Biochimica et Biophysica Acta, 643 (1981) 101—114

BBA 79192

FREEZE-FRACTURE STUDY OF CARDIOTOXIN ACTION ON AXONAL MEMBRANE AND AXONAL MEMBRANE LIPID VESICLES

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(Received October 31st, 1980)

Key words: Cardiotoxin; Lipid vesicle; Phospholipase A_2, Freeze fracture; (Axonal membrane)

Summary

Freeze-fracture electron microscopy was used to follow morphological changes induced by *Naja mossambica mossambica* venom cardiotoxins on crab axonal membranes and their lipids.

It was shown that the extent of morphological changes depended drastically on the purity of cardiotoxin preparations and on their nature. Highly purified cardiotoxin induced mainly fusion of membrane or lipid vesicles. The extent of fusion and other morphological changes depended on the nature of cardiotoxin used: $V^{II}4$ cardiotoxin induced only fusion while $V^{II}1$ led to further modifications of membranes and liposomes. The most spectacular morphological changes were observed with axonal membranes treated with cardiotoxin containing traces of venom phospholipase A_2. At low cardiotoxin concentration (10^{-7}—10^{-5} M) important intramembrane particle aggregation was observed and at higher concentrations (more than 10^{-4} M) intramembrane particles disappeared from the membrane and were found in solution. The membrane vesicles, devoid of intramembrane particles, were observed to fuse rapidly into liposome-like aggregates. These morphological changes are interpreted as being due to the removal of intrinsic membrane proteins from the membrane by the combined action of cardiotoxin and phospholipase A_2.

Abbreviations: Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid; SDS, sodium dodecyl sulphate.

Introduction

Cardiotoxins are the most abundant constituents of cobra venom [1—3]. They are proteins, each composed of about 60 residues in a single polypeptide chain cross-linked by four disulfide bridges [4,5]. They affect various kinds of cell, causing marked permeability changes of the cell membranes and consequently imparing both the function and the structure of membranes [4,6—10].

The molecular mechanisms of cardiotoxin action on membranes and different lipids have been extensively studied in the past years [11—14]. In the case of crab axonal membranes, it has been shown that cardiotoxins first associate rapidly and reversibly with essentially negatively charged membrane lipids, then, in the second step, induce a rearrangement of the membrane structure leading to, among other things, irreversible deactivation of $(Na^+ + K^+)$-ATPase [11].

The main purpose of the present work was to follow by freeze-fracture electron microscopy the morphological changes induced by cardiotoxins on axonal membranes and on axonal membrane lipid vesicles. We were able to show that the extent of the morphological changes depended drastically on the purity and the nature of cardiotoxin preparations. Highly purified cardiotoxins induced mainly fusion of membranes or lipid vesicles. Cardiotoxin containing traces of venom phospholipase A_2 induced intramembrane particle aggregation, leading eventually to their complete removal from the membrane. The latter results are interpreted in terms of combined action of cardiotoxin and phospholipase A_2.

Materials and Methods

Axonal membranes and axonal membrane lipids were prepared from the crab *Cancer pagurus*, as described previously [11]. The membranes were suspended in a 12.5 mM Tes buffer at pH 7.5 containing 0.32 M sucrose at a concentration of 1.5 mg of membrane proteins per ml of solution. Lipids were extracted from axonal membranes (2 mg of membrane protein in 1.3 ml of Tris/sucrose buffer) with chloroform/methanol (2 : 1, v/v). After evaporation of the solvent, the lipid fraction was suspended in 1.5 ml of 2 mM Tes buffer at pH 7.4 containing 100 mM NaCl 2 mM histidine 0.1 mM EDTA. Lipid vesicles were prepared from this suspension, as described by Papahadjopoulos [15], at a final phospholipid concentration of 4.4 mM.

Cardiotoxins were purified from *Naja mossambica mossambica* venom as described previously [7,10]. The present work was carried out with two cardiotoxins $V^{II}1$ and $V^{II}4$, according to present nomenclature [5]. Phospholipase A_2 contamination of $V^{II}1$ and $V^{II}4$ cardiotoxin were 0.02% and less than 0.003%, respectively, (calculated using the value for the specific activity of *Naja mossambica mossambica* phospholipase A_2 of 379 units per mg of protein).

For freeze-fracture electron microscopy the suspensions of membranes and lipid vesicles and the solutions of cardiotoxins were prepared in buffers containing 25—30% of glycerol. Small drops of the preparations were deposited on conventional Balzers gold planchetts and rapidly frozen in Freon-22 (at —160°C) or in liquid propane. Fracturing and replication were done with a Balzers BAF 301 freeze-etching unit using platinum-carbon shadowing. The repli-

cas, after digestion of organic material with chromic acid and washing with distilled water, were observed in a Philips 301 electron microscope.

Results

Untreated preparations of axonal membranes are composed of small vesicles showing on both their fracture faces very low densities of randomly distributed intramembrane particles (Fig. 1). In contrast to most biological membranes, the distribution of particles between exoplasmic (EF) and protoplasmic (PF) fracture faces are similar (Fig. 1B).

When cardiotoxins are added to axonal membrane preparations containing 0.1 mM EDTA, to inhibit any possible phospholipase activity, the first morphological change observed is the aggregation (Fig. 2B) followed by fusion of membrane vesicles into larger vesicles and eventually into liposome-like structures (Fig. 2C). The morphological changes induced by $V^{II}4$ cardiotoxin never exceed the step of aggregation and fusion but that induced by $V^{II}1$ cardiotoxin may lead to further disintegration of the membranes. An example of such a disintegration is shown on Fig. 2D which represents a typical image observed in preparations of axonal membranes treated with $V^{II}1$ cardiotoxin at concentrations corresponding to more that 1 mol of cardiotoxin per 2 mol of negatively charged lipids (Phosphatidylserine and phosphatidylinositol, which account,

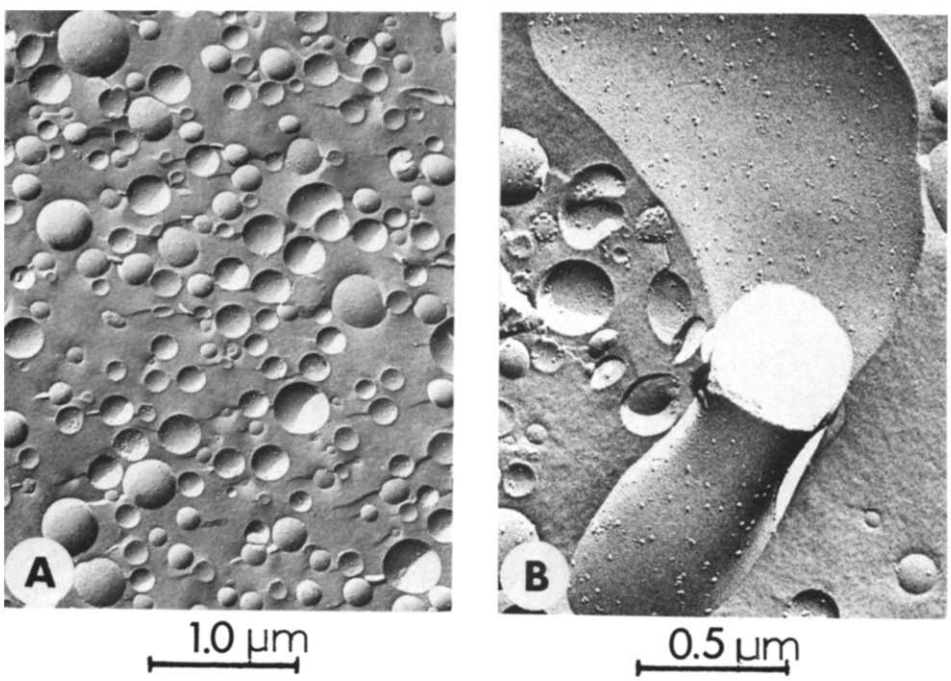


Fig. 1. Freeze-fracture images of crab axonal membrane vesicles. A, Low magnification view of the preparation. B, Higher magnification view of a large piece of axonal membrane, showing both EF (concave) and PF (convex) fracture faces. Note the very low amount of intramembrane particles and their similar distribution on both fracture faces.

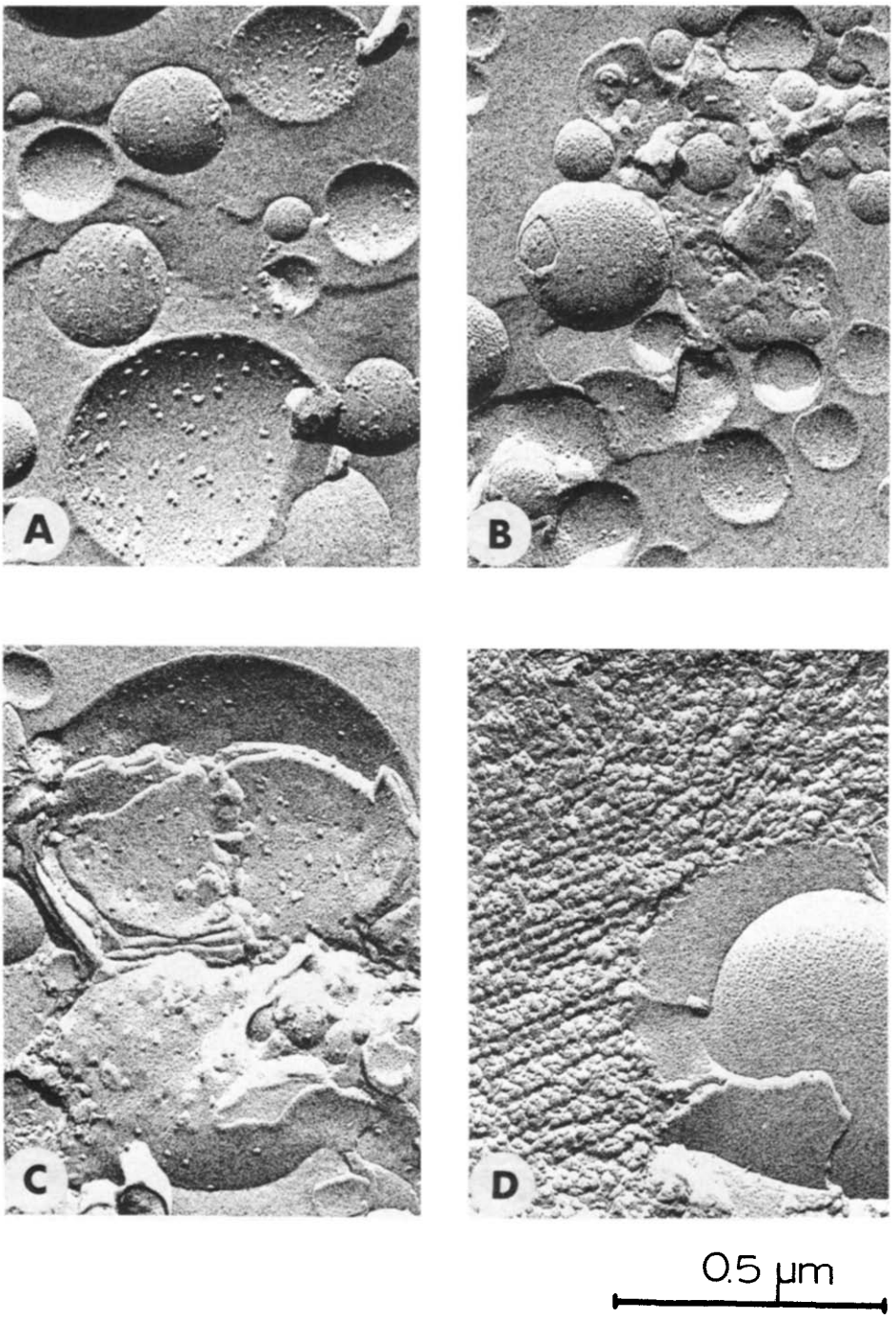


Fig. 2. Freeze-fracture images of V^{II}_1 cardiotoxin-treated crab axonal membranes. A, Axonal membrane vesicles before the addition of cardiotoxin. B, Axonal membrane 1 min after the addition of cardiotoxin (1 mol of cardiotoxin per 4 mol of negatively charged lipids). Note the beginning of aggregation of membrane vesicles. This aggregation is very clearly seen on low magnification images of the preparation as separated domains containing aggregated vesicles and vesicle-free domains. C, The same as B, 30 min later. Note the presence of highly aggregated, fused membrane vesicles. D, The same as B, 24 h later. Note the presence of highly aggregated, partly ordered, particulate material and smooth vesicles.

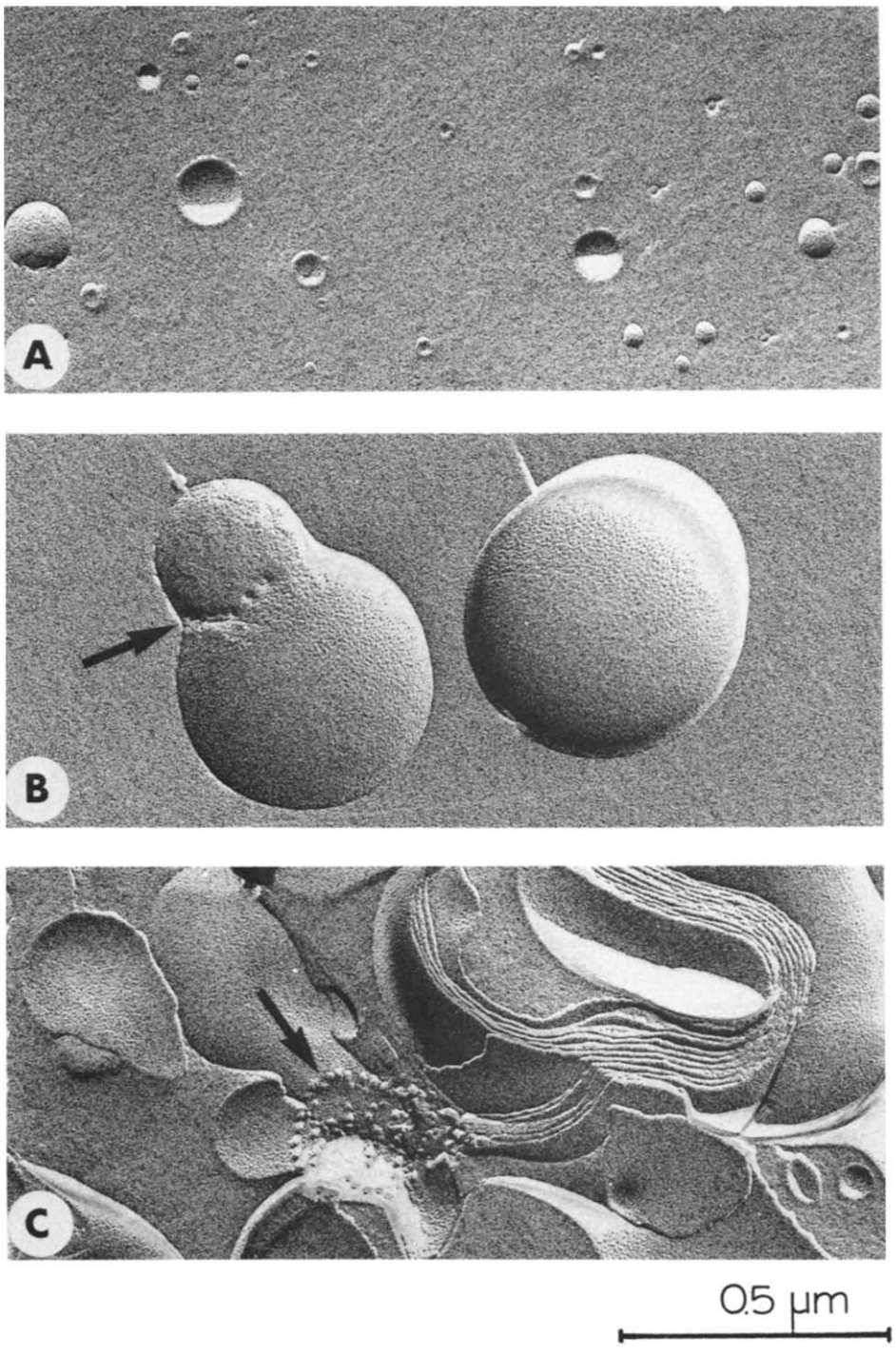


Fig. 3. Crab axonal membrane lipid vesicles treated with $V^{II}4$ cardiotoxin. A, Preparation of lipid vesicles. B, Lipid vesicles, 30 min after the addition of cardiotoxin. Note the presence of so-called 'lipidic particles' [16,19] in the region of fusion between two vesicles (arrow). C, The same as B, 24 h later. Note the presence of large liposomes and some 'lipidic particles' (arrow).

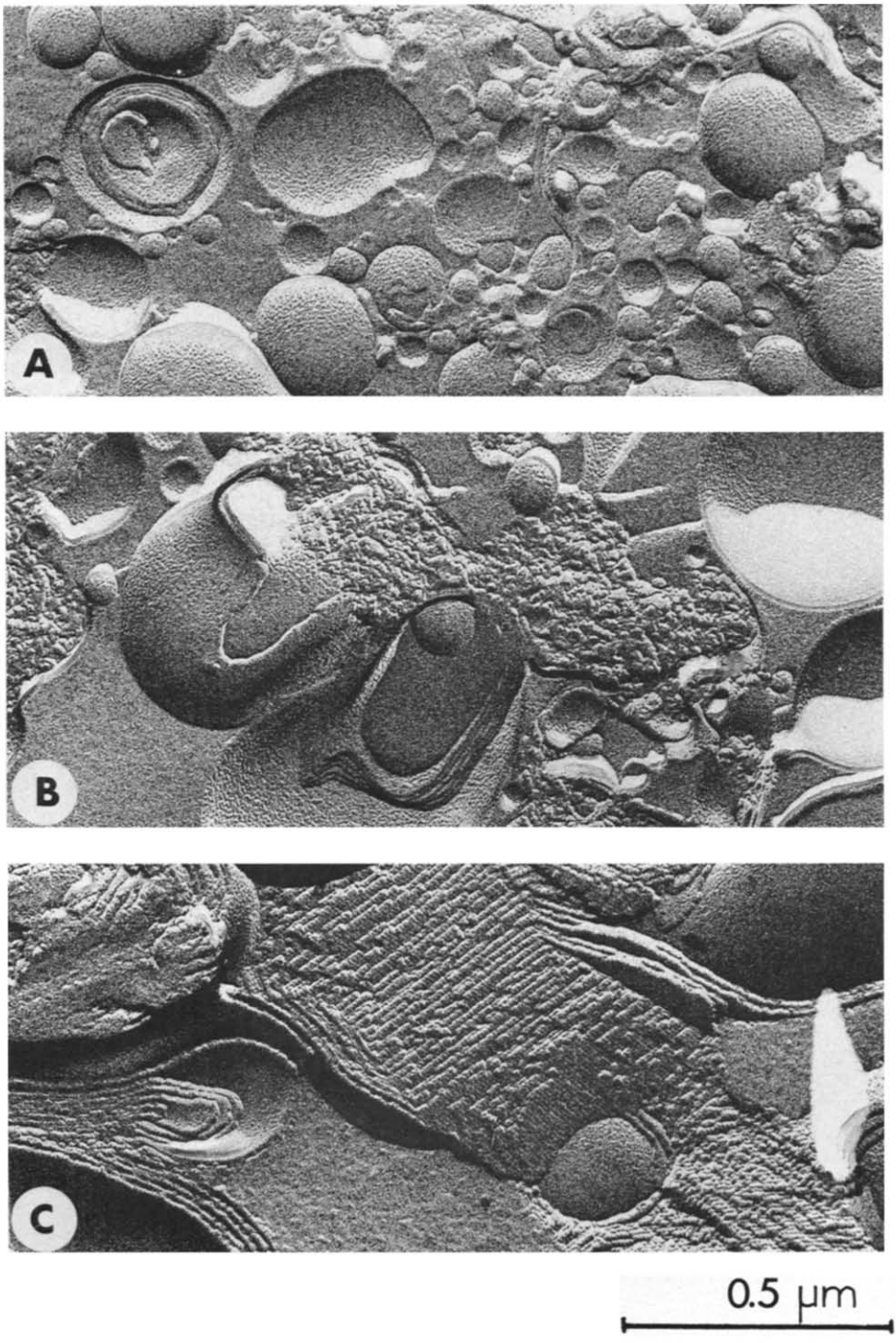


Fig. 4. Crab axonal membrane lipids treated with cardiotoxin $V^{II}1$. A, Lipid vesicles 1 min after the addition of cardiotoxin (pure lipid vesicles are shown in Fig. 3A). Note the presence of aggregated, fused vesicles. B, The same as A, 30 min later. Note the presence of large liposomes and of small pieces of aggregated lamellae. C, The same as A, 24 h later. Note the presence of large liposomes and of a highly ordered region morphologically similar to hexagonal H_{II} phase [17,20].

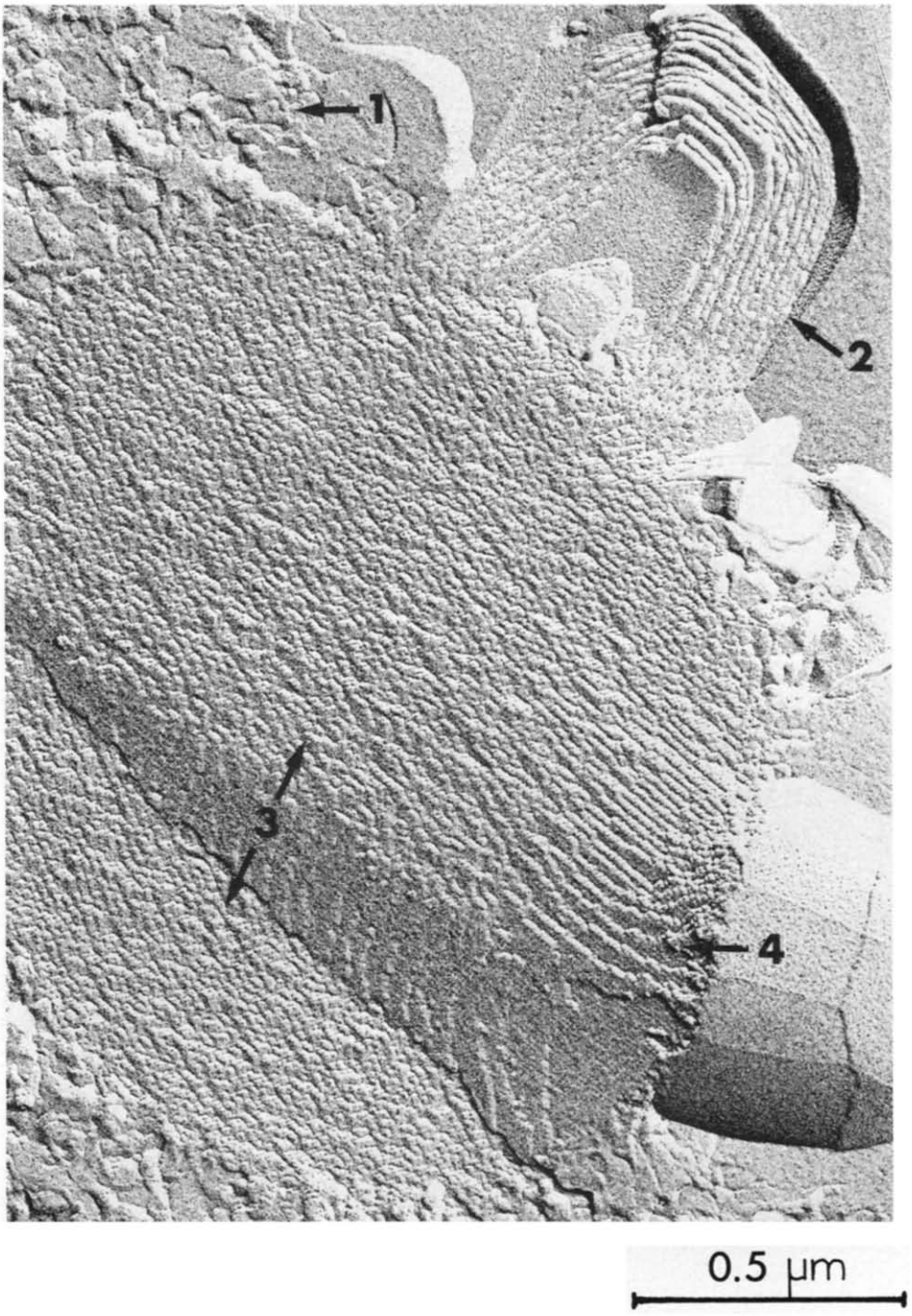


Fig. 5. Freeze-fracture image of a large portion of lipid cardiotoxin $V^{II}1$ precipitate obtained after 24 h incubation. Note the presence of four distinct regions indicated by arrows: 1, small pieces of lamellae; 2, large liposomes (lamellar phase); 3, ordered particulate regions: 4, highly ordered regions, morphologically similar to hexagonal H_{II} phase [17,20].

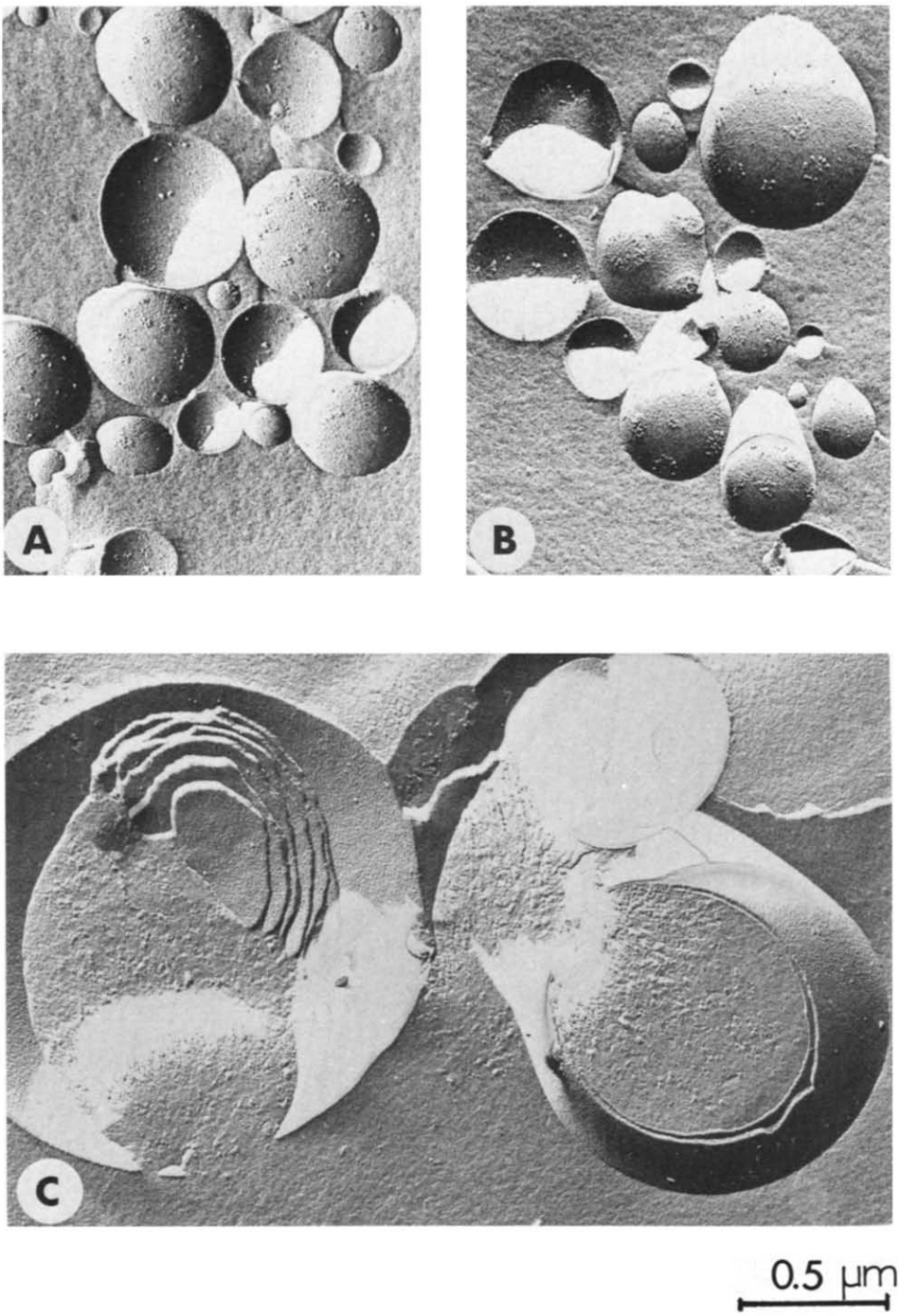


Fig. 6. Crab axonal membrane vesicles treated with V^{II}_4 cardiotoxin containing about 0.03% of venom phospholipase A_2. A, Incubation in 10^{-5} M solution of cardiotoxin. B, Incubation in 10^{-4} M solution of cardiotoxin. C, Incubation in $5 \cdot 10^{-4}$ M solution of cardiotoxin. Note the presence of aggregated intramembrane particles in A and B, and of large liposome-like aggregates, almost free of particles, in C. Note also the presence of small particles in solution both inside and outside of fractured vesicles in C.

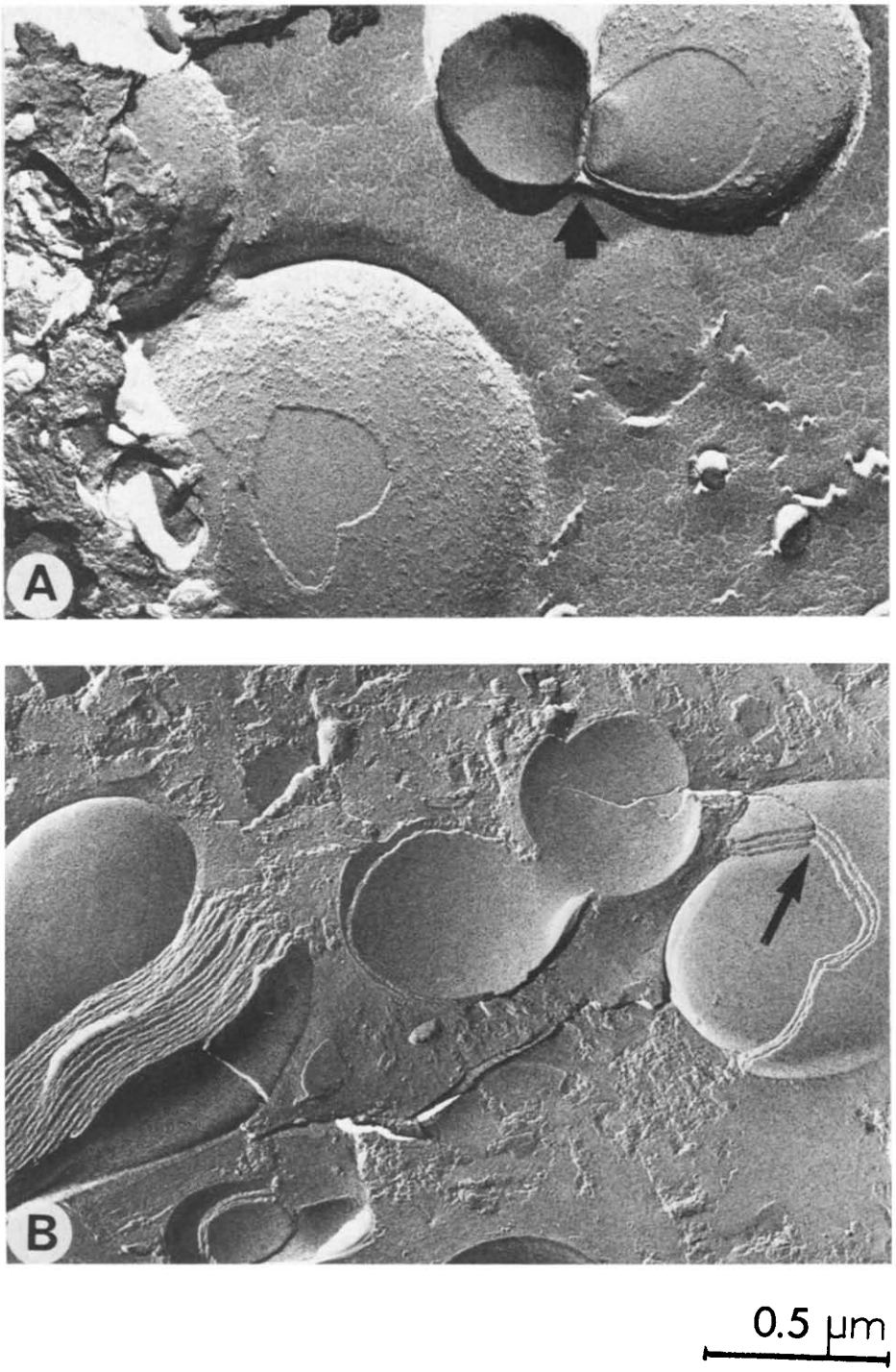


Fig. 7. Freeze-etching (A) and freeze-fracture (B) images of $V^{II}4$ cardiotoxin-treated crab axonal membranes. The concentration of cardiotoxin (containing about 0.03% of venom phospholipase A_2) was $5 \cdot 10^{-4}$ M, corresponding to about 1 mol of cardiotoxin per mol of negatively charged membrane lipids. A, Etched sample. Note smooth convex and concave fracture faces (arrow) and particulate surfaces exposed after sublimation of ice. These particulate surfaces correspond probably to the deposition of soluble materials during sublimation of ice. B, Centrifugation pellet of the sample. Note smooth fracture faces of liposome-like material and the presence of particulate material in solution. Note also that the oblique fractures of stacked vesicles take place every two membranes (arrow), indicating the presence of non-equivalent neighbouring hydrophobic or hydrophilic layers (see Results and Discussion).

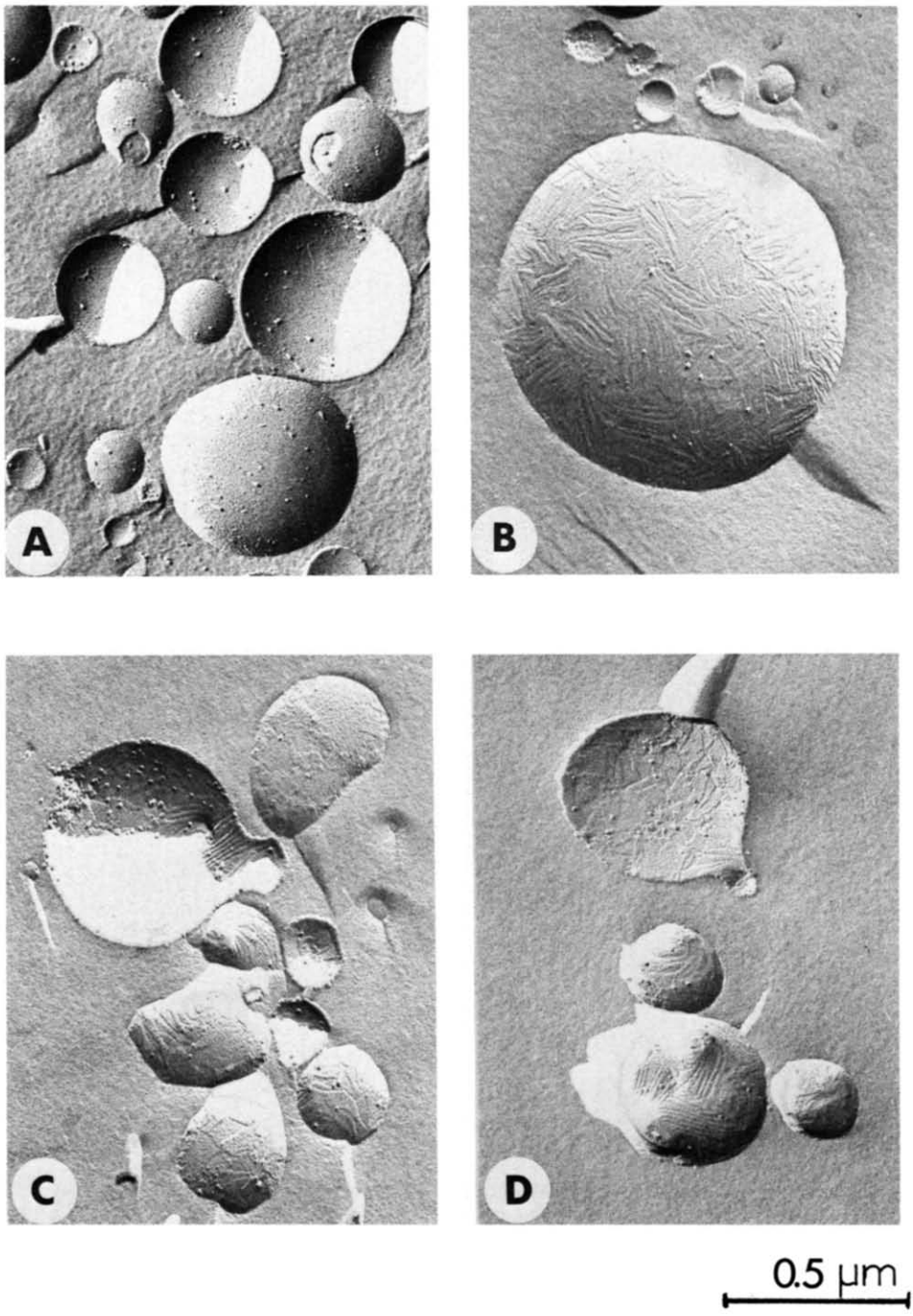
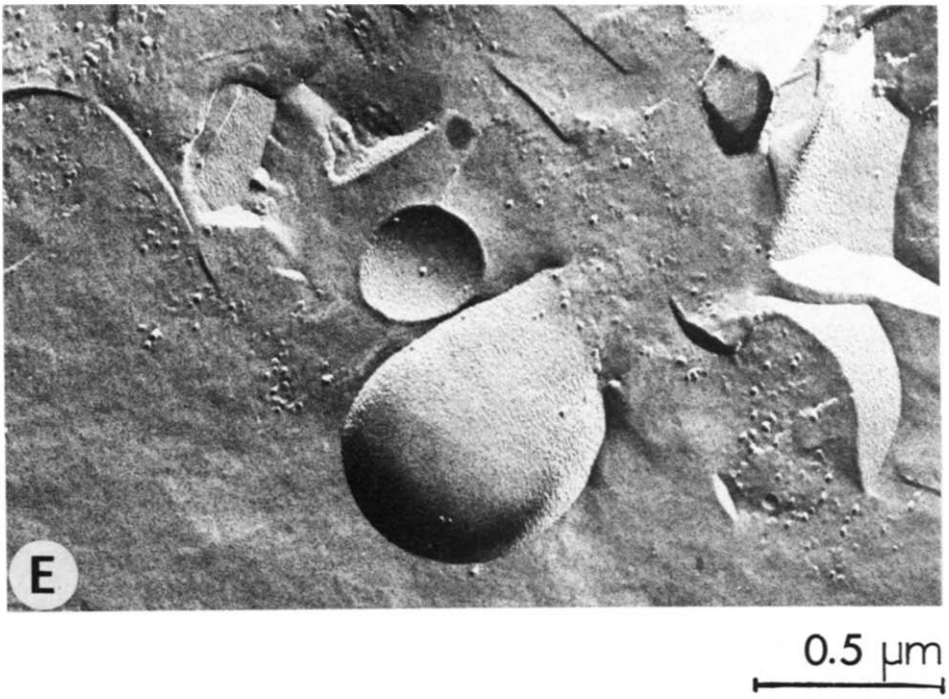


Fig. 8. Kinetic study of the action of $V^{II}4$ cardiotoxin containing about 0.03% of venom phospholipase A_2 on crab axonal membranes. The concentration of cardiotoxin correspond to 1 mol of cardiotoxin per mol of negatively charged membrane lipids. A, Axonal membrane vesicles before the addition of cardiotoxin. B, Axonal membrane vesicles, 1 min after the addition of cardiotoxin. C, The same as B, 2 min later. D, The same as B, 4 min later. Note peculiar morphological changes of the membranes: mainly the appearance of rippled surfaces and small vesicular excrescences. E, The same as B, 25 min later. Note the presence of membrane vesicles displaying smooth fracture faces and the presence of particles in solution.



respectively, for 13.5% and 1.1% of total membrane lipids). Such preparations are composed of highly aggregated particulate material and of a few large smooth vesicles, devoid of intramembrane particles.

The morphological changes induced by $V^{II}4$ cardiotoxin on axonal membrane lipid vesicles are shown in Fig. 3. As in the case of the corresponding membrane vesicles, the main morphological changes are aggregation and fusion of lipid vesicles, leading eventually to large liposomes (Fig. 3C). The fusion is often accompanied by the appearance of small 'lipidic particles' (see arrows in Figs. 3B and C), as has already been observed with other lipid vesicles [16].

More drastic morphological changes are induced by $V^{II}1$ cardiotoxin (Figs. 4, 5 and 6), the action of which leads to further disintegration of the rapidly formed liposomes into small pieces of lamellae (arrow 1 in Fig. 5) or into particulate material which forms partly or highly ordered structures (Figs. 4C and 5).

The most spectacular morphological changes of the axonal membranes are induced by cardiotoxins containing small amounts of venom phospholipase A_2, in the absence of EDTA. These changes are illustrated on images shown in Fig. 6, 7 and 8.

$V^{II}4$ cardiotoxin (containing about 0.03% of venom phospholipase A_2) induces, at low concentrations (10^{-7}—10^{-5} M), an aggregation of intramembrane particles which is accompanied by the appearance of excrescences on axonal membrane vesicles at the concentration of about 10^{-4} M (Fig. 6B). At a

still higher cardiotoxin concentration (more than 10^{-4} M), intramembrane particles disappear from the fracture surfaces of the membranes and appear in solution (Fig. 6C). The particle-depleted vesicles fuse very rapidly, giving rise to large liposome-like structures showing smooth fracture faces and somewhat particulated external surfaces, when exposed after deep etching (Fig. 7A). When such preparations are centrifuged, the resulting pellet is composed of highly aggregated particulate material and stacked membranes displaying only smooth fracture faces and unusual fracture properties, since most (if not all) oblique fractures show pairs of membranes (arrow in Fig. 7B). These types of images have been observed in lipid-containing complex lamellar structures, in which two neighbouring lamellae differ by their hydrophobic or hydrophibic layers [17].

The images corresponding to the kinetic studies of the morphological changes induced by high concentration of $V^{II}4$ cardiotoxin containing about 0.03% of phospholipase A_2 on axonal membrane vesicles are shown in Fig. 8. Almost immediately after mixing, the fracture faces of membrane vesicles are covered with randomly distributed ripples. A few minutes later, the membrane vesicles undergo further changes leading to the appearance of many excrescences (Figs. 8C and D). These excrescences disappear later, with concomitant extrusion of intramembrane particles into the solution (Fig. 8E). All these results can be interpreted on the basis of combined action of cardiotoxin and phospholipase A_2 (see Discussion).

Discussion

Crab axonal membrane preparations are particularly well suited for freeze-fracture morphological studies since they are composed of medium-sized vesicles (mean diameter of about 0.3 μm), which show well-separated, randomly distributed intramembrane particles of different sizes. We used this preparation to study the morphological changes induced by many different toxins (neurotoxins and cardiotoxins), and saw that most toxins induce visible morphological perturbations of the membrane. The most pronounced changes were induced by cardiotoxins. The results are all in favor of a direct interaction of cardiotoxins with membrane lipids. The morphological changes induced by cardiotoxins ($V^{II}1$ and $V^{II}4$) on membranes and on lipid vesicles are very similar. In both cases aggregation and fusion of vesicles is observed with $V^{II}4$ cardiotoxin while with $V^{II}1$ cardiotoxin additional changes of both, membranes (Fig. 2D) and lipids (Fig. 5) occur.

Some understanding of the mechanism of cardiotoxin action on membranes can be obtained from the study of its interaction with membrane lipid vesicles. The images of gradual morphological changes induced by $V^{II}1$ cardiotoxin (Figs. 4 and 5) allow the definition of different steps in the transition from liposomes to highly ordered, probably hexagonal H_{II}, structure. The fact that this transition is very slow (30 min to many hours) and never complete (the liposomes are always seen together with new structures) suggests that this liposome hexagonal H_{II} phase-transition is probably due to the segregation of different lipid species into separate domains. Cardiotoxin may be involved in a gradual removal of acidic lipids from the original liposomes into cardiotoxin

lipid associations displaying a different structure from the remaining lipids. In the case of intact axonal membranes, cardiotoxin may produce the rearrangement of membrane lipids by inducing the segregation of acidic lipids, which may lead to perturbations of membrane function and morphology. The morphological changes observed immediately after the mixing of axonal membranes with cardiotoxin (rippled fracture surface in Fig. 8) may well correspond to such rearrangements. In the latter case, however, small amounts of venom phospholipase A_2 are also present and, under those conditions, the images could be due to the combined action of cardiotoxin and phospholipase. This combined action of cardiotoxin and phospholipase A_2 leads, indeed, to very spectacular morphological changes of membranes (Figs. 6, 7 and 8).

The most interesting aspect of these changes is, in our opinion, the extrusion of the intra-membrane particles from the membranes into the solution. This can be interpreted as being due to the removal of intrinsic membrane proteins from the membrane in the form of association with some lysolipids, obtained by action of phospholipase A_2. The action of cardiotoxin may be to protect part of the membrane lipids against the action of phospholipase A_2, preventing the complete disintregation of membrane vesicles. This hypothesis is not in contradiction with the known property of cardiotoxin to enhance the phospholipase A_2 activity on the membranes [7,18], since its action is probably inducing the rearrangement of membrane lipids within the membrane in such a way that some lipids may become more sensitive to the phospholipase action and others, on the contrary, may become protected. The cardiotoxin molecules are probably firmly bound only to the external surface of membrane vesicles. This conclusion is based on the images obtained after centrifugation of cardiotoxin-treated membranes (fractures involving pairs of membranes, Fig. 7B). After centrifugation, the stacking of assymetric vesicles leads, indeed, to the existence of two different polar layers on each side of a given membrane: in our case, one with and another without cardiotoxin.

The fracture properties of these two different layers being different, the final result is that for all oblique fractures the resistance for crossing each polar layer will be different, leading, in consequence, to differences in the extent of fractures of two adjacent membranes (see also the discussion in Ref. 17).

The above presented results on combined cardiotoxin-phospholipase A_2 action may be of interest for better understanding of the mechanism of solubilization of intrinsic membrane proteins by detergent, since they indicate that intrinsic membrane proteins can be removed from the membranes as detergent-protein micelles (in this case lysolipid-protein micelles) without solubilization of all membrane lipids. This last conclusion needs, however, further confirmation by a direct biochemical analysis of the vesicular and particulate materials. In the case of axonal membranes, the preliminary results obtained by SDS-gel electrophoresis showed that after centrifugation of cardiotoxin/phospholipase A_2-treated membranes, both the supernatant (which shows only particles in solution) and the pellet (which shows stacked vesicles and particulate material, Fig. 7B) contain all axonal membrane-bound polypeptides. This last result suggests that cardiotoxin/phospholipase A_2 mixtures can be used for isolation of some intrinsic membrane proteins. We were able, indeed, to isolate in this way well-defined rhodopsin-lysolipid micelles from rod outer segment membranes [21].

Finally, the results obtained on combined cardiotoxin phospholipase A_2 action may be of interest for understanding of the physiological action of the cobra venom itself.

Acknowledgements

We would like to thank J.C. Dedieu for his excellent technical assistance throughout this work. We would also like to thank Drs. M. Fosset and H. Schweitz for the preparations of axonal membranes and cardiotoxins and for many helpful discussions and Dr. L. Aggerbeck for critical reading of the manuscript. This investigation was supported by Délégation Générale à la Recherche Scientifique et Technique, Comité de Membranes Biologiques.

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