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CORRELATION BETWEEN CHANGES IN THE MEMBRANE ORGANIZATION AND SUSCEPTIBILITY TO PHOSPHOLIPASE C ATTACK INDUCED BY ATP DEPLETION OF RAT ERYTHROCYTES

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SUMMARY

About 20 and 43 % of the total membrane phospholipids are hydrolized in fresh rat erythrocytes by treatment with phospholipase C (*Bacillus cereus*), or both sphingomyelinase and phospholipase C, respectively, without causing cell lysis. Treatment of ATP-depleted cells with phospholipase C alone results in 50 % hydrolysis and extensive lysis. Depletion of ATP causes a marked increase in the aggregation of intramembranous particles accompanied by a similar increase in the smooth area between the particle clusters as revealed by the freeze-etch technique. Such changes are not induced by extensive phospholipid hydrolysis in absence of cell lysis in fresh cells.

Based on these and additional data, it is suggested that the membrane phospholipid organization can be divided into 3 types: phospholipids exposed to phospholipase C; phospholipids protected against phospholipase C by presence of sphingomyelin; phospholipids which can be exposed following alteration of the protein-lipid interactions. Such alterations which might be induced by a variety of means, including ATP depletion, might result in clustering of intramembranous particles and increase of the free lipid bilayer phase of the membrane.

INTRODUCTION

The fluid model of Singer and Nicholson [1] predicts that the lipid bilayer phase of the plasma membrane is exposed toward the medium surrounding the cell and thus potentially available to attack by phospholipases. Results obtained in different laboratories support this view and indicate that the lipid bilayer is asymmetric, sphingomyelin usually being located on the outer face of the membrane [2, 3]. It was also shown that, in cells whose sphingomyelin content amounts to about 22–30 % of the total membrane phospholipid (human, pig), phospholipase C can not reach the

* Preliminary results of this work were published in the annual meeting of the Israel Society for Electron Microscopy [19].

phospholipids unless sphingomyelin is first hydrolyzed. In this case phospholipid hydrolysis by the combined effect of sphingomyelinase and phospholipase C resulted in cell lysis [2]. Recent results demonstrated that in chicken erythrocytes an important part of the membrane phospholipids is not available to phospholipase C (*Bacillus cereus*) attack, even after hydrolysis of the sphingomyelin. In sphingomyelinase-treated chicken erythrocytes, as well as in non-treated toad erythrocytes, the majority of the phospholipids became available to phospholipase C attack and the cells lyse only if they have been depleted of ATP [3]. Thus it was concluded that the availability of the membrane phospholipids to phospholipase C present in the outer medium is affected not only by the relative content of sphingomyelin but also by structural factors, which can be reversibly altered upon changing the ATP level of the cells [3, 4]. This ATP-dependent reversible alteration of membrane structure has been so far described for avian and amphibian nucleated erythrocytes. However, the phenomenon might be of a more general nature.

In the present work the susceptibility of the membrane phospholipids to phospholipase C and the accompanying structural changes as revealed by the freeze-fracture technique have been investigated in fresh and ATP-depleted rat erythrocytes in which sphingomyelin amounts to only 15 % of the total membrane phospholipids, as in the toad (amphibian, nucleated) erythrocytes. The results are in agreement with the concept that ATP-dependent structural alterations of the membrane are responsible for exposure of the majority of membrane phospholipids to hydrolytic attack.

MATERIALS AND METHODS

Rat erythrocytes were obtained by heart puncture using heparin (100 units/ml blood) as an anticoagulant. Depletion of ATP, cell handling, treatment with phospholipases, phospholipid extraction and separation, and determination of lipid content and hydrolysis, were carried out as described before [3, 4].

Phospholipase C (*B. cereus*) was purchased from Makor Chemicals, Jerusalem, Israel.

Sphingomyelinase was prepared by the procedure described by Colley et al. [2] with several modifications. The enzyme was concentrated from the supernatant of the *Staphylococcus aureus* Hood 46 NCTC 20345 culture medium by adsorption on cellulose powder (Whatman CF 11) suspended in 70 % saturated $(NH_4)_2SO_4$ solution. The enzyme was eluted from the cellulose powder after packing in a column and washing with distilled water. The eluted material was precipitated several times with the same concentration of $(NH_4)_2SO_4$, the final precipitate was dissolved in distilled water, freed from particulate material by centrifugation (15 000 $\times g$ for 10 min) and finally concentrated by dialysis against a solution of 50 % glycerol at 4 °C for 4 h.

The dialysate was loaded on a Sephadex G-100 column (5 cm $\times$ 1.7 m) suspended in 50 % glycerol solution containing 10 mM Tris · HCl, pH 7.4, 5 mM $CaCl_2$ and 5 mM $MgCl_2$. The enzyme was eluted with a similar solution (4–5 ml/h) at room temperature. The enzyme-containing factions (0.9–1.1 l) were pooled and concentrated using an Amicon ultrafiltration apparatus equipped with a Diaflo membrane UM-10. The final preparation contained 50 units/mg protein of sphingomyelinase activity and no phospholipase C or hemolytic activity (Gazitt, Y., Loyter, A. and Ohad, I., in preparation).

For preparation of freeze fractured cells, the erythrocytes were washed by centrifugation and suspended in a solution containing 135 mM KCl, 5.4 mM NaCl and 0.8 mM $MgSO_4$ in 30 mM phosphate buffer, pH 7.8, and fixed by addition of glutaraldehyde (Ladd Research Industries) to a final concentration of 1 % (v/v). Fixation was carried out at room temperature for 10 min. The cells were then centrifuged, resuspended in 15 % glycerol in water (packed cells : glycerol, 1 : 3, v/v) for 10 min and then centrifuged ($1000 \times g$ for 5 min) and finally resuspended in 30 % glycerol as above. After 10 min the cells were packed by centrifugation ($1000 \times g$ for 5 min) frozen on a gold support in liquid freon 22 for 10 s, then transferred to liquid N^2 and stored until further processing for 30 min. Fractures were obtained with a Balzer's Freeze fracture apparatus equipped with electron gun evaporators for both platinum shadowing and carbon coating and using a 4 sample-holder stage. In all cases control and treated cells were fractured and coated in the same run. The thickness of the platinum and carbon deposit was kept as near constant as possible in all preparations, using a crystal monitor. The fractures were etched for 1 min before coating. The replicas were cleaned by floating for 10 min on a concentrated hypochlorite commercial cleaning solution. Micrographs were obtained with a Philips EM 300 microscope operated at 80 kV. Negative plates (X 75 000) were scanned in a Beckman densitometer equipped with analytrol attachment suitable for film density recording. The slit width was adjusted to about 30 % of the particle diameter. For each sample representative negatives were selected from at least 100 negatives obtained from 10 different experiments. Negatives were taken from membranes fractured over surfaces of 2–3 μm diameter. Only flat surfaces were used for estimation of particle count and distribution.

RESULTS

Hydrolysis of phospholipids by sphingomyelinase and phospholipase C in fresh and ATP-depleted rat erythrocytes

Treatment of fresh or ATP-depleted cells with sphingomyelinase resulted in about 50 % hydrolysis of the sphingomyelin without lysis. When fresh cells were treated with phospholipase C, 20 % of the total phospholipid content was hydrolized, including 38 % of the total phosphatidylethanolamine and 30 % of the phosphatidylcholine (Fig. 1). As in the case of treatment with sphingomyelinase alone, phospholipase C treatment did not lyse fresh cells. Combined treatment with both sphingomyelinase and phospholipase C resulted in the hydrolysis of over 43 % of the total membrane phospholipids, including 53 % of the total phosphatidylcholine and phosphatidylethanolamine content. However, even in this extreme case, the cells were not lysed at 37 °C (Fig. 1). In all these treatments phosphatidylserine and phosphatidylinositol were only slightly affected.

When ATP-depleted cells were treated with phospholipase C in the absence of sphingomyelinase extensive lysis occurred (80 %), accompanied by hydrolysis of 50 % of the phospholipids under our experimental conditions. The combined action of sphingomyelinase and phospholipase C on ATP-depleted cells caused an increased lysis (89 %) and phospholipid hydrolysis reached 74 %. The extent of lipid hydrolysis when accompanied by cell lysis should be considered only as a qualitative indication of the changes occurring in the membranes as compared with advanced hydrolysis in the absence of lysis in fresh cells.

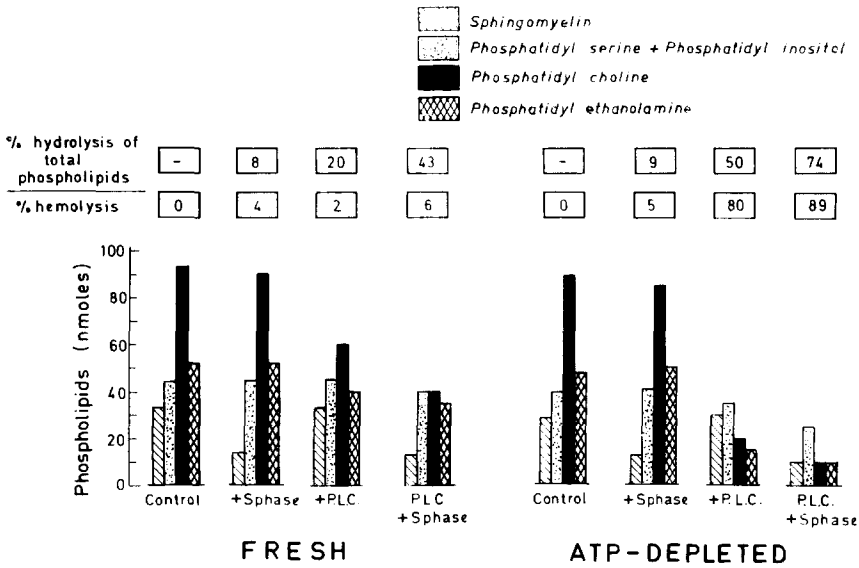


Fig. 1. Susceptibility of fresh and ATP-depleted rat erythrocytes to phospholipase C and sphingomyelinase. The enzyme concentrations were as follows: sphingomyelinase (Sphase), 0.05 I.U./ml; phospholipase C (PLC), 0.4 I.U./ml. Aliquots of 80 μl of the phospholipids extracted from equal amounts of cells (5 ml, 10 % cells v/v) were used for chromatographic separation and phospholipid phosphorous determination. The enzyme concentration and time of incubation were the minimum required to give almost a maximal effect on the ATP-depleted cells. The fresh cells were not affected even if the enzyme concentration was increased 2- to 3-fold. The time of incubation was not extended beyond 1 h in order to avoid effects due to partial depletion of ATP.

Changes in the structure of the rat erythrocytes membrane upon ATP depletion

The fact that phospholipids could be hydrolized to different extents in the fresh and ATP-depleted cells might indicate the existence of differences in the organization of the membrane of the ATP-depleted cell. In order to find out whether such difference could be detected, cells were freeze fractured before and after ATP depletion and replicas of the fractured cells examined by transmission electron microscopy.

Two types of surface could be found in preparations of fresh cells. One surface showed a relatively smooth and even area on which few particles of about 80–100 Å diameter were present. The distribution of the particles appeared to be random and clusters of a few particles (2–4) were only occasionally observed. The areas between the particles appeared to be essentially flat and one could barely distinguish faint contours of underlying gentle protrusions delineating areas whose diameter varied between 250 and 500 Å (Fig. 2). A second type of membrane surface was found on which the number of particles was much higher (Fig. 3, Table I). The particles in this case formed aggregates or clusters of varied number and shape and degree of packing ranging from loose contact to dense packing. The clusters were evenly dispersed over the whole membrane area and appeared to be arranged in such a way as to leave open spaces between them of somewhat regular shape and size (about 130 Å in diameter) (Fig. 3, Table II). These surfaces correspond, respectively, to the outer surface of the cell and a fracture plane passing through the membrane, as seen in human erythrocytes [6].

The same types of surface were also observed in preparations of ATP-depleted cells. However, the fracture face (within the membrane) was found to differ from that found in fresh cells. The particles appeared to be more aggregated and more tightly packed and to leave bigger open spaces (about 280 Å in diameter) with a smooth surface between them (Fig. 5, Table II).

In addition, the protrusions of the smooth areas of the outer face were more evident as compared to fresh cells (Fig. 4).

Change in the average number of free or single particles over a certain randomly selected linear distance might serve as an indication for a change in the degree of aggregation provided that the average number of particles per unit area remains constant. Since the total number of particles/μm^2 is similar in the membrane fracture face of both fresh and ATP-depleted cells (Table I), one could use such measurements as a semi-quantitative indication for particle clustering. The results shown in Table III demonstrate that a decrease of about 50 % occurs in the number of free particles in fracture faces of ATP-depleted cells as compared to fresh cells.

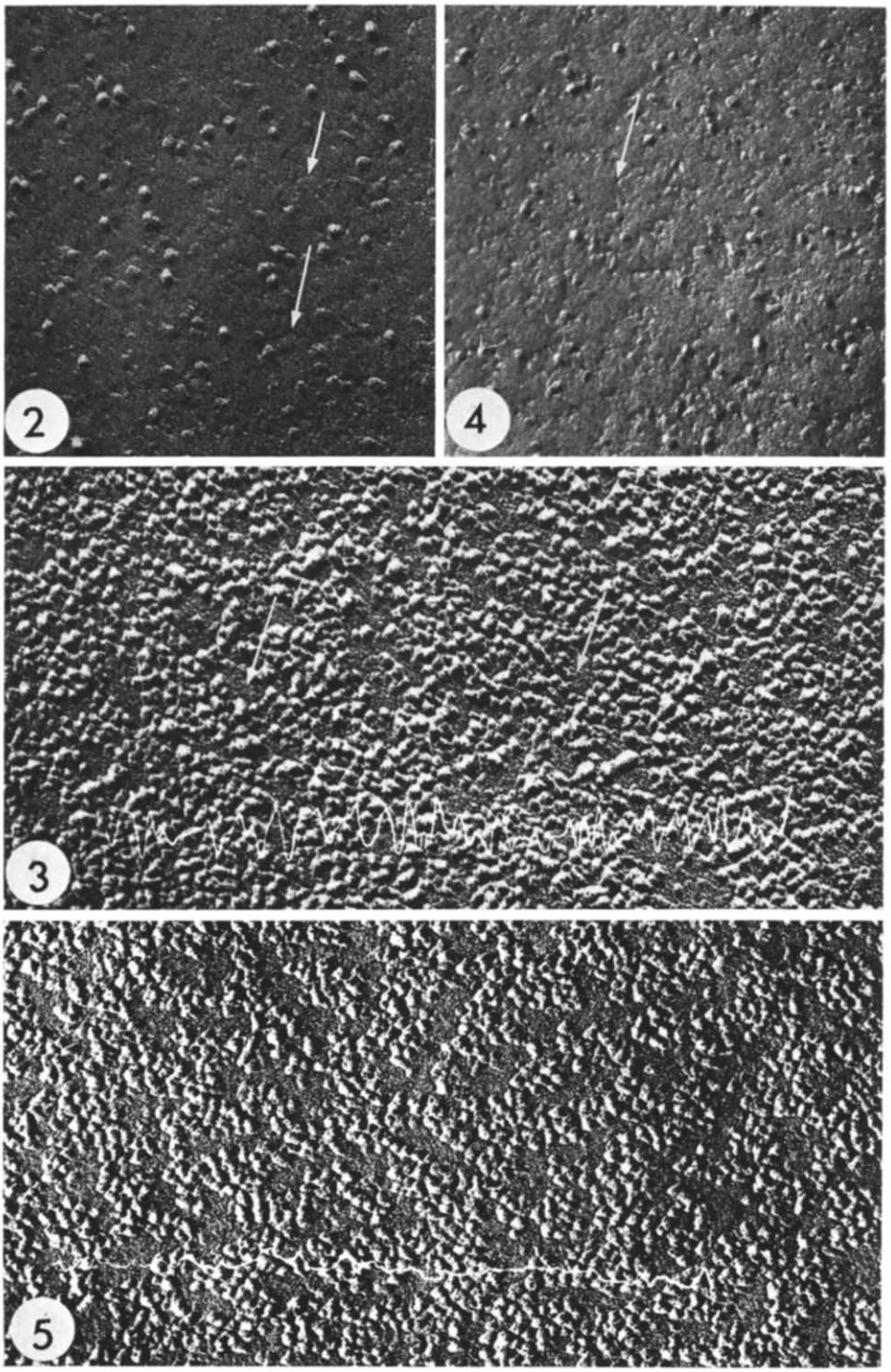
Changes in the organization of fresh and ATP-depleted cells following treatment with phospholipase C and sphingomyelinase

The rat erythrocyte offers a unique opportunity of studying the changes in the ultrastructure of its membrane following hydrolysis of a major part of its phospholipids in absence of cell lysis. As shown above, 43 % of the total phospholipid content of the fresh cell can be hydrolyzed by the combined use of sphingomyelinase and phospholipase C without lysis. However, hydrolysis is enhanced and lysis is extensive if ATP-depleted cells are treated with phospholipase C alone (Fig. 1).

Examination of freeze-fractured fresh cells after treatment with phospholipase C showed already marked changes as compared with untreated cells (Fig. 6, compare with Fig. 2). The smooth area of the outer surface disclosed a pattern of mild convex regions or shallow protrusions of an average diameter of 250–500 Å, which seems to be an enhancement of the same type of structure, barely seen on the surface of untreated cells (compare with Fig. 2). However, no difference was found in the appearance of the inner fracture face (Fig. 7). The intramembranous particles in phospholipase-treated cells (Figs. 7, 9 and 11) appear to be somewhat smaller than those of untreated cells. Since no accurate measurements have yet been made, this observation will not be further considered here.

The particles seen on the outer face of ATP-depleted cells treated with phospholipase C, which induced hydrolysis of 60 % of the phospholipids and 80 % lysis, appear to be clustered and located around slightly protruded smooth surfaces of the same type as those seen in fresh cells following treatment with phospholipase C (Fig. 8, compare with Fig. 6). The inner fracture faces of the membranes of these cells also show a stronger aggregation of the particles (Fig. 9) as compared with that of the phospholipase C-treated fresh cells (Fig. 7). In addition, the number of particles per surface area appears to be significantly increased (Table I, see also Tables II and III).

Treatment of the cells with both enzymes, sphingomyelinase and phospholipase C, results in small additional changes in the appearance of the outer surface of fresh cells (Fig. 10). However, distinct changes are revealed in the inner fracture face of the membrane of these cells (Fig. 11). In this case the smooth regions found between the numerous aggregated particles appear to be projected outward,



Figs 2, 3, 4 and 5. See opposite page for legends.

TABLE I

DENSITY OF PARTICLES PER μm^2 OF MEMBRANE IN FRESH AND ATP-DEPLETED RAT ERYTHROCYTES FOLLOWING TREATMENT WITH PHOSPHOLIPASES

The numbers represent averages of measurements made on at least 8 randomly selected areas of 6×6 cm for the outer face and 16 areas of 1.5×1.5 cm for the fracture face, using in all cases micrographs at a final magnification of $\times 150\,000$.

Membrane face		Non treated	Phospholipase C	Phospholipase C + sphingomyelinase
Fresh cells	Outer face	350 ± 25	365 ± 50	440 ± 20
	Fracture face	3410 ± 400	3200 ± 370	2270 ± 300
ATP-depleted cells	Outer face	350 ± 30	520 ± 30	310 ± 45
	Fracture face	3410 ± 590	4030 ± 450	3200 ± 350

TABLE II

AVERAGE LINEAR DISTANCE OF THE SMOOTH AREAS FOUND BETWEEN PARTICLES ON THE INNER FRACTURE FACE OF FRESH AND ATP-DEPLETED RAT ERYTHROCYTE MEMBRANES BEFORE AND AFTER TREATMENT WITH PHOSPHOLIPASES

The numbers represent averages of measurements in Å length of smooth surface along random lines of 1 μm actual length drawn across the micrographs ($\times 150\,000$). The data were calculated from at least 5 independent measurements for each micrograph.

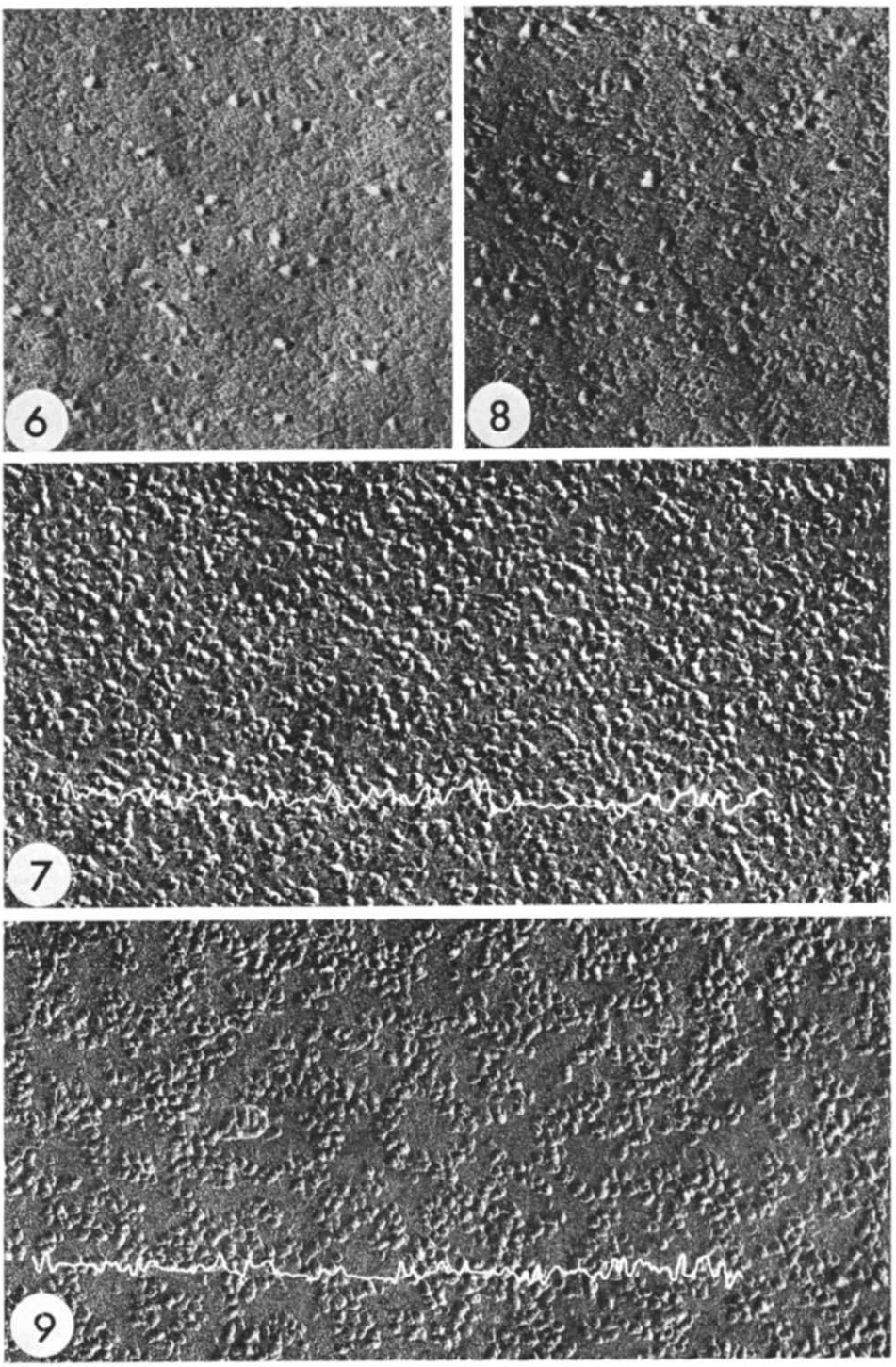
Treatment	Fresh cells	ATP-depleted cells
Non treated	130 ± 30	280 ± 70
Phospholipase C	180 ± 50	290 ± 85
Phospholipase C + sphingomyelinase	180 ± 70	270 ± 65

Fig. 2. Outer face of a fresh erythrocyte membrane showing particles scattered over a smooth surface. Notice that the smooth surface is not entirely flat and one can distinguish faint protrusions and ridges (arrows). $\times 150\,000$.

Fig. 3. Fracture face of a fresh erythrocyte membrane showing numerous particles distributed in clusters of varying numbers and small stretches of smooth area between them (arrows). The white track at the bottom is a typical densitometer recording over 660 nm actual length of the membrane. Notice that only short parts of the densitometer trace are flat; these parts represent the smooth area between the particles $\times 150\,000$.

Fig. 4. Outer face of an ATP-depleted cell showing few particles scattered over a relatively smooth surface. The ridges or protrusions seen over the smooth area are more evident than in the same surface of the fresh cells (see Fig. 2) $\times 150\,000$.

Fig. 5. Membrane fracture face of an ATP-depleted cell showing a strong aggregation of particles which leaves larger stretches of smooth area between the clusters. The white track at the bottom represents a densitometer recording along a line 660 nm actual length. Notice the increase in the length of the densitometer trace which is flat and corresponds to the smooth area (compare with Fig. 3) $\times 150\,000$.



Figs 6, 7, 8 and 9. See opposite page for legends.

TABLE III

AVERAGE NUMBER OF ISOLATED PARTICLES ALONG 1 μm DISTANCE ACROSS THE INNER FRACTURE FACE OF FRESH AND ATP-DEPLETED RAT CELLS

The data were obtained by counting isolated single particles across random lines of 1 μm actual length. At least 5 measurements were made for each micrograph $\times$150 000.

Treatment	Fresh cells particles/μm	ATP-depleted cells particles/μm
Non treated	30$\pm$4	17$\pm$2
Phospholipase C	23$\pm$4	12$\pm$2
Phospholipase C + sphingomyelinase	12$\pm$3	11$\pm$2

giving rise to roundish protrusions of regular and even distribution throughout the cell membrane and having an average diameter of 180 Å (Table II).

The short shadows of these projections, when compared to their diameters, indicate that they are not spherical but rather flat, of a shallow oval vertical cross section. In addition there is a marked decrease in the number of particles per unit area as can be seen from Table I.

The outer surface of ATP-depleted cells treated with both enzymes, which have caused 74 % lipid hydrolysis and 89 % cell lysis, reveals clearly visible protrusions on the smooth outer face of the cell (Fig. 12), more pronounced than those found on the surface of fresh cells treated in a similar way (compare with Fig. 10). In addition, smooth depressions of similar size could be seen. Also a slight decrease in the number of particles per unit area was detected (Table I).

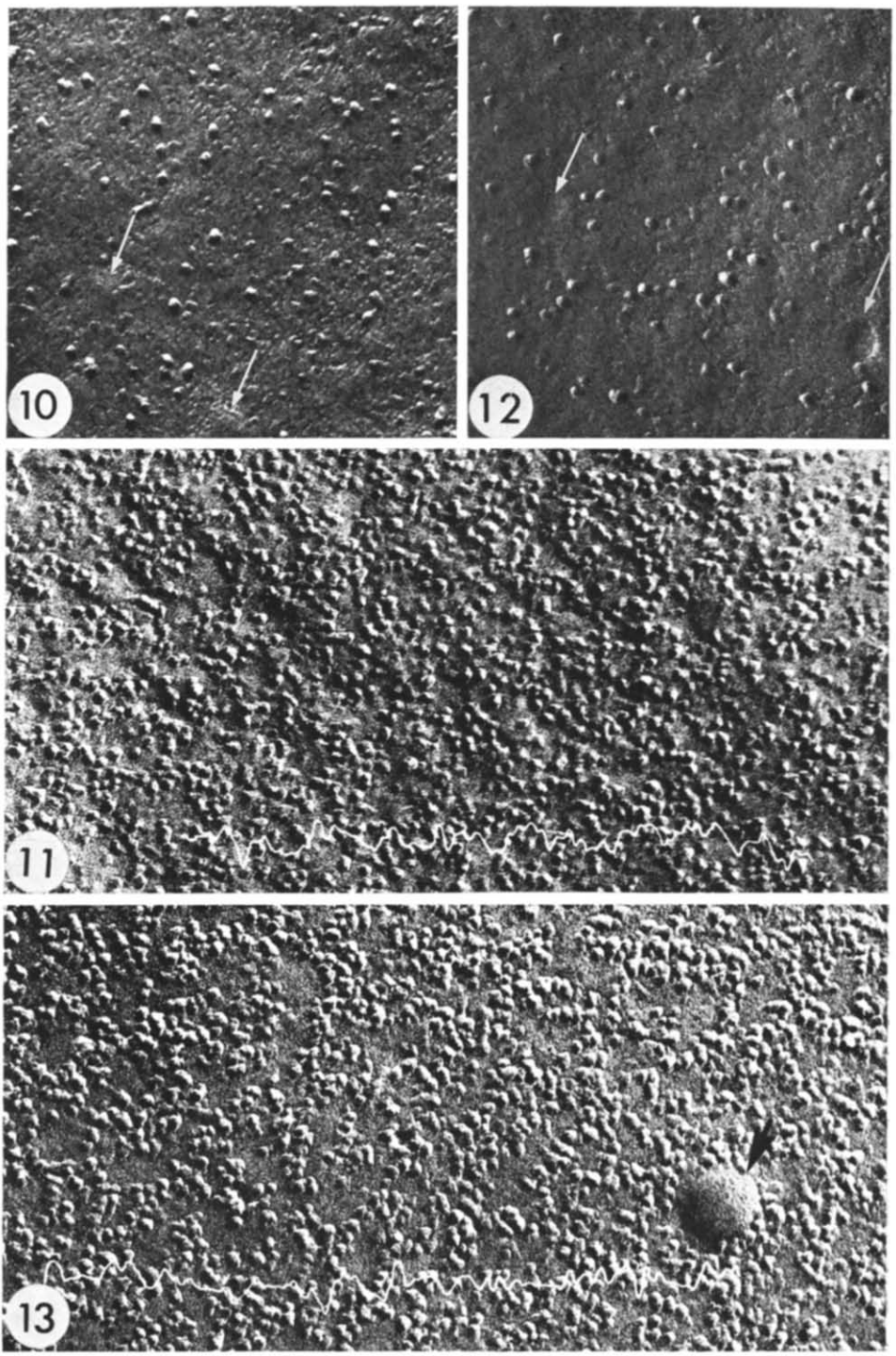
The membrane inner fracture face of ATP-depleted cells treated with both enzymes shows some aggregation of the particles (Table II) and round-shaped protrusions with smooth surfaces of about 750–1000 Å in diameter. The elevation of these projections over the surface of the fracture face is variable, ranging from shallow to almost spherical. Their distribution over the surface is not even (Fig. 13, Table I, compare with Figs. 5 and 11). The density of the particles per unit area is increased compared to that of the fresh cells treated in a similar way. For comparison, representative densitometer tracings of membrane fracture faces obtained from fresh and ATP-depleted cells, before and after treatment with phospholipases, are given in Fig. 4.

Fig. 6. Outer face of a fresh cell treated with phospholipase C. Notice the enhancement of the protrusions clearly defined in the smooth area regions (compare with Figs. 2 and 4) $\times$150 000.

Fig. 7. Membrane fracture face of a fresh cell after treatment with phospholipase C. The fracture face is very similar to the one seen in the control cells (compare with Fig. 3) $\times$150 000.

Fig. 8. Outer face of a phospholipase C-treated ATP-depleted cell. Notice the appearance of clusters of particles, accompanied by an increase in the density of the protrusions (compare with Fig. 6) $\times$150 000.

Fig. 9. Membrane fracture face of a phospholipase C treated ATP-depleted cell. Notice the increase in the degree of clustering (compare with Fig. 5) $\times$150 000.



Figs 10, 11, 12 and 13. See opposite page for ledgends.

DISCUSSION

The data presented in this work show that at least 20 % of the membrane phospholipids are readily hydrolized by phospholipase C in the rat erythrocyte membrane and similar results have been previously reported also from experiments carried out with toad cells [3]. Since in both these cases sphingomyelin represents only 14–15 % of the total phospholipids, it appears that protection of phospholipids against phospholipase C by sphingomyelin [2, 7] is variable from species to species and might be correlated with the ratio sphingomyelin/total phospholipids of the membrane (cf also Ref. 3). However, this is not the only factor which prevents phospholipase C attack. Depletion of the cells of ATP has a dramatic effect on the availability of phospholipids to phospholipase C. This effect is either to increase the total amount of phospholipids directly hydrolyzable by phospholipase C for the rat erythrocytes and toad cells [3], or to reveal phospholipids unavailable even after hydrolysis of sphingomyelin, in cells in which the content of sphingomyelin is higher, such as chicken erythrocytes [3, 4].

Following phospholipid hydrolysis the organization of the lipid bilayer which seals the cell against leakage of its content is altered, diacylglycerol droplets are formed and the seal breaks. This was considered to be the case in human and pig cells [2]. However, cell lysis does not occur after hydrolysis of the readily available, sphingomyelin non-protected lipids, which might amount to as much as 20–30 % of the total membrane phospholipid in toad cells [3] and, most strikingly, not even after hydrolysis of the 43 % of the total membrane phospholipids carried out by the combined effect of sphingomyelinase and phospholipase C on the rat erythrocytes. In these cells, as well as in chicken [3], lysis occurs only after phospholipid hydrolysis following ATP depletion. Thus it appears that in some species lipids whose organization is affected by the ATP level of the cell, as revealed by their resistance to hydrolysis, play also a major role in sealing the cell against lysis.

Changes in the organization of the membranes due to depletion of ATP are readily detected by the freeze-etch technique. The most distinct differences are found in the inner fracture face. The main finding is that depletion of ATP causes an increase in the degree of aggregation or clustering of the intramembrane particles and as a result the average dimensions of the remaining smooth areas increase accordingly. However, the total smooth area does not increase, nor does the total number of particles per membrane area which remains constant in the range of 3300 per

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Fig. 10. Outer face of a cell treated with sphingomyelinase and phospholipase C. Notice the presence of protrusions of 250–500 Å (arrows) (compare with Figs 2 and 4) ×150 000.

Fig. 11. Membrane fracture face of a cell treated with sphingomyelinase and phospholipase C. Notice the reduction in the density of particles, in addition to the appearance of regular protrusions in the smooth areas between the particles ×150 000.

Fig. 12. Outer face of an ATP-depleted cell treated with sphingomyelinase and phospholipase C. Notice the regular protrusions of 500 Å diameter, which may represent small droplets of diacylglycerols ×150 000.

Fig. 13. Membrane fracture of an ATP-depleted cell, treated with sphingomyelinase and phospholipase C. Notice the change in the distribution of the particles with the appearance of regularly shaped protrusions with a diameter of about 1000 Å ×150 000.

μm^2. This value is close to that obtained in other systems, such as human erythrocytes [8].

It is generally accepted that the intramembranous particles represent protein or lipoprotein complexes while the smooth areas represent stretches of free lipid bilayer [9]. The clustering of the proteins and increase in the size of the free lipid bilayer area observed in the rat erythrocyte membrane following ATP depletion of the cell can be regarded as responsible for the increase in the susceptibility of phospholipids to phospholipase C. One can consider that the interactions of polar or hydrophobic type, between membrane proteins and lipids, might prevent lipid exposure to phospholipase C attack [10, 11]. Evidence for lipid immobilization by binding to proteins and changes in peptide conformation due to their interaction with lipids have been reported [12, 13]. Thus presence of numerous protein particles interspersed within the lipid phase might stabilize the whole layer against phospholipase C. Depletion of ATP, which causes a decrease in the phosphorylation of membrane proteins (Gazitt, Y., Loyter, A and Ohad, I., in preparation) and eventually alter the pattern of S-S bonds (Gazitt, Y., Loyter, A. and Ohad, I., unpublished data), might alter the interaction between proteins and lipids [16]. This will reduce the protective effect to a shorter range away from the protein foci and on the one hand allow the proteins to cluster, while on the other hand increase the dimensions of areas of free bilayer phase. Support for this concept can be found in the case of human erythrocytes in which the intramembrane particles in both fresh and ATP-depleted cells appear to be clustered to the same extent as in the rat ATP-depleted cells (Gazitt, Y., Loyter, A. and Ohad, I., in preparation) (compare also Fig. 14), both being lyzed by the action of sphingomyelinase and phospholipase C [2]. However, further increase in clustering of the intramembrane particle in human cells can be achieved by exposing ghosts to

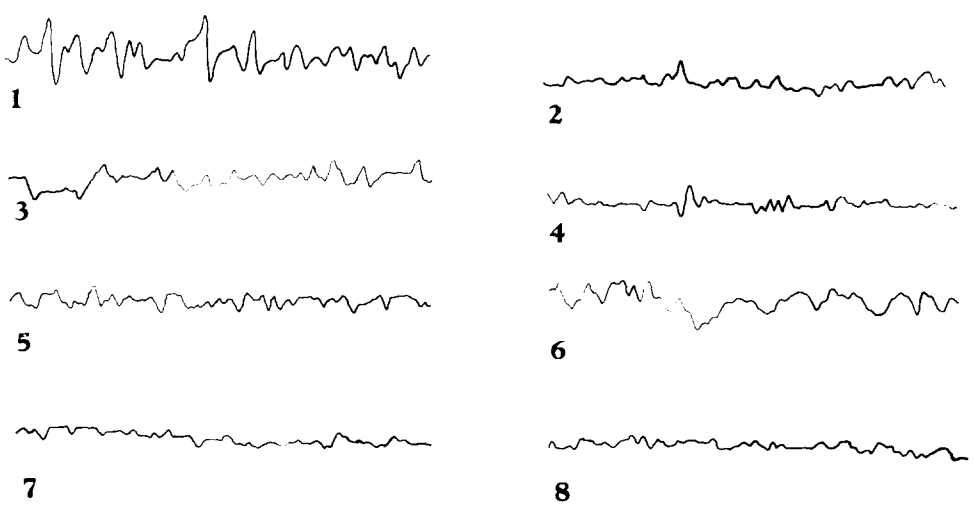

Fig. 14. Representative densitometer tracings of the intramembrane fracture face of cells following different treatments. (1, 2) fresh and ATP-depleted cells, respectively; (3, 4) same following treatment with phospholipase C; (5, 6) same, following treatment with phospholipase C and sphingomyelinase. For comparison tracings of fresh and ATP-depleted human cells are also given (7, 8). Densitometer tracings were obtained by scanning negatives of micrographs magnified ×75 000 along randomly selected lines of an actual length of 415 nm. Flat streches of the tracings represent smooth surface.

acidic pH or by increasing the ionic concentration, both treatments being supposed to alter polar protein-lipid interactions [16].

The outer smooth area of the membrane face of rat cells is faintly ridged. These ridges or shallow protrusions are enhanced in ATP-depleted cells and seem to delineate the pattern of the underlying clusters of intramembranous particles. Alteration of the structure of the outer cell surface following treatment of the cells with phospholipases has been reported [17]. The results presented here show that even in the case of extreme phospholipid hydrolysis (43 %) in absence of cell lysis, the general appearance of the intramembrane face is not significantly changed. This is in agreement with the concept that the lipid-protein and protein-protein interaction in this phase of the membrane depends on the properties of the proteins which maintain the stability of this phase toward hydrolysis as well as the resistance of the cell to lysis. The changes observed in the degree of clustering and number of particles per unit area of membrane following treatment of a cell with phospholipases could be due to changes in the total membrane area [18] and/or shifting of the fracture plane within the membrane. These phenomena could be the result of changes in the packing and the formation of droplets containing diacylglycerols and ceramides.

Based on the results presented in this paper and previous work [3], one can tentatively describe the phospholipid organization in the erythrocyte membrane as consisting of several different types: (a) phospholipids exposed to phospholipase C; (b) phospholipids protected against phospholipase C by sphingomyelin; (c) phospholipids which are not exposed because of their interaction with the membrane proteins, which can be altered by changing the ATP level of the cell. The ratio of these types varies in different cells, ranging from cells in which the c-type is apparently missing (human) to cells in which the a-type is missing (chicken). Resistance of the cells to phospholipase-induced lysis appears to be correlated with the presence of c-type organization. Changes in the metabolism or other factors which will cause a reduction of this phase will, accordingly, render the cells susceptible to lysis when treated with phospholipases. In this respect one can consider the lipids organized in this way to be equivalent to the membrane intrinsic proteins, while the lipids of the types a and b are equivalent to the peripheral membrane proteins.

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