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ELECTRON MICROSCOPY OF *BACILLUS SUBTILIS* PROTOPLAST MEMBRANE AFTER TREATMENT WITH PHOSPHOLIPASE A_2 AND PHOSPHOLIPASE C

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SUMMARY

The effects of phospholipase A_2 and phospholipase C on *Bacillus subtilis* protoplast membrane have been studied by electron microscopy and by chemical methods. Phospholipase A_2 (from porcine pancreas) almost quantitatively converted cardiolipin, phosphatidylethanolamine, phosphatidylglycerol and lysylphosphatidylglycerol to fatty acids and lysoderivatives. The fatty acids like the lysophospholipids remained in the membrane. Phospholipase C (from *Bacillus cereus*) hydrolyzed about 80% of the phosphatidylethanolamine and about 40% of the cardiolipin. Electron microscopy has been carried out with respect to general morphology of the affected protoplasts, the occurrence of a triple-layered membrane structure in thin sections, and the ultrastructure of membrane fracture faces upon freeze fracturing. Phospholipase A_2 treatment resulted in fragmentation of the protoplasts. In all cases the triple-layered membrane profile was preserved in thin sections. The membrane fracture faces appeared normal, *i.e.* they showed a convex face with many particles and a concave face with few particles. This indicated that the hydrophobic interior of the membrane was not too much damaged after incubation with phospholipases, presumably because of the stabilizing action of membrane proteins.

INTRODUCTION

Several electron microscopy studies have been made of the interaction of phospholipase C (EC 3.1.4.3) with biological membranes. These investigations refer to muscle microsomes[1], erythrocyte ghosts[2,3], intact erythrocytes[4], purified myelin[5], and axonal membrane[6]. The most detailed experiments have been carried out on erythrocyte membranes.

In the present work, we have studied the effects of pure phospholipase A_2 (EC 3.1.1.4) and pure phospholipase C on the ultrastructure of *Bacillus subtilis* protoplast membrane. Thin sections have been made to study the general appearance of the affected protoplasts and possible alterations in the triple-layered membrane profile. The technique of freeze fracturing was used to examine membrane fracture faces. It may be recalled that freeze fracturing exposes the presumed hydrophobic interior of membranes (see ref. 7 for a recent review). The detailed chemical effects

of phospholipid hydrolysis by the two enzymes will be reported elsewhere. (Op den Kamp, J. A. F., Kauerz, M. Th. and van Deenen, L. L. M., unpublished.)

MATERIALS AND METHODS

Preparation of cells and protoplasts

B. subtilis, strain Marburg, was grown aerobically at 37 °C with continuous shaking. The growth medium was composed of 10 g yeast extract (Difco), 10 g peptone (Difco), 5 g NaCl, and 400 mg Na_2HPO_4 per liter water (pH 6.8). When phospholipid analysis was carried out the media were supplied with [^{32}P]orthophosphate (200 μCi per 250 ml). Cells were harvested in the stationary phase of growth by centrifugation, washed with 0.01 M Tris buffer, pH 7.2, containing 0.32 M sucrose and 0.0015 M $MgCl_2$, and finally suspended in the same buffer at a concentration of 1 g cells (wet weight) per 15 ml. The cells were incubated with 0.1 ml of lysozyme (6% (w/v) solution, Worthington Biochemical Corp.) and 0.1 ml pancreatic deoxyribonuclease (1 mg/ml, Calbiochem.) per 10 ml suspension at 37 °C until protoplast formation was completed as judged by phase-contrast microscopy.

Incubation with phospholipases

Phospholipase A_2 was purified from porcine pancreas[8] and phospholipase C from *Bacillus cereus*[9]. The enzymes were used as will be described elsewhere (Op den Kamp, J. A. F., Kauerz, M. Th. and van Deenen, L. L. M., unpublished). In order to obtain maximal hydrolysis, a large excess of both enzymes had to be used. Samples of 0.5 ml of the protoplast suspension (corresponding to about 100 nmoles of phospholipid) were incubated in the presence of 0.001 M $CaCl_2$ with 25 units of phospholipase A_2 or 15 units of phospholipase C at 37 °C for 20 min. For freeze fracturing large scale incubations were carried out (up to 4 ml of protoplast suspension). Samples for phospholipid analysis were incubated in the same way and then analyzed (Op den Kamp, J. A. F., Kauerz, M. Th. and van Deenen, L. L. M., unpublished).

Thin-sectioning

Protoplasts or their fragments were collected by centrifugation for 15 min at $16000 \times g$ in a Sorvall RC2B centrifuge at 4 °C. They were resuspended in 2% (w/v) agar in the acetate–veronal buffer of Michaelis, pH 6.0, containing 0.01 M $MgCl_2$ (fixation buffer). The specimens contained in agar blocks were processed further according to the Ryter–Kellenberger technique[10], which includes (in our case) overnight fixation in 1% (w/v) OsO_4 in fixation buffer in the presence of tryptone, post-treatment with uranyl acetate, and embedding in Vestopal W (Jaeger, Geneva, Switzerland). In some experiments the specimens were prefixed with 5% (v/v) glutaraldehyde in fixation buffer. No clear-cut differences were noted with either fixation procedure. Sections were cut with glass knives on an LKB ultratome III. No post-staining of sections was applied.

Freeze fracturing

Freeze-fracture replicas were made with either a Denton DFE-3 freeze-fracture apparatus (Denton Vacuum Inc., Cherry Hill, N. J.) or with a Balzers apparatus (Balzers AG, Liechtenstein). Both instruments gave satisfactory results. No freeze

protecting agent was used. Filled specimen holders were frozen in solid N_2. Fracturing and shadow-casting were carried out at −100 °C. Shadow-casting, replication, and cleaning of the replicas were done according to Moor and Mühlethaler[11].

Electron microscopy

Electron micrographs were taken with Philips EM 200 or EM 300 electron microscopes operating at 80 kV. In this report micrographs of freeze-fractured specimens have been printed without reversal of contrast, *i.e.* the shadows appear white. The direction of shadowing has been indicated by an arrow in the lower right hand corner of the relevant electron micrographs.

RESULTS

Phospholipid hydrolysis

It was shown (Op den Kamp, J. A. F., Kauerz, M. Th. and van Deenen, L. L. M., unpublished) that the four main phospholipids in *B. subtilis* protoplast membrane, cardiolipin, phosphatidylethanolamine, phosphatidylglycerol, and lysylphosphatidylglycerol are hydrolyzed equally well by phospholipase A_2. It was also clear that the final amount of phospholipid which is hydrolyzed depends mainly on the initial phospholipase A_2 concentration. Therefore, to obtain a virtually complete hydrolysis

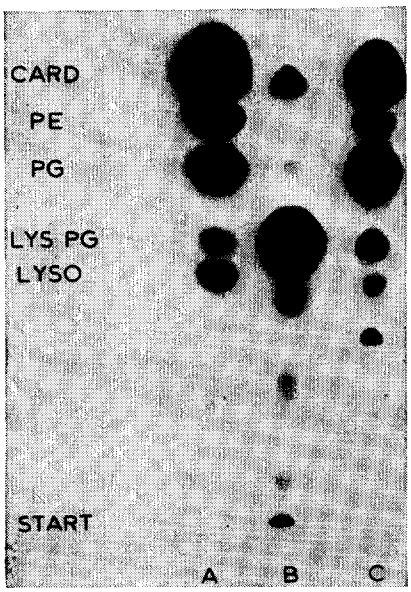

Fig. 1. Autoradiogram of *B. subtilis* phospholipids. ^{32}P-labeled *B. subtilis* protoplasts were incubated without enzyme (A) and with phospholipase A_2 (B) and phospholipase C (C) as described in the text. The lipids were extracted with chloroform–methanol (1:2, v/v) and separated on silicagel impregnated paper in diisobutylketone–acetic acid–water (8:5:1, v/v/v) (Op den Kamp, J. A. F., Kauerz, M. Th. and van Deenen, L. L. M., unpublished). The chromatogram was exposed to Kodak X-ray film for 3 days. The compounds are: CARD, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; LYS PG, lysylphosphatidylglycerol; LYSO, 1-acyl lysophospholipids.

of the phospholipids we used a large excess of phospholipase A_2 and furthermore incubation was carried out at 37° C instead of room temperature. Fig. 1 shows that most of the phospholipid is degraded under these conditions and that only small amounts of cardiolipin and phosphatidylglycerol remain. A quantitative representation of the action of phospholipase A_2 is presented in Table I. Phosphatidylethanolamine is hydrolyzed nearly completely, whereas cardiolipin and phosphatidylglycerol are degraded at least up to 92 and 95%, respectively.

TABLE I

HYDROLYSIS OF *B. SUBTILIS* PHOSPHOLIPIDS BY PHOSPHOLIPASES

Incubation of the protoplasts and extraction and separation of the phospholipids was carried out as described in the legend of Fig. 1. Radioactive spots on the chromatogram were cut out and counted in the liquid scintillation counter. The data are expressed as percentages of total lipid phosphorus present before incubation. After phospholipase A_2 treatment the amount of lysylphosphatidylglycerol could not be measured, whereas after phospholipase C treatment phosphatidylglycerolphosphate is formed which cochromatographs with phosphatidylglycerol.*

	No enzyme	*Phospholipase A_2*	*Phospholipase C*
Cardiolipin	56	4	35
Phosphatidylethanolamine	16	0.5	3
Phosphatidylglycerol	21*	1	35
Lysylphosphatidylglycerol	7	—	6

The lysophospholipids which are formed appeared to remain in the membrane material. When the protoplasts were centrifuged for 15 min at $16000 \times g$ after the phospholipase treatment no phospholipid could be detected in the supernatant. To check the fate of the released fatty acids, *B. subtilis* was cultivated in the presence of [^{14}C]palmitic acid. A substantial amount of this fatty acid appeared to be incorporated in the bacterial lipids. Preliminary experiments indicate that phospholipase A_2 treatment of these ^{14}C-labeled protoplasts results in the formation of both [^{14}C]-lysophospholipids and ^{14}C free fatty acids. The free fatty acids like the lysophospholipids remained associated with the protoplasts.

Phospholipase C is known to preferentially hydrolyse phosphatidylethanolamine in *B. subtilis* protoplast membrane (Op den Kamp, J. A. F., Kauerz, M. Th. and van Deenen, L. L. M., unpublished). As is shown in Fig. 1 and Table I more than 80% of the initial amount of phosphatidylethanolamine is degraded, whereas only 40% of the cardiolipin is hydrolyzed. The degradation of the latter phosphoglyceride results in the formation of diglyceride and phosphatidylglycerolphosphate, which co-chromatographs with phosphatidylglycerol and is responsible for the increase in radioactivity at that position on the chromatogram. The phosphatidylglycerolphosphate remains associated with he protoplast and therefore the loss of lipid phosphorus from the membrane is rather limited (20%) and is caused mainly by degradation of phosphatidylethanolamine.

Morphology

Electron microscopy has been carried out with respect to the general morphology of the affected protoplasts, the occurrence of a triple-layered membrane structure in thin sections and the ultrastructure of the fracture faces in freeze-fractured membrane preparations.

The general appearance of thin-sectioned lysozyme and deoxyribonuclease-treated protoplasts is shown in Fig. 2. The cells possess a variable amount of cell

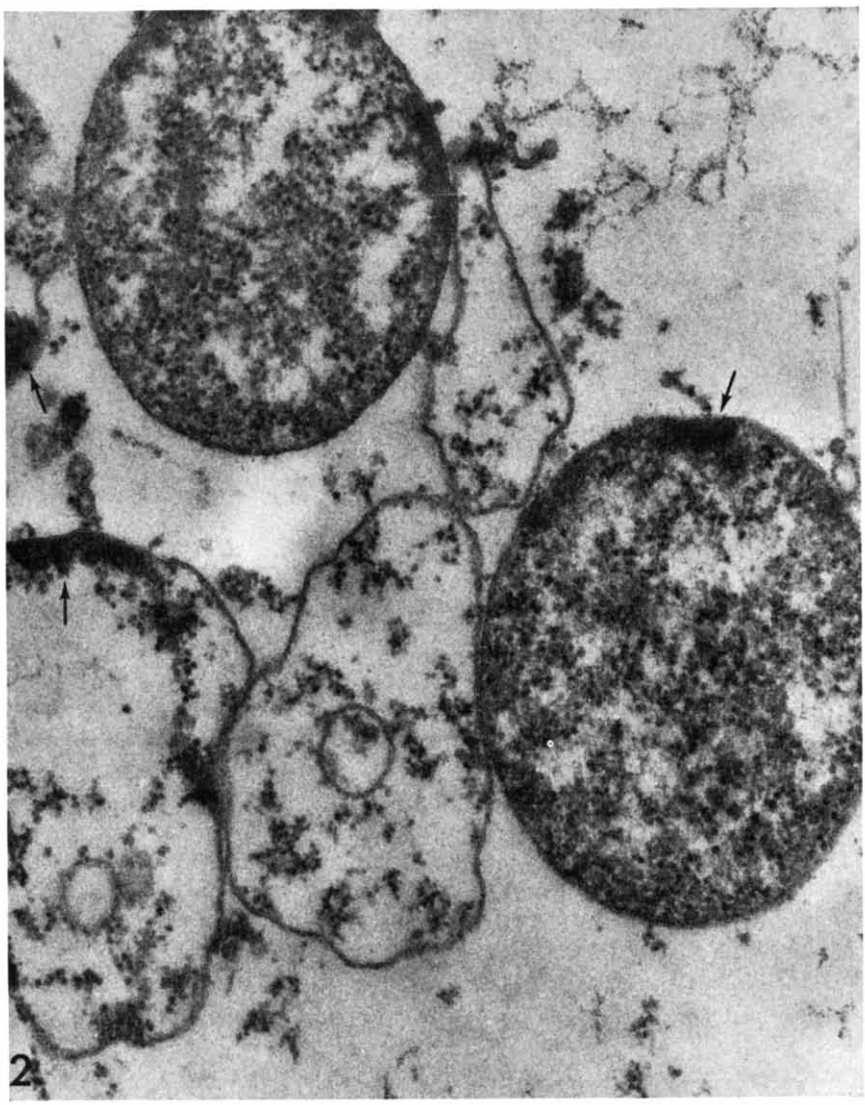

Fig. 2. Thin-sectioned *B. subtilis* protoplasts. Note (arrows) electron dense areas near cytoplasmic site of the plasma membrane. Some protoplasts appear rather empty. We believe this to be due to the fact that protoplasts were prepared from stationary-phase cells. Fixation in glutaraldehyde and OsO_4 tetroxide. ×47000.

contents and the triple-layered image of the plasma membrane is clearly visible. A consistent feature is the presence of electron dense areas (arrows) on the cytoplasmic side of the plasma membrane. These areas are also present when the glutaraldehyde pre-fixation step is omitted. Freeze-fractured control protoplasts can be seen in Fig. 3.

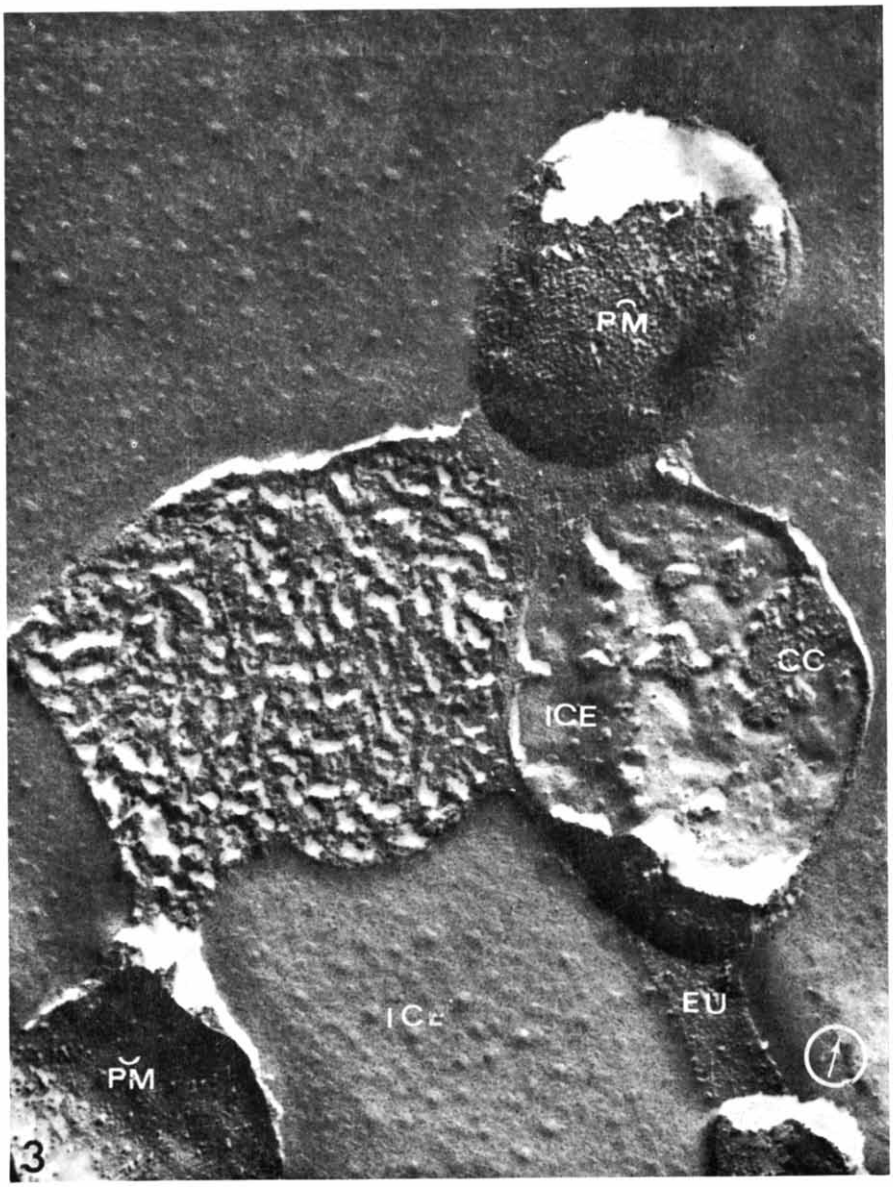

Fig. 3. Freeze-fractured *B. subtilis* protoplasts. Note convex ($\widehat{PM}$) and concave ($\widecheck{PM}$) fracture faces of the protoplast membrane. Ice crystals (ICE) are seen around compressed cell content (CC) in the cross-fractured and rather empty protoplast to the right. Larger ice areas interrupted by eutectic regions (EU) surround the freeze-fractured protoplasts. The arrow in the lower right-hand corner in this and subsequent freeze-fracture replicas depicts the direction of shadowing. ×52000.

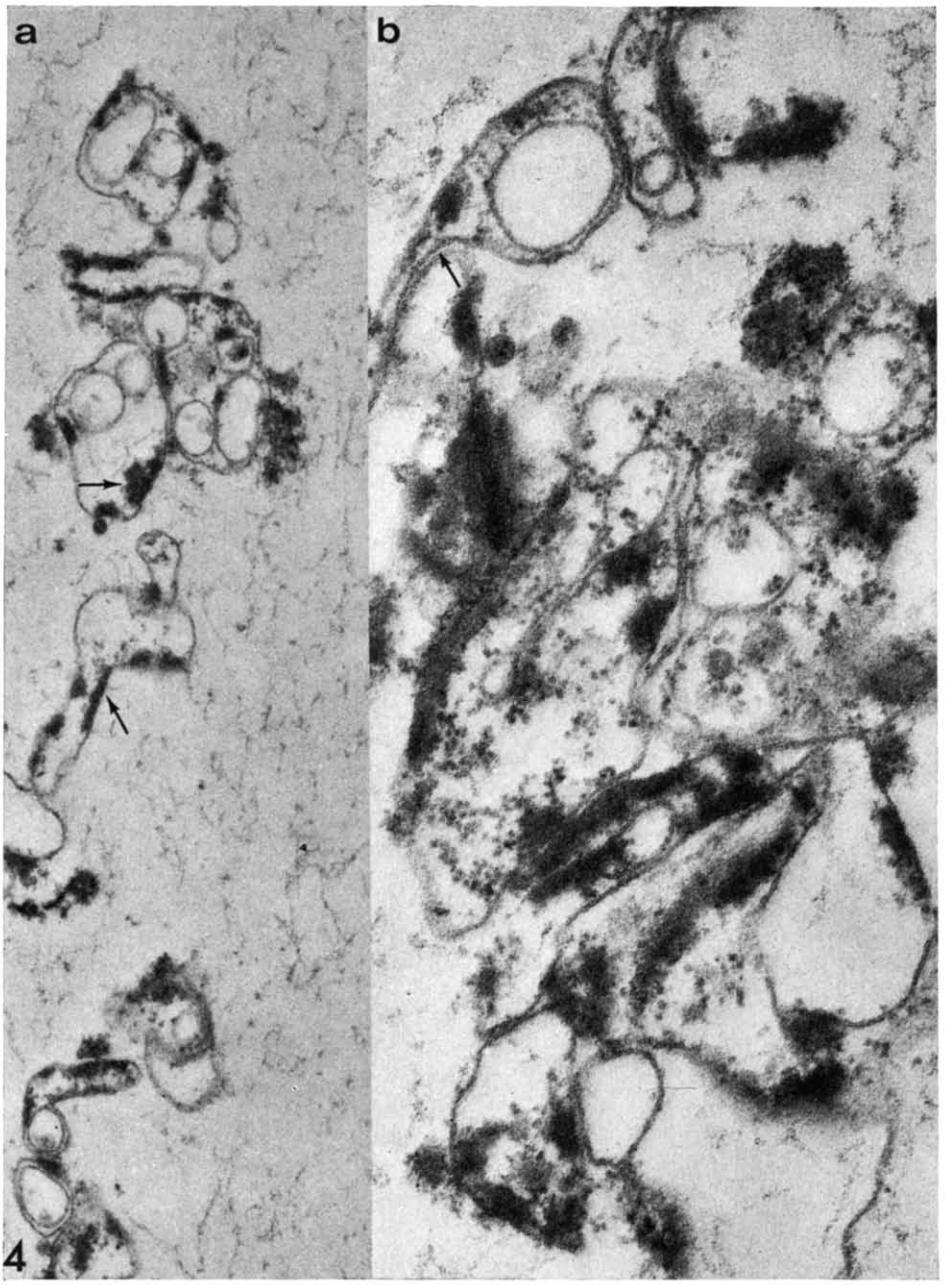

Fig. 4. Thin-sectioned phospholipase A_2-treated protoplasts. Note fragmentation of protoplasts and electron dense areas near the plasma membrane (a, and *cf.* Fig. 2). At higher magnification the triple-layered image of the plasma membrane is still visible (b). The inverse bent plasma membrane (arrow in b) could give rise to a convex face with few particles upon fracturing (*cf.* Fig. 5b). Fixation in glutaraldehyde and OsO_4 (a) $\times 27\,500$. (b) $\times 63\,000$.

The fracture faces have the same appearance as those from intact *B. subtilis* cells[12,13] *i.e.* a convex face with many particles and a concave face with few particles. Protoplasting does not seem to alter the particle distribution on either membrane face in a significant way. We have also noticed that protoplasts are cross-fractured more readily than intact rods. That is to say that in the latter case fracture faces of membranes are more frequently observed.

Treatment of protoplasts with phospholipase A_2 results in their fragmentation (Fig. 4a). In earlier experiments we demonstrated that complete fragmentation can be obtained only with a large excess of enzyme as has been applied in this study. Suboptimal enzyme concentration renders the cells leaky without causing fragmentation

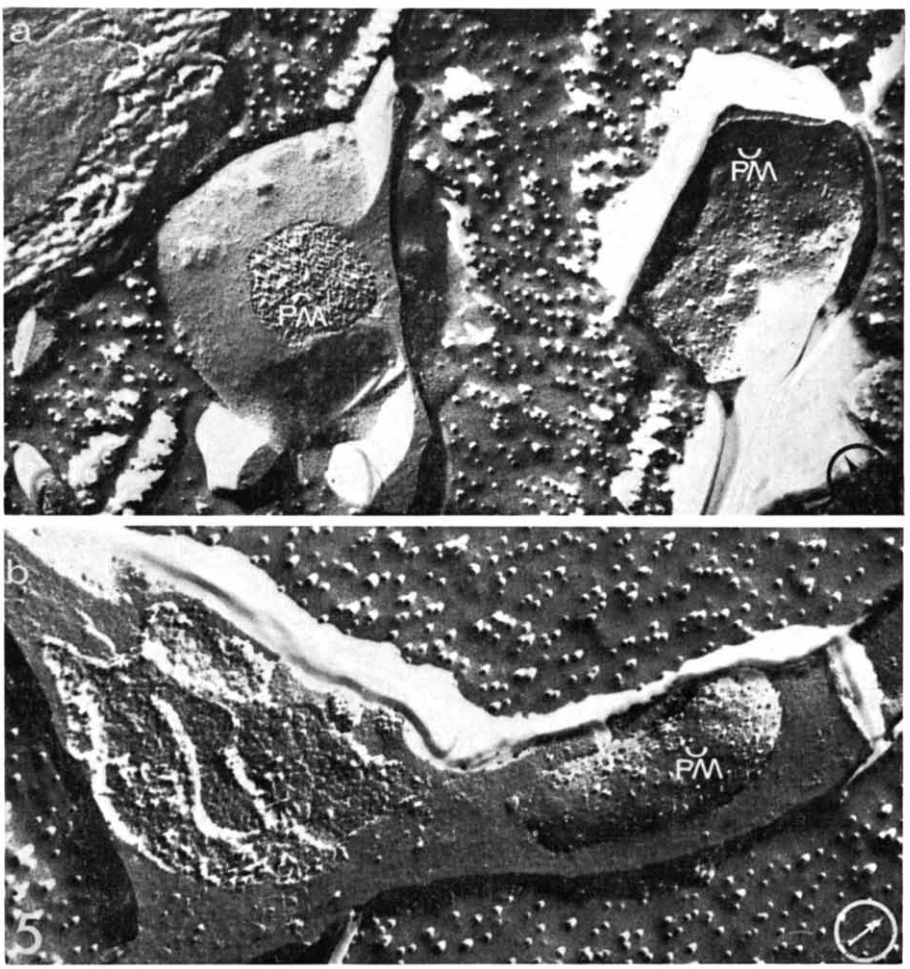

Fig. 5. Freeze-fractured phospholipase A_2-treated protoplasts. The membrane fracture faces ($\widehat{PM}$ and $\widecheck{PM}$) are similar to those of the control cells (Fig. 3). At the left in (a) the etched outer surface of the plasma membrane is visible. In (b) a cross-fractured membranous structure can be seen together with a convex face ($\widecheck{PM}$) with few particles (*cf.* text for interpretation). (a) ×50000. (b) ×52000.

(Op den Kamp, J. A. F., Kauerz, M. Th. and van Deenen, L. L. M., unpublished). The electron dense areas near the plasma membrane are conspicuous (*cf.* refs 2–4). However, since similar areas are present in the control preparations (Fig. 2), it is unlikely that, in this case, they resulted from the action of phospholipase A_2. We can further note (Fig. 4b) that the triple-layered appearance of the plasma membrane has been maintained; also the morphology of the membrane fracture faces seems to be unchanged (Fig. 5). Nevertheless, in the right corner of Fig. 5b we can observe a convex face with few particles, whereas one would expect many particles. This is an exceptional situation. We assume that in such a case an inversely bent membrane fragment has been fractured. For instance, a structure as indicated by the arrow in Fig. 4b.

Treatment of protoplasts with phospholipase C does not seem to affect their integrity. As far as membrane structure is concerned no basic difference could be observed when compared to control cells. This is especially clear when freeze-fractured specimens are examined (not shown).

We may mention that during freeze fracturing control cells and enzyme-treated cells were always run together in order to obtain freeze-fracture replicas of comparable degree of etching and quality of shadow-casting.

DISCUSSION

In this study we report the effects of phospholipase A_2 and phospholipase C on the morphology of *B. subtilis* protoplast membrane. The first enzyme almost quantitatively converted cardiolipin (diphosphatidylglycerol), phosphatidylethanolamine, phosphatidylglycerol and lysylphosphatidylglycerol to free fatty acids and lysoderivatives (Op den Kamp, J. A. F., Kauerz, M. Th. and van Deenen, L. L. M., unpublished; and Fig. 1). The second enzyme hydrolyzed about 80% of the phosphatidylethanolamine and about 40% of the cardiolipin (Op den Kamp, J. A. F., Kauerz, M. Th. and van Deenen, L. L. M., unpublished; and Fig. 1).

Digestion with phospholipase A_2

B. subtilis membrane morphology after phospholipase A_2 treatment, as studied by thin-sectioning and freeze fracturing, is indistinguishable from that of non-treated membranes. This can be explained by the fact that the breakdown products remained in the membrane and by "the persistence of organized protein layers"[15]. It appears that disruption of the binding of fatty acids to their lysophospholipids did not result in a dramatic disturbance of the hydrophobic interior of the membrane, as judged by freeze fracturing. The protoplasts, however, are lysed. It has been suggested that the lytic action was caused by fatty acids and by lysophospholipids that were produced by phospholipase A_2 hydrolysis (Op den Kamp, J. A. F., Kauerz, M. Th. and van Deenen, L. L. M., unpublished).

Digestion with phospholipase C

Before discussing our results it seems worthwhile to mention briefly results of earlier morphological studies on the effect of phospholipase C on erythrocyte membranes. This seems further relevant because freeze-fractured erythrocyte membranes[16] and *B. subtilis* plasma membrane[12,13] have similar fracture faces. In addition, direct

evidence for the concept of membrane splitting by freeze fracturing has thus far been given for the erythrocyte[17,18] and *B. subtilis* membrane[19] exclusively.

In phospholipase C-treated erythrocyte ghosts it was observed in thin sections that the typical triple-layered image of the membrane was preserved, although the ghosts appeared considerably reduced in size[3,4,20]. Also, electron-dense droplets were observed in the vicinity of the ghost membranes[2–4]. These droplets were interpreted to represent accumulations of diglyceride that arose by phospholipase C hydrolysis[2,3]. Similar results have been obtained with intact erythrocytes[4]. However, doubts have been cast on the purity of the enzyme preparations that were employed[21]. It was found[21] that intact erythrocytes are not attacked by phospholipase C, whereas ghosts are. Thus considered, the afore-mentioned electron microscopy studies should be viewed with some caution. Nevertheless, our results with phospholipase C-treated *B. subtilis* protoplast membrane are similar, *i.e.* the triple-layered image of the thin-sectioned plasma membrane is maintained. We find in addition that the fracture faces of the membrane interior are unchanged after phospholipase C treatment. This can be explained by the finding that the decrease in total amount of phospholipid as based on lipid phosphorus determinations was only 20% because phosphatidylglycerolphosphate was formed out of cardiolipin and remained associated with the membrane (*cf.* Results). Our experiments further indicate that the diglycerides formed were retained in the protoplasts. This latter observation contrasts with experiments on erythrocytes in which, after phospholipase C incubation, a phospholipid–phosphorus loss of around 70% was observed[2,3,20] concomitant with electron dense spots near the affected membrane[2–4].

Thin-sectioning versus freeze fracturing

Thin-sectioning seems to have its limitations in establishing to which extent membrane structure has been preserved after chemical digestion. This appears most strikingly in the case of phospholipid-depleted membranes where the unit membrane-like structure could still be seen in thin sections[15,22,23]. It is not to be expected that phospholipid-depleted membranes will give normal, if any, fracture faces upon freeze fracturing when the hydrophobic interior has been damaged (*cf.* ref. 14). An array of smooth plaques was observed on the convex face of phospholipase A-treated and freeze-fractured erythrocyte membranes[24]. The plaques were thought to arise from a deflection of "the hydrophobically-guided fracture plane" because of newly formed polar regions introduced by phospholipase A treatment. Thus far, we have not observed plaque-like structures in our material.

Conclusion

One may ask whether our experiments suggest the acceptance of a particular membrane model, be it of the subunit type or a more or less modified Danielli–Davson model. Freeze fracturing (membrane splitting) results, in general, seem most compatible with a bilayer structure. The accessibility of phospholipids for enzymes suggests their presence at the membrane surface as has been pointed out by Lenard and Singer[20]. However, one can also envisage that the phospholipases penetrate through membrane pores, whether pre-existing or formed upon arrival of the enzyme at the membrane surface. Extensive lipid areas on the surface of the membrane are not necessarily involved. The degradation products of phospholipase A_2 treatment

probably remain at their place with the aid of protein framework[15]. To test this possibility experiments are now carried out on liposomes prepared from *B. subtilis* protoplasts.

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