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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

XXXVII. EVIDENCE FOR LATERAL AGGREGATION OF RHODOPSIN MOLECULES IN PHOSPHOLIPASE C-TREATED BOVINE PHOTORECEPTOR MEMBRANES

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Summary

Photoreceptor membranes derived from isolated bovine rod outer segments, are subjected to treatment with phospholipase C (*Bacillus cereus*). This results in varying degrees of hydrolysis of the membrane phospholipids into diglycerides and water soluble phosphate esters without loss of rhodopsin. Electron microscopic observations of thin sections and freeze-fractured preparations indicate extrusion of diglycerides from the membranes and their coalescence to lipid droplets, beginning at 20% hydrolysis of phospholipids. After 90% hydrolysis of phospholipids membranous structures are still present. The rhodopsin is located in these structures, presumably in the form of two-dimensional lateral aggregates. This explains the cross-fracturing of the membranous structures, regularly observed upon freeze-fracturing of the phospholipase-treated photoreceptor membranes.

Introduction

Phospholipase treatment of the photoreceptor membrane of bovine rod outer segments has previously enabled us to demonstrate that the presence of phospholipids, including the amino group containing phospholipids, is not essential for the maintenance of the typical absorbance spectrum of rhodopsin [1,2]. On the other hand, phospholipase C treatment of these membranes renders rhodopsin susceptible to more complete proteolytic degradation, involving loss of 500 nm absorbance [3]. This suggests that phospholipase treatment, while not affecting the rhodopsin molecule internally, can alter the

microenvironment of the rhodopsin molecule.

In this and the following paper we have studied in more detail the effects of phospholipase C treatment on the microenvironment of rhodopsin and the resulting changes in its properties. Here we report the results of an electron-microscopic study of the morphology of the photoreceptor membrane of bovine rod outer segments before and after treatment with phospholipase C from *Bacillus cereus*.

Methods and Materials

Rod outer segments are isolated according to de Grip et al. [4]. The rod outer segments-containing band is removed from the sucrose gradient and is diluted with an equal volume 0.1 M Tris · HCl (pH 7.2), added dropwise. After centrifugation (4°C, 30 min, 8000 rev./min, rotor SS34, Sorvall RC2B), the resulting sediment is twice washed with 0.05 M Tris-maleate (pH 7.0) under identical sedimentation conditions and is finally resuspended in 0.1 M Tris-maleate (pH 7.0). This procedure results in rod outer segments with a molar phospholipid/rhodopsin ratio of 60—70, which show only relatively mild lysis (Fig. 1).

Phospholipase C (*B. cereus*, EC 3.1.4.3) is isolated essentially according to the method of Otnaess et al. [5] and is free of proteolytic activity.

A fresh outer segment suspension is incubated with 0.25 units of phospholipase C per nmol rhodopsin during 1 h at room temperature under mild agitation. During this treatment about 90% of the membrane phospholipids is hydrolyzed to diglycerides and water-soluble phosphate esters, as measured by phosphate determinations in the supernatant [6]. Residual phospholipid consists mainly of phosphatidylserine. Rhodopsin remains spectrally completely intact. Intermediate degrees of phospholipid hydrolysis are obtained by shorter incubation periods, after which the enzyme activity is inhibited by addition of 10 mM EDTA and cooling to 0°C.

For electronmicroscopic observation the membranes are sedimented (4°C, 30 min, 8000 rev./min, rotor SS34, Sorvall RC2B). For thin sectioning, the pellets of treated and untreated preparations are fixed in 2% glutaraldehyde in cacodylate buffer (pH 7.2) and then post-fixed in 1% osmium tetroxyde. After dehydration the samples are embedded in Vestopal W. Thin sections are stained with uranylacetate and lead citrate and then examined in a Philips 300 or 301 electronmicroscope.

For the freeze-fracturing technique, small drops of pellets of enzyme-treated or untreated photoreceptor membranes are placed on gold discs. Some of these preparations are fixed in a solution of glutaraldehyde (1%) and paraformaldehyde (1%) in cacodylate buffer [7], impregnated with 10% glycerol for 30 min and then rapidly frozen in melting Freon 22. Others are frozen immediately. No morphological differences could be detected between fixed or unfixed preparations. The samples are stored in liquid nitrogen. Fracturing and replication is performed in a Balzers apparatus BA 360M or BAF 300 at —150°C. An electron gun system is used for the evaporation of platinum and carbon. The preparations are examined in a Philips 300 or 301 electronmicroscope.

Results and Discussion

Observation of thin sections of phospholipase C-treated photoreceptor membranes reveals electron dense droplets in close contact with clustered membranous profiles (Fig. 2), while untreated preparations are completely free of this material (fig. 1). These droplets very likely represent diglycerides, resulting from the enzymatic phospholipid hydrolysis. They closely resemble the droplets resulting from phospholipase C treatment of erythrocyte ghosts [8—13]. Biochemical analysis of the latter droplets, isolated by density gradient centrifiguation, indicates that they are rich in neutral lipids [9,10]. Apparently, the reduction of the amphipathic character of the lipids through removal of the polar head groups results in their extrusion from the membrane. The diglycerides formed in one membrane, possibly together with those of proximate membranes, coalesce to spherical structures (droplets) with the least possible exposure to the aqueous phase. The droplets are partially in contact with residual membranous structures. The process of extrusion and coalescence may start at a critical concentration of diglycerides in the membrane. In membranes where about 20% of the phospholipids have been hydrolyzed droplets begin to be visible, whereas after 40% hydrolysis of the phospholipids distinct droplets are invariably present. Upon further hydrolysis there is a tendency towards an increase in the size of the droplets.

The size and shape of the vesicular membranous structures are too variable to allow estimation of the reduction in surface area upon extrusion of the diglycerides. Therefore, our assumption that nearly all diglycerides are gathered in the droplets is only based on the analogy to erythrocyte membranes.

Replicas of freeze-fractured phospholipase C-treated membranes show droplets of similar size and frequency as found in thin sections (Figs. 2 and 4). There is some indication of a regular (layered) structural organization within these droplets (Fig. 6). However, no inclusions are seen with the dimensions of the intramembranous particles (probably rhodopsin) visible on fracture faces of untreated preparations (PF in Fig. 3) and on the edges of cross-fractured phospholipase C-treated membranes (Figs. 4 and 6). Thus the enzymatic hydrolysis of the phospholipids into diglycerides and water-soluble phosphate esters appears to result in the effective removal of phospholipids from the membranous structure.

Consequently all rhodopsin molecules must reside in the residual membranous structures, which are visible in thin sections of phospholpase C-treated membranes, even when 90% of the phospholipids are hydrolyzed (Fig. 2). These structures seem to be more dilated and fragmented than those in untreated preparations. The triple-layer aspect is more pronounced in the lipid depleted membranes than in the native membranes, which is inidicative of an increase in the thickness of the membrane elements (insets in Figs. 1 and 2).

This is a remarkable observation. The bilayer, which normally comprises the rhodopsin molecules, would be expected to be absent when after 90% hydrolysis only about 7 phospholipid molecules remain per rhodopsin molecule. This number of phospholipid molecules per rhodopsin molecule is too small to allow the phospholipids to surround each rhodopsin molecule completely in the form of a bilayered annulus. Apparently the loss of hydrophobic shielding

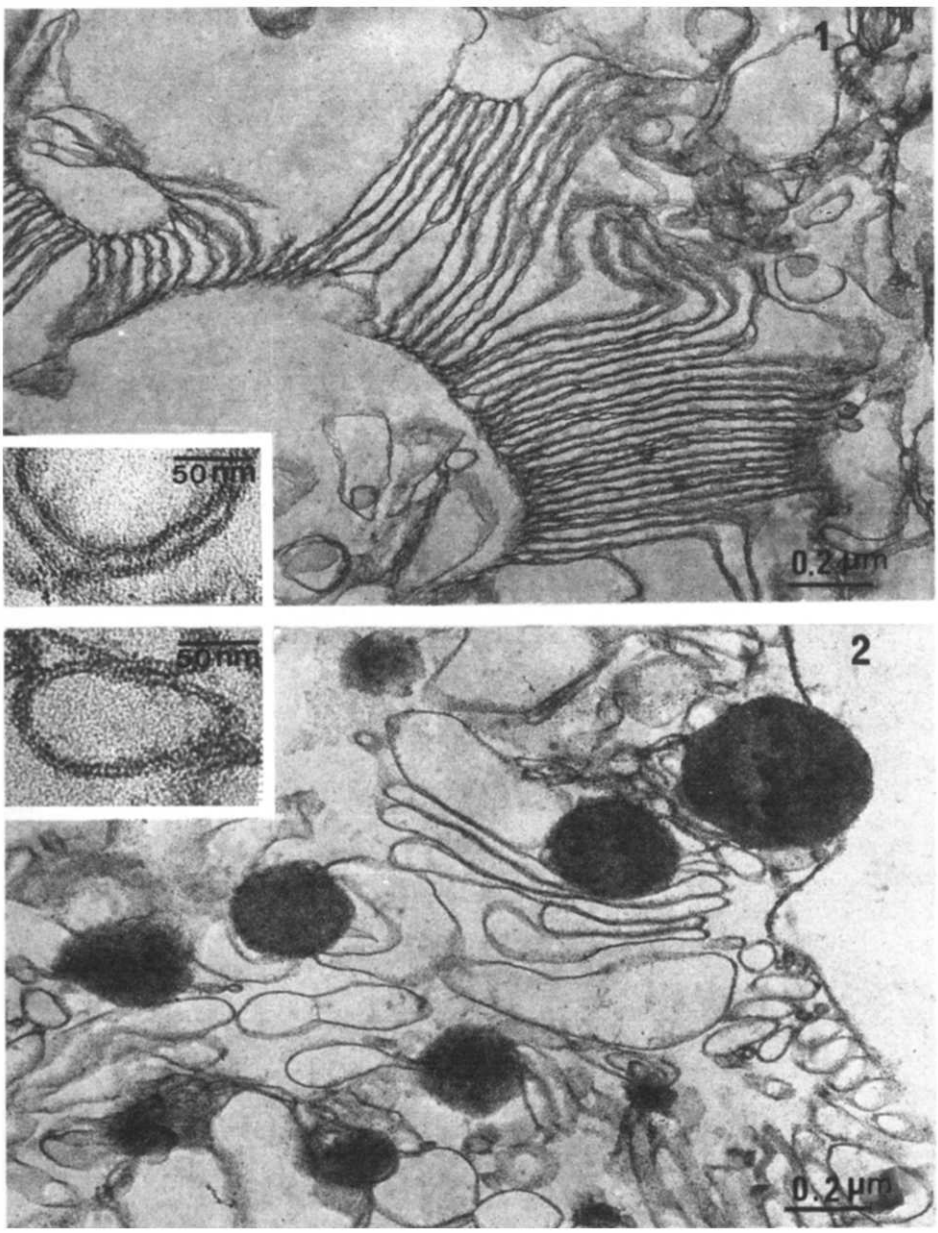

Fig. 1. Thin section of isolated rod outer segments, exhibiting mild lysis. The triple layered aspect is not very pronounced (insert showing two membranes).

Fig. 2. Thin section of phospholipase C-treated rod outer segments after hydrolysis of about 90% of the phospholipids. The triple layered aspect is clearly observable (insert).

is compensated by lateral contacts between the hydrophobic surfaces of the protein molecules, rather than by a breaking up of their two-dimensional arrangement. Electrophoretic analysis of the photoreceptor membrane preparations confirms the earlier finding that rhodopsin accounts for at least 85%

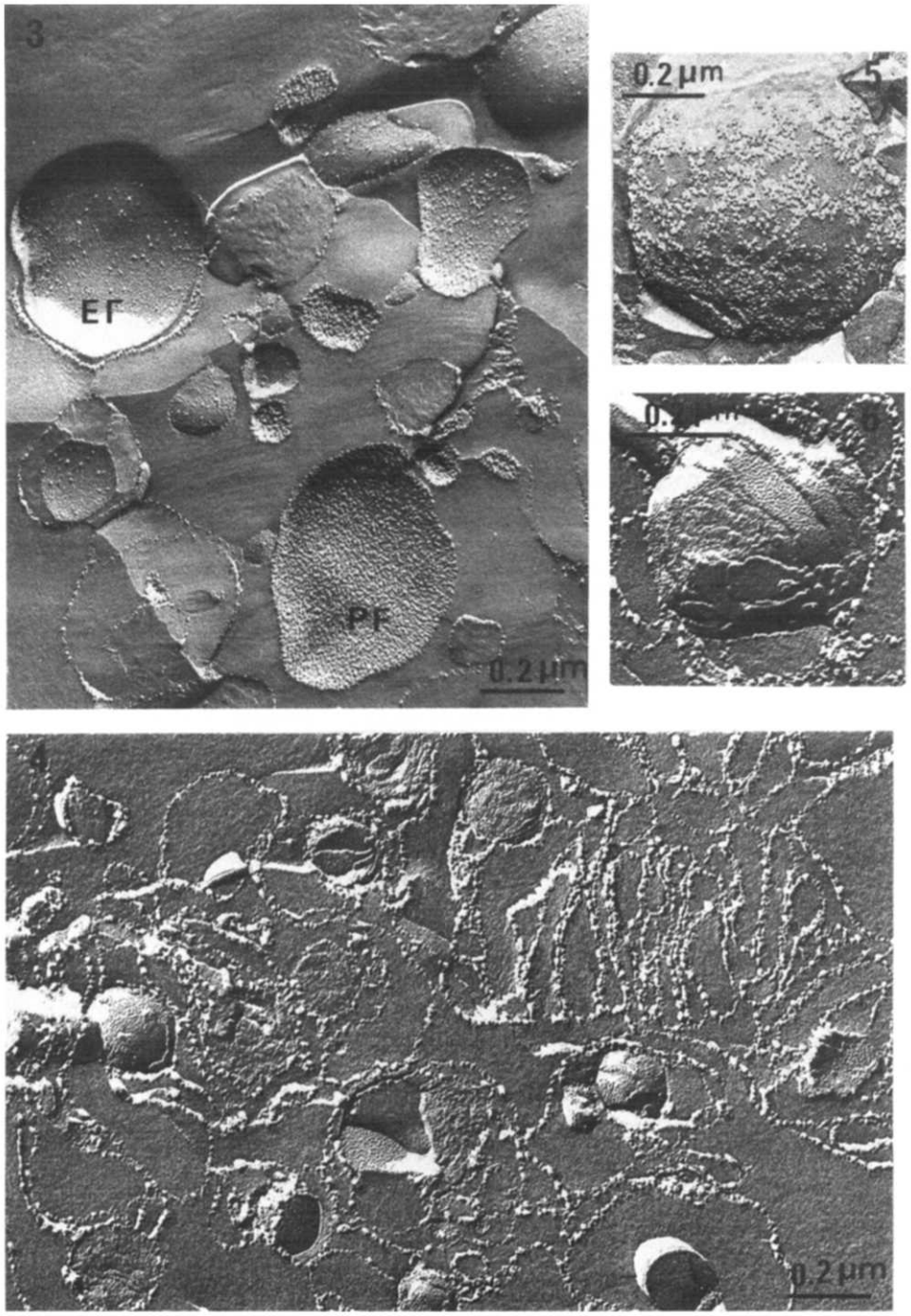

Fig. 3. Freeze-fracture replica of native photoreceptor membranes. PF refers to what is probably the cytoplasmic fracture face of the membrane (cf. Olive and Benedetti [22], Corless et al. [23]). EF indicates the rather smooth intradiscal fracture face of the membrane. Nomenclature according to Branton et al. [24].

Fig. 4, 5 and 6. Freeze-fracture replicas of phospholipase C-treated photoreceptor membranes. The preparations are fixed and impregnated with glycerol prior to freezing (see Materials and Methods). Fig. 4 represents a preparation in which about 90% of the phospholipids is enzymatically hydrolyzed. Fig. 5 shows an example of one of the rare cases in which a phospholipase C-treated membrane is laterally fractured (40% phospholipid hydrolysis). In those cases segregation of proteins and lipids is invariably observed. Fig. 6 depicts a lipid droplet in a preparation as presented in Fig. 4.

of all protein present [14]. Hence, direct rhodopsin to rhodopsin approximation must have occurred in the membranous structures remaining after 90% hydrolysis of the phospholipids. We shall use the term 'lateral aggregation' for this process.

This view seems to be supported by the observation that freeze-cleavage of preparations, subjected to extensive phospholipid hydrolysis, invariably leads to cross-fracturing of the membranous structures (Fig. 4) and to appearance of rhodopsin particles at the edges of the cross-fractured structures (Figs. 4 and 6). This is in contrast to untreated membranes, which are usually laterally fractured, i.e., along their internal hydrophobic core (Fig. 3). It has been reported that cross-fracturing of both artificial phospholipid bilayers and biomembranes is favoured when less lateral interaction occurs between the fatty acyl chain [15—17]. These observations indicate that in our case a lipid bilayer arrangement does no longer determine the nature of the membrane cleavage, as is also observed for phospholipase C-treated erythrocyte membranes [12]. An increase of cross-fracturing as a result of cross-linking between lipids and proteins due to fixation [18] is very unlikely, since we do not see any difference in cross-fracturing between fixed and unfixed preparations, either in native photoreceptor membranes [19] or in phospholipase C-treated membranes.

Even at 40% hydrolysis of the phospholipids, lateral fracturing is rather infrequent, but when it does occur, segregation between lipids and protein particles is observed (Fig. 5). Apparently, a more or less random distribution is no longer maintained in this case, which may or may not be related to the increase in particle size that seems to accompany increasing levels of phospholipid hydrolysis. Segregation is a well known phenomenon and may result from temperature-sensitive lateral phase separations, as shown for instance for rhodopsin in artificial membranes [20]. Clustering of intramembranous particles may however also be initiated by proteolytic and by lipolytic treatment of biological membranes [12,21]. Apparently any perturbation of membrane components can affect the interrelation between (certain) lipids and proteins, giving rise to an aversion to (specific) lipids and consequent clustering of the proteins. However, lateral fracturing is observed too rarely to justify the conclusion that segregation and increase of particle size invariably occur at intermediate levels of phospholipid hydrolysis.

Nevertheless, even if random dispersion of the rhodopsin particles were maintained during diglyceride formation, the conversion of 90% of all phospholipids must involve the formation of two-dimensional (lateral) aggregates of rhodopsin molecules, since all rhodopsin molecules are confined to the residual membranous structures.

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