Biochimica et Biophysica Acta, 644 (1981) 127–133
Elsevier/North-Holland Biomedical Press

BBA 79265

ISOLATION OF RHODOPSIN BY THE COMBINED ACTION OF CARDIOTOXIN AND PHOSPHOLIPASE A_2 ON ROD OUTER SEGMENT MEMBRANES

EMILIO A. RIVAS, MARC LE MAIRE and TADEUSZ GULIK-KRZYWICKI

Centre de Génétique Moléculaire du C.N.R.S., 91190 Gif-sur-Yvette (France)

(Received December 12th, 1980)

Key words: Cardiotoxin; Phospholipase A_2; Rhodopsin isolation; Membrane protein; (Rod outer segment)

Freeze-fracture electron microscopy was used to follow morphological changes induced by *Naja mossambica mossambica* venom V_4^{II} cardiotoxin in rod outer segment membrane preparations. The extent of the morphological changes depended on the purity of the cardiotoxin. Pure cardiotoxin had no detectable effect upon the preparation, but, when contaminated with venom phospholipase A_2, led to a rapid disintegration of the membrane vesicles. With trace amounts (up to about 0.5% of the cardiotoxin) of phospholipase A_2, the membrane vesicles disintegrated into smooth lamellae and particles in solution. These two components were separated by centrifugation. The pellet, which showed the presence of smooth lamellae and aggregated particles, was composed of unbleached rhodopsin, initial membrane lipids, lysolipids and cardiotoxin. The supernatant, which showed only the presence of dispersed particles, was composed of unbleached rhodopsin, lysolipids and cardiotoxin. With cardiotoxin containing larger amounts of phospholipase A_2 (more than 0.5% of the cardiotoxin), membrane vesicles were disintegrated into large aggregates of amorphous material, composed of bleached rhodopsin, initial membrane lipids, lysolipids and cardiotoxin. These results confirm our previous observation on the release of integral membrane proteins from membrane vesicles by the action of cardiotoxin containing traces of phospholipase A_2 (Gulik-Krzywicki, T., Balerna, M., Vincent, J.P. and Lazdunski, M. (1981) Biochim. Biophys. Acta 643, 101–114) and suggest its possible use for isolation and purification of integral membrane proteins.

Introduction

The actions of cardiotoxins, the principal protein components of cobra venom [2], on cells, membranes and lipids have been extensively studied in recent years [3–9]. Their mechanism of action seems to be quite well understood; they bind rapidly and reversibly to membrane lipids, essentially negatively charged lipids; then, they induce a rearrangement of the membrane structure leading to, among other changes, a marked alteration in the permeability of the cell membrane [10–13]. When the cardiotoxins contain traces of venom phospholipase A_2, more drastic changes of membrane structure occur, and, eventually, complete disintegration of the membrane results when relatively high concentrations of cardiotoxins are used [1].

In our recent study of cardiotoxin action on crab axonal membranes [1], we showed that V_4^{II} cardiotoxin, containing traces of venom phospholipase A_2, produces morphological changes of the membrane which can be interpreted as due to the gradual removal of intrinsic membrane proteins from the membrane vesicles. The main purpose of the present work was to extend the previous investigations to other membranes and to try to isolate and characterize the fractions containing solubilized intrinsic membrane proteins.

We report here the results obtained with rod outer segment membrane preparation, which is particularly suitable for such a study because of its rather simple lipid and protein composition. We show that combined V_4^{II} cardiotoxin-phospholipase A_2 action leads to the disintegration of the membrane

vesicles and that, at very small phospholipase A_2 concentrations, it is possible to isolate from the incubation medium a water-soluble form of unbleached rhodopsin in association with lysolipids and cardiotoxin.

Materials and Methods

Rod outer segment membranes were prepared as described previously [14]. Pure V_4^{II} cardiotoxin containing less than 0.003% of phospholipase A_2 [1] and its mixtures with venom phospholipase A_2 as well as pure phospholipase A_2 were generously provided by Dr. M. Lazdunski. Rod outer segment membranes were treated with cardiotoxin and/or phospholipase in the following way: the fresh or lyophilized rod outer segment membranes (1–4 mg with about 0.6 μmol lipids per mg membrane) were suspended in 1 ml of 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.5), 50 mM NaCl and incubated 1 h at 20°C in the presence or in the absence of phospholipase or cardiotoxin. The amounts of pure phospholipase or cardiotoxin (pure or with phospholipase) added were chosen so that the final molar ratio rod outer segment lipids/phospholipase or rod outer segment lipids/cardiotoxin were about 80/1 or 2–7/1 respectively. In some experiments, the mixtures were then centrifuged for 30 min at $8000 \times g$ to remove large debris (pellet) and the supernatant further analyzed. Lipid extraction from rod outer segment membranes was carried out with a chloroform/methanol mixture (2 : 1 v/v). The lipids were washed twice, according to Folch et al. [15] and concentrated under nitrogen before being analyzed by thin-layer chromatography on silica gel (Merck) using $CH_3Cl/CH_3OH/NH_4OH$ (28%), 65 : 35 : 5 v/v. The lipids were revealed by iodine vapour or by 3% formaldehyde in concentrated H_2SO_4. Phospholipids were identified on the plates after spraying with the Vaskovsky reagent [16]. Lipids containing free amines were detected with ninhydrin. The identity of each phospholipid was established by comparison of its migration distance with those of known standards (egg yolk phosphatidylcholine, prepared according to Singleton et al. [17], phosphatidylserine and phosphatidylethanolamine (Lipid Products)). The presence of lysolipids in cardiotoxin-treated rod outer segment membrane fractions was detected by comparison of their TLC chromatograms with those of cardiotoxin- and/or phospholipase A_2-treated lipid standards. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis according to Weber and Osborn [18], and their concentrations were measured by the method of Lowry et al. [19]. Rhodopsin spectra were recorded on a Carry 14 spectrophotometer after centrifugation as described above.

For freeze-fracture electron microscopy, the samples were prepared in buffers containing 25 to 30% of glycerol. The small drops of preparations were deposited under red light on conventional Balzers gold planchets and rapidly frozen in Freon-22 (at −160°C). Fracturing and replication were done with Balzers BAF 301 freeze-etching unit using platinum carbon shadowing. The replicas, after digestion of organic material with chromic acid and washing with distilled water, were observed in Philips 301 electron microscope.

Results

Rod outer segment membrane preparations are composed of very large membrane vesicles which exhibit on both of their fracture faces rather high concentrations of intramembrane particles (Fig. 1A). These intramembrane particles reveal the presence of rhodopsin, which is the main intrinsic membrane protein present in that membrane [14]. When rod outer segment membranes were treated with pure V_4^{II} cardiotoxin in the presence of 0.1 mM EDTA, to avoid any possible phospholipase activity, no changes of the membrane morphology could be detected, even after prolonged incubations. When the same membrane preparation was incubated with cardiotoxin containing less than 0.5% of phospholipase A_2, in the absence of EDTA, drastic morphological changes of the preparation were observed (Fig. 1B, Fig. 2 and Fig. 3). During the first minutes of incubation the main change was the appearance of particles in solution, followed by the appearance of pieces of lamellae displaying smooth fracture faces (Fig. 1B). After a few hours of incubation the preparation was composed almost exclusively of particulate material and of small pieces of smooth lamellae. When this preparation was centrifuged

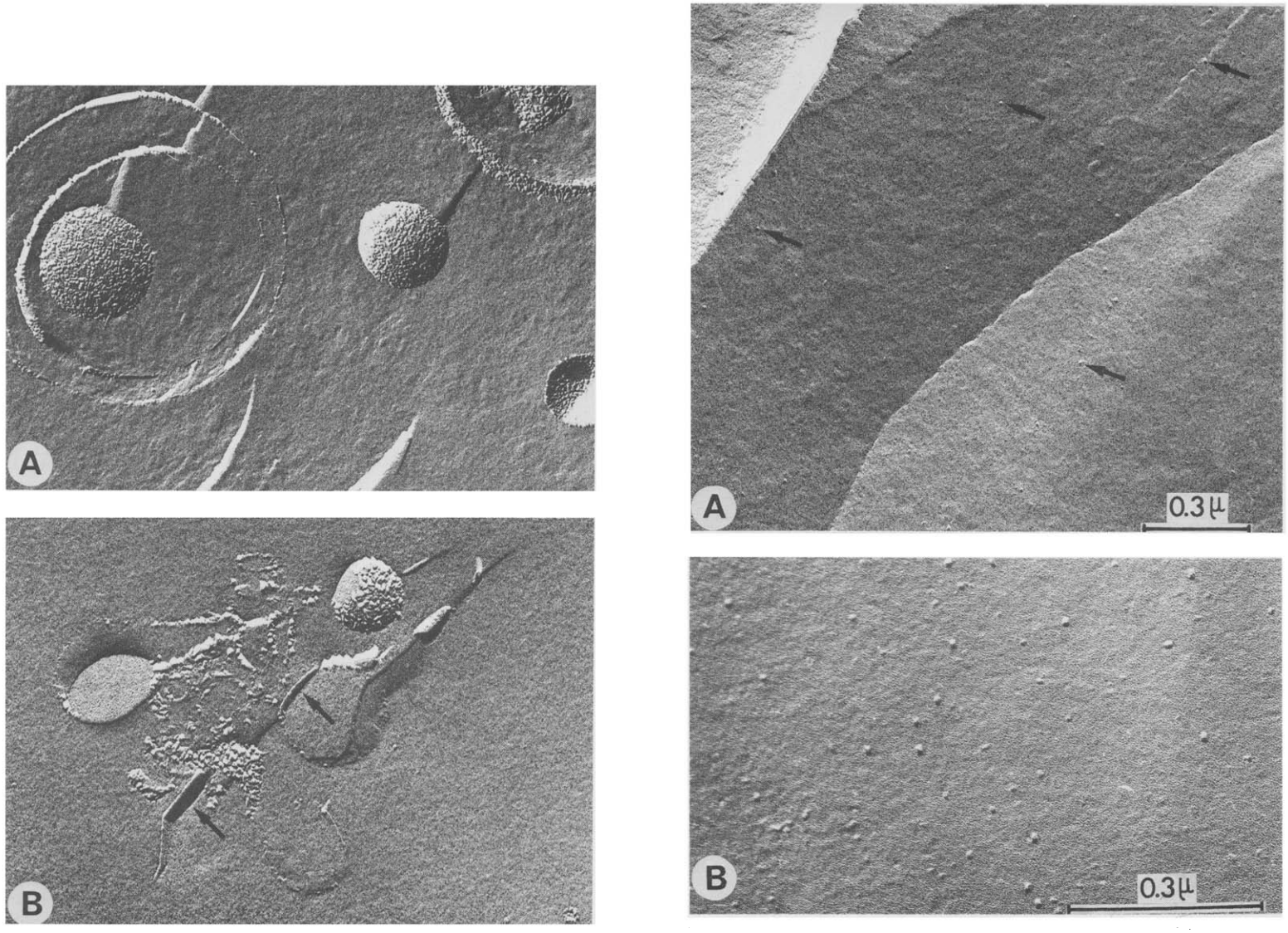
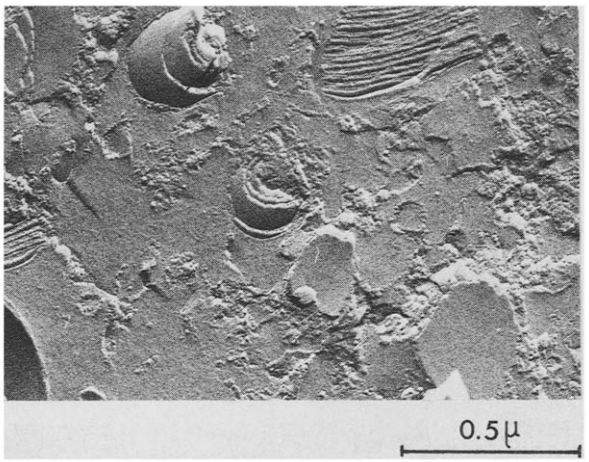


Fig. 1. Freeze-fracture image of rod outer segment membrane preparation (A) and of the same preparation five minutes after the addition of cardiotoxin containing about 0.04% venom phospholipase A_2 (B). Note the presence of large membrane vesicles with densely-packed intramembrane particles in A and the appearance of smooth lamellae (arrows) and of the particles in solution in B.

Fig. 2. Freeze-fracture images of the supernatant obtained after centrifugation of the rod outer segment membranes incubated overnight with cardiotoxin containing about 0.04% venom phospholipase A_2. Note the presence of clearly visible, randomly dispersed, small particles (arrows).



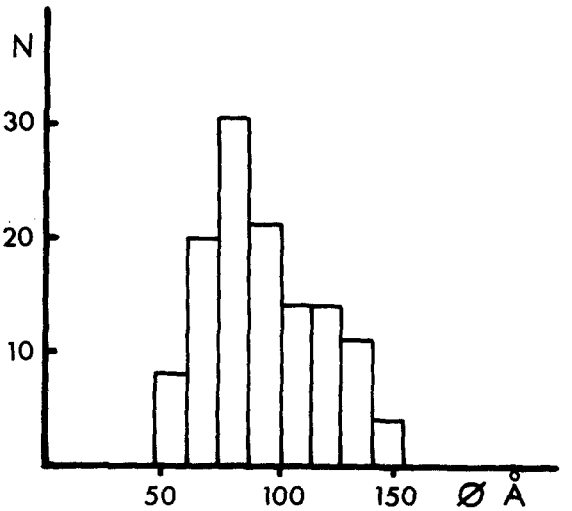


Fig. 3. Freeze-fracture image of the pellet obtained after centrifugation (30 min, 8000 × *g*) of the rod outer segment membranes incubated overnight with cardiotoxin containing about 0.04% cobra venom phospholipase A_2. Note the presence of smooth lamellae and of aggregated, particulate material.

Fig. 4. Size distribution of particles for the preparation shown in Fig. 2. N, the number of particles, ϕ, the dimension of a particle as measured in the direction perpendicular to its shadow. The measurements were done on micrographs similar to those shown in Fig. 2B.

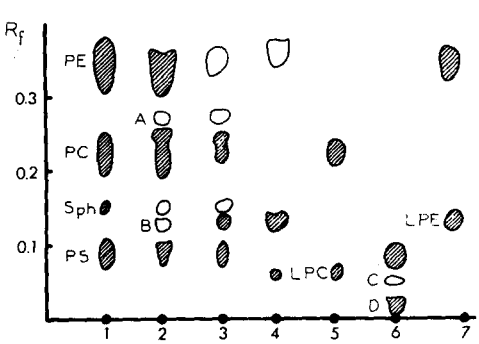
(30 min, 8000 × *g*), the supernatant showed only randomly dispersed small particles (Fig. 2) and the pellet was composed of aggregated particulate material and of small pieces of smooth lamellae (Fig. 3). The distribution of sizes of these particles, as measured in a direction perpendicular to their shadows, is shown in Fig. 4. The mean value of the particle size is close to 85 Å, which corresponds to a Stokes' radius of about 45 Å, according to the calibration curve obtained with proteins of known Stokes' radii [20]. This last value is very close to the previously reported value of Stokes' radius for rhodopsin detergent micelles [14].

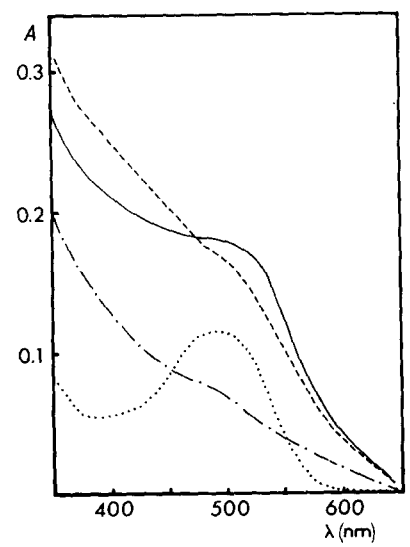
The lipid composition of the supernatant was analyzed only qualitatively. The results are shown in Fig. 5, together with the thin-layer chromatograms of the lipid extracted from native rod outer segment and from the centrifugation pellet. As can be seen from this figure, the main lipid components of the supernatant are lysolipids (and free fatty acids).

As far as the protein composition of the supernatant is concerned, only rhodopsin and cardiotoxin were detected by polyacrylamide gel electrophoresis in the presence of SDS. Rhodopsin is present in its native, unbleached form, as indicated by its absorption spectra shown in Fig. 6.

The cardiotoxin-phospholipase A_2 action described above is not observed in preparations containing more than 0.5% of phospholipase A_2. In the latter case, cardiotoxin-phospholipase A_2 action induces a complete disintegration of the membrane vesicles, leading to the appearance of only highly aggregated, particulate material (Fig. 7A), containing bleached rhodopsin (Fig. 6). The rhodopsin is also bleached by the action of pure bee venom phospholipase A_2 (Fig. 6) but, in contrast to cardiotoxin-phospholipase A_2, its action does not induce the disintegration of the rod outer segment membrane vesicles (Fig. 7B).

Fig. 7. (Right-hand figure.) Freeze-fracture image of rod outer segment membranes incubated overnight with cardiotoxin containing about 5% venom phospholipase A_2 (A) and of the same preparation incubated with bee venom phospholipase A_2. Note the presence of highly aggregated particulate material in A and of densely particulated vesicles in B.





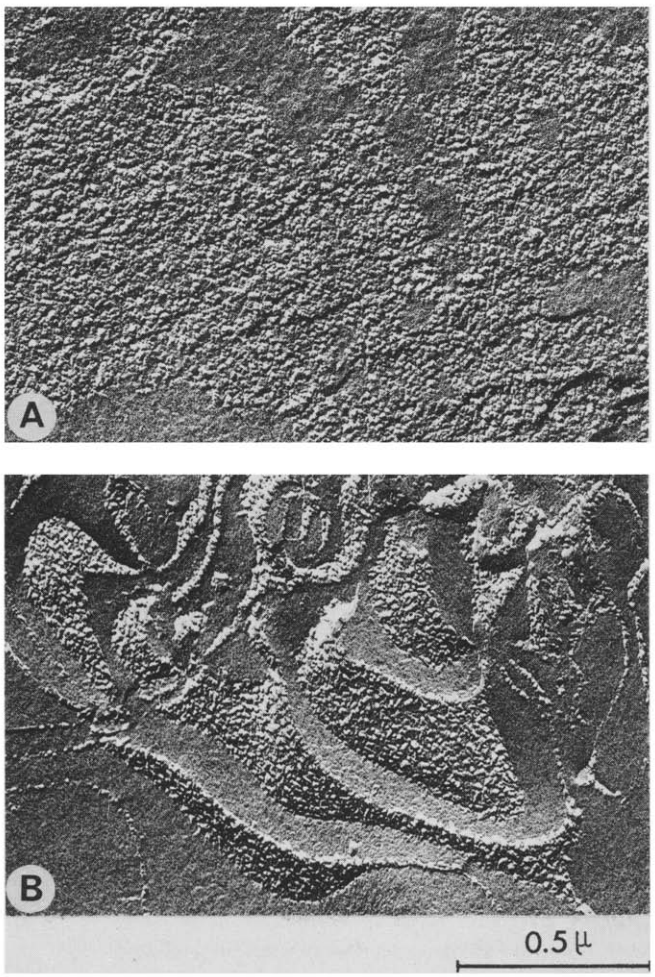


Fig. 5. (Top left.) Effects of cardiotoxin containing 0.04% phospholipase A_2 on the lipid composition of rod outer segment. The lipids were prepared and analyzed qualitatively by thin-layer chromatography on silicagel as described under Methods. The spots with little material are circled but not hatched (average of six experiments). Line 1: standards; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin; PS, phosphatidylserine. Line 2: rod outer segment untreated, A; unidentified, B; lysophosphatidylethanolamine present as a contaminant in some preparations. The three main lipids in rod outer segment are phosphatidylethanolamine (approx. 38%), phosphatidylcholine (approx. 37%), phosphatidylserine (approx. 13%); Sph and others are minor components [21–23]. Line 3 and 4: rod outer segment treated with cardiotoxin containing 0.04% phospholipase A_2 (see Materials and Methods); line 3, pellet; line 4, supernatant. Line 5, 6, and 7: standards (respectively phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine) treated with cardiotoxin containing 0.04% phospholipase A_2. LPC, lysophosphatidylcholine and LPE, lysophosphatidylethanolamine. In line 6, PS interacts with cardiotoxin to give a spot (D) near the origin; another spot (C) appears which could be phosphatidic acid (phosphore positive, ninhydrin negative). The free fatty acids which were observed in lines 4, 5, 6 and 7 are not shown on this figure. Note that in the supernatant of cardiotoxin-treated rod outer segment (line 4) the lysolipids LPE and LPC are the main components.

Fig. 6. (Bottom left.) Absorption spectra of different preparations of rod outer segment membranes. ——— 3.95 mg rod outer segment membrane alone; - - - - - - 3.15 mg rod outer segment membrane plus 0.7 mg bee venom phospholipase A_2; · — · — · 1.05 mg rod outer segment membrane plus 2.5 mg cardiotoxin containing 4% phospholipase A_2; · · · · · · 2.7 mg rod outer segment membrane plus 1.34 mg cardiotoxin containing 0.04% phospholipase A_2. After a 10 min exposure to the 500 nm beam the absorbance at this wavelength was only reduced by 0.5% (i.e. little bleaching). Note the high A values at 350 nm in the first three preparations due to light scattering (non-solubilized membranes or aggregated materials). By contrast note the existence of a peak at 500 nm for the last preparation, reflecting the presence of solubilized intact rhodopsin.

Discussion

In our recent work on the interactions between *Naja mossambica mossambica* venom cardiotoxins and crab axonal membranes [1], we made the observation that cardiotoxins containing traces of venom phospholipase A_2 were capable of extracting intrinsic membrane proteins from that membrane. The main purpose of this work was to characterize further this combined cardiotoxin-phospholipase A_2 action on different membrane preparations. The rod outer segment membrane was chosen because of its simple protein and lipid composition, thus offering the possibility of rapid chemical characterisation of different components in the isolated fractions. The results presented here show that the combined cardiotoxin-phospholipase A_2 action on rod outer segment membranes is quite similar to that observed with axonal membranes. In both cases, indeed, prolonged action led to the disintegration of the membranes and to the appearance of particles in solution, when traces of phospholipase A_2 are present. In the case of rod outer segment membranes, we were able to isolate these particles. The chemical analysis of the solution containing these isolated particles shows the presence of lysolipids (Fig. 5), with rhodopsin and cardiotoxin being the only protein components. The distribution of the sizes of these particles (Fig. 4) is similar to those obtained with pure, standard proteins of known size [20]. If one assumes that the observed particles reflect the presence of rhodopsin lysolipid micelles, then a comparison of their mean size with mean sizes of particles corresponding to standard proteins of known dimensions gives a Stokes' radius of about 45 Å for the rhodopsin-lysolipid micelle (see the calibration curve in Ref. 20). This value is quite close to the Stokes' radius of 49 Å reported for rhodopsin detergent micelles [14]. It is probable, then, that the observed particles do in fact correspond to rhodopsin-lysolipid micelles, but complete characterization will require more quantitative biochemical and morphological work. We believe that these results may be of some interest for the understanding of the mechanism of combined cobra venom cardiotoxin-phospholipase A_2 action on biological membranes. The action of cardiotoxin may be to protect some of the membrane lipids against the action of phospholipase A_2, preventing the complete disintegration of the membrane. This hypothesis is not in contradiction with the known property of cardiotoxin of enhancing the phospholipase A_2 activity on the membranes [6,24], 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. As far as the solubilization of membrane proteins is concerned, much more work is needed for better characterization of the isolated complexes and, in particular, their comparison with detergent solubilized proteins, in order to decide if the cardiotoxin-phospholipase A_2-induced solubilization can be of any use for purification and physicochemical characterization of intrinsic membrane proteins.

Acknowledgements

We would like to thank Dr. M. Lazdunski for the gift of the cardiotoxin and phospholipase A_2 used in this work, Dr. L. Aggerbeck for many fruitful discussions and help in preparing this manuscript and J.C. Dedieu for his excellent technical assistance throughout this work. This investigation was supported by Délégation Générale à la Recherche Scientifique et Technique, Comité de Membranes Biologiques.

References

1 Gulik-Krzywicki, T., Balerna, M., Vincent, J.P. and Lazdunski, M. (1981) Biochim. Biophys. Acta 643, 101–114
2 Lee, C.Y., Lin, J.S. and Wei, J.W. (1971) in Toxins of Animal and Plant Origin (De Vries, A. and Kochwa, E., eds.), pp. 307–317, Gordon and Breach, New York
3 Lee, C.Y. (1972) Annu. Rev. Pharmacol. 12, 265–286
4 Louw, A.I. (1974) Biochim. Biophys. Acta 336, 470–480
5 Condrea, E. (1974) Experientia 30, 121–129
6 Louw, A.I. and Visser, L. (1977) Biochim. Biophys. Acta 498, 143–153
7 Dimari, S.J., Lembock, K.J. and Chatman, V.B. (1975) Biochim. Biophys. Acta 393, 320–334
8 Lin Shian, S.Y. and Lee, C.Y. (1977) Biochem. J. 161, 229–232
9 Vincent, J.P., Schweitz, H., Chicheportiche, R., Fosset, M., Balerna, M., Lenoir, M.C. and Lazdunski, M. (1976) Biochemistry 15, 3171–3175

10 Vincent, J.P., Balerna, M. and Lazdunski, M. (1978) FEBS Lett. 85, 103–108
11 Dufourcq, J. and Faucon, J.P. (1978) Biochemistry 17, 1170–1176
12 Zaheer, A., Noronha, S.H., Hospathankar, A.V. and Braganca, B.B. (1975) Biochim. Biophys. Acta 394, 293–303
13 Faucon, J.F., Dufourcq, J., Courtaud, F. and Rochat, H. (1979) Biochim. Biophys. Acta 554, 332–339
14 Osborn, H.B., Sardet, Ch. and Helenius, A. (1974) Eur. J. Biochem. 44, 383–390
15 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
16 Vaskovsky, V.E. and Kostetsky, E.Y. (1968) J. Lipid Res., 9, 396
17 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil. Chem. Soc., 42, 53–56
18 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
20 Le Maire, M., Møller, J.V. and Gulik-Krzywicki, T. (1981) Biochim. Biophys. Acta 643, 115–125
21 Anderson, R.E. and Maude, M.B. (1970) Biochemistry 9, 3624–3628
22 Borggreven, J.M.P.M., Daemen, F.J.M. and Bonting, S.L. (1970) Biochim. Biophys. Acta 202, 374–381
23 Nielsen, N.C., Fleischer, S. and McConnell, D.G. (1970) Biochim. Biophys. Acta 211, 10–19
24 Louw, A.I. and Visser, L. (1978) Biochim. Biophys. Acta 512, 163–171