaques, this treatment produces the alterations described in ref. 20, *i.e.* particle-free membrane areas, bordering on smooth regions 3000–10000 Å diameter, possibly localized accumulations of neutral lipids.

We reason that the fracture plane shifts at sites whose internal polarity has been increased by the membrane perturbants. Thus if untreated membranes (A,B,C) normally exhibit one internal fracture plane (F_A, F_B, F_C), with two faces, phospholipase A treatment, *etc.* tends to cause alternation of the fracture planes between adherent membrane faces, *i.e.* from F_A to F_B, F_B to F_C, etc. producing the observed plaques. These are usually 80–100 Å thick and attain greater widths only with more extreme membrane perturbation, when the fracture plane occasionally appears to cross two or more membranes perpendicularly at one site (*e.g.* F_A to F_C and F_A to F_D; Fig. 7; arrow). At still higher levels of saponin, lysolecithin and sodium dodecyl sulphate, the balance between polar and apolar forces in the membranes is sufficiently disturbed that the membranes no longer guide the fracture plane; the same is true for acetone-extracted membranes[23,27].

Trypsinization also increases the polarity at its sites of action, by generating new COO^- and NH_3^+ groups, but does not affect the fracture plane detectably, suggesting that the alternations occur in lipid domains and that the plaques represent more tightly associated regions of high protein content. The trypsinization data are also otherwise significant, particularly since (a) there are few, if any –S–S– linkages in ghost peptides[28] and (b) our electrophoretic monitoring of peptidolysis is done in the presence of the randomizing detergent, sodium dodecyl sulphate, as well as dithiothreitol, to reduce the rare –S–S– existing. The fact that, modest peptidolysis produces no morphologic changes, and that such are minor even when all major membrane peptides are severely degraded, shows that membrane proteins, like in water-soluble ones, are stabilized primarily by hydrophobic forces; these are disrupted only after addition of sodium dodecyl sulfate.

The influence of trypsin and sodium dodecyl sulfate on the membrane particles suggests that these at least partly protein. However, although TILLACK *et al.*[29] recently suggested that the membrane-associated particles are most likely lipid-associated glycoprotein, we consider this unlikely, since it is established that the glycopeptides of human erythrocytes are particularly sensitive to proteolysis[12,30,31]. Also, the particles of sheep erythrocyte membranes are not altered even after the most severe trypsinization.

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