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REVERSIBLE MODULATION OF RHODOPSIN PHOTOLYSIS IN PURE PHOSPHATIDYLSERINE MEMBRANES

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Bovine rhodopsin is reconstituted into artificial membranes of bovine spinal cord phosphatidylserine (PS). The influence of cations (La^{3+}, Ca^{2+}, Mn^{2+}, Mg^{2+}) and variation in lipid/protein ratio (100:1; 30:1; 10:1) on structure and photolytic behaviour (in particular the metarhodopsin I → II transition) of these reconstituted preparations is investigated. For comparison, rhodopsin reconstituted in endogenous photoreceptor membrane lipids has been studied under similar conditions. The following observations have been made: (1) Photolytic behaviour or structure of rhodopsin reconstituted in endogenous lipids is not affected by the cations studied. Lowering the lipid/protein ratio below 30, however, markedly affects the metarhodopsin I → II transition. Decreasing the lipid/protein ratio then simultaneously increases the percentage of very slowly decaying metarhodopsin I. (2) In the absence of cations, rhodopsin reconstituted in PS shows normal photolytic behaviour. However, lowering the lipid/protein ratio has much less influence on the metarhodopsin I → II transition than incase of endogenous lipids. (3) At the high lipid/rhodopsin ratio (100:1), Ca^{2+} induces a phase separation in preparations reconstituted in PS and formation of so-called 'cochleate cylinders', a highly condensed Ca^{2+}-PS phase. A concomitant effect on the metarhodopsin I → II transition rate is observed. This effect is reasonably specific for Ca^{2+} ($Ca^{2+} \gg La^{3+} > Mn^{2+} - Mg^{2+}$) and is completely reversed by incubation with EDTA. (4) At the lower lipid/rhodopsin ratios, Ca^{2+} affects the structure and photolytic behaviour of rhodopsin reconstituted in PS only slightly. Now, only La^{3+} shows any significant effect ($La^{3+} \gg Mn^{2+} > Ca^{2+} > Mg^{2+}$). (5) We conclude that interaction of PS with rhodopsin has a strong electrostatic component. Solvation of rhodopsin in the lipid bilayer is enhanced by PS relative to the average endogenous lipid and consequently PS minimizes protein-protein contacts, even at low lipid volumes.

Introduction

The rod photoreceptor membrane, primary site of light absorption through the visual pigment rhodopsin, is one of the most unsaturated membranes of the vertebrate organism. Up to 50% of its phospholipid acyl chains are polyunsaturated [1,2]. At this moment, it is unclear which process requires this high degree of unsaturation. Although two known functional properties of rhodopsin are expressed only in the presence of unsaturated phospholipids, one 'natural' double bond per acyl chain is sufficient for full activity [3,4]. Upon reconstitution of bovine rhodopsin in saturated phospholipids, the rate of the metarhodopsin I → metarhodopsin II transition in its photolytic sequence is slowed down at least 3 orders of magnitude [3]. In addition, and probably related, no light-induced activation of the rod cGMP phosphodiesterase through the GTP-binding protein is observed in such systems [5]. These phenomena are probably due to the increased order of more

Abbreviations: PS, phosphatidylserine; Pipes, 1,4-piperazinediethanesulphonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

saturated bilayer membranes. In a more rigid environment, the metarhodopsin I → II transition, which is probably accompanied by a transient increase in volume [6], appears to become thermodynamically and/or kinetically much more unfavourable. This is observed not only in saturated phospholipid bilayers, but also in lipid-depleted rhodopsin [7] and in photoreceptor membranes which are 'artificially' rigidified by dehydration or glutaraldehyde fixation [8]. Reversible modulation of this metarhodopsin I → II transition in one system has not been demonstrated so far.

Phosphatidylserine bilayers are well known to undergo a dramatic change in properties upon binding of certain bivalent cations like Ca^{2+} [9,10], comprising shrinkage of the bilayer with considerable rigidification and conversion of vesicles or liposomal structures into tightly packed cylinder-like structures ('cochleate cylinders' [9]). These transformations are completely reversible upon 'recapture' of the cation with a suitable chelating agent. Hence, this system seems an interesting environment in which to study the above-mentioned rhodopsin parameters. We therefore investigated structure and photolytic behaviour of rhodopsin reconstituted in phosphatidylserine with respect to the effect of Ca^{2+} and of lipid/rhodopsin ratio. The results indicate that addition of calcium indeed induces phenomena similar to those observed for pure phosphatidylserine bilayers, leading to a phase separation with a concomitant slowing down of the metarhodopsin I → II transition, but only at higher lipid/protein ratios (at least 60 : 1). At low ratios (no more than 30 : 1), calcium produces no marked effects on either structure or photolytic behaviour preparations reconstituted in PS. Apparently, calcium is not able to break up strong rhodopsin-phosphatidylserine interactions. Possible implications of these observations are discussed.

Materials and Methods

Isolation and purification of rhodopsin

The procedures for isolation of bovine rod outer segments, purification of rhodopsin and reconstitution of rhodopsin in lipids are described in detail elsewhere [11]. Except for electron microscopy, experiments are performed under dim red light or in darkness and under nitrogen. In short, bovine eyes were obtained from a local slaughterhouse and dissected within 2 h after slaughtering. Following mild homogenization of the retinas, the homogenate was filtered through nylon gauze (125 mesh) and the filtrate layered on top of a continuous sucrose gradient (23–36% (w/w) sucrose). The rod outer segments isolated after centrifugation for 1.5 h at $100\,000 \times g$ and 10°C were lysed by three washings with water, yielding a pellet of photoreceptor membranes. The final yield was 15–20 nmol of rhodopsin per retina, with an A_{280}/A_{500} ratio of 2.0–2.2. The opsin content was always less than 10%, usually less than 5%. For purification of rhodopsin, photoreceptor membranes were solubilized in detergent buffer (an ice-cold solution of 20 mM Pipes/120 mM NaCl/10 mM KCl/3 mM $CaCl_2$/2 mM $MgCl_2$/0.1 mM EDTA/0.5 mM dithioerythritol (pH 6.5), containing 20 mM nonylglucose (0.6% w/w) as detergent [12]). Rhodopsin was purified by affinity chromatography over Con A-Sepharose (Pharmacia, Uppsala, Sweden), using 0.2 M α-methylmannoside (Sigma, St. Louis, MO, U.S.A.) in the same Pipes buffer as the eluent [13]. If concanavalin A contamination was present in the purified rhodopsin, it was reduced by passage through a Sephadex G-50 column, which at the same time removed α-methylmannoside. The resulting rhodopsin solutions had an A_{280}/A_{500} ratio of between 1.6 and 1.8 and still contained small amounts of concanavalin A (under 1%). These amounts did not interfere with the present study, but could be completely removed by passage over a column containing immobilised antibodies against concanavalin A.

Reconstitution

For reconstitution, both photoreceptor membrane lipids and bovine spinal cord phosphatidylserine (Lipid Products, South Nutfield, U.K.) have been used in this study. The latter was pure by TLC. Its fatty acid composition was checked by GLC and found to consist primarily of 18 : 0 (36%), 18 : 1 (36%), 18 : 3 (10%), 20 : 2 (1%), 20 : 3 (2%), 22 : 5 (1%) and 22 : 6 (2%). This is comparable to egg PC and sufficient to maintain rhodopsin's functionality. Lipids are prepared for reconstitution by dissolving the required amount under

nitrogen in the same ice-cold detergent buffer, using a detergent/lipid ratio of about 10. If the free Ca^{2+} concentration is to be in the μM range, 6 mM EDTA is included in all buffers. The lipid solution is mixed under nitrogen with the rhodopsin solution, incubated for a short time (5–10 min) on a bath sonicator under ice cooling, and kept on ice for 30–60 min. Reconstitution can then be achieved either slowly by dialysis over 24 h against various changes of ice-cold detergent-free buffer, or rapidly by dilution of the rhodopsin/lipid mixture with detergent-free buffer to a final nonylglucose concentration below 3 mM. The first procedure yields large, very heterogeneous multilamellar structures (liposomes), while the latter one yields smaller (50–150 nm diameter) largely monolamellar vesicles (De Grip, W.J., unpublished data). As judged by density centrifugation and electron microscopy, reconstituents are fairly homogeneous with respect to particle distribution. The reconstituents are collected by centrifugation at $100\,000 \times g$ (4°C; 0.5 h for liposomes; overnight for vesicles) washed once and stored at −70°C under nitrogen. The lipid/protein ratio of the sediments were checked by phosphorus [14] and rhodopsin assay and found not to deviate significantly from the ratio originally put together. This indicates that no selective losses occur during reconstitution. Final yields vary between 70 and 95%. The two types of reconstituted preparation give similar results in the present study with respect to photolytic behaviour and calcium response but preparations obtained by dialysis more clearly show structural differences produced by variation in lipid/rhodopsin ratio. All electronmicrographs shown therefore derive from this type of reconstitution.

Electronmicroscopy

Electronmicroscopy is performed on samples fixed in darkness by addition of glutaraldehyde to a final concentration of 2%, and stored in the cold. For thin-sectioning, samples are washed with buffer, postfixed in 1% OsO_4, dehydrated and embedded in Vestopal W. Thin sections are stained with uranylacetate and lead citrate. For freeze-fracturing, small pellets of material are placed on gold discs, rapidly frozen in melting Freon 22 and stored in liquid nitrogen. Fracturing and replication is performed with a Balzers BA 301 at −150°C. For the evaporation of platina and carbon, an electron gun system is used. All preparations are examined in a Philips 300 or 400 electronmicroscope.

Spectroscopy

Spectroscopy is performed on membrane suspensions either in a Pye-Unicam SP-1750-B Ultraviolet-Visible Spectrophotometer, using the front cuvette position in order to minimize loss of scattered light, or in an Aminco Bowman DW-2A spectrophotometer in which an opal glass had been inserted between cuvette and photomultiplier. Spectra are recorded from 650 to 250 nm (Pye Unicam) or vice versa (Aminco Bowman) with a scan speed of 4–5 nm/s and a bandwidth of 0.5 nm. Reconstituted preparations are suspended by vortexing under nitrogen to a final concentration of 10–15 μM in rhodopsin, in 20 mM Pipes (pH 6.5) with or without 1 mM EDTA as required and in addition containing 30% (w/v) sucrose so as to increase its density and prevent rapid sedimentation of larger liposomal material. Temperature varied between 19 and 21°C. The photolytic reaction cascade is initiated by flashing with a Xenon flash-gun equipped with a 530 cut-off filter (Schott OG-530). At various time intervals after the flash, spectra are recorded until a stable pattern is observed (20–40 min). Then a 1 M solution of hydroxylamine, buffered at pH 6.5, is added to a final concentration of 50 mM, after which another spectrum is taken. This allows calculation of the amount of rhodopsin bleached by the flash, since hydroxylamine converts all photolysis intermediates and products into retinaloxime ($\lambda_{max} = 365$ nm) and opsin, but leaves rhodopsin intact [11]. A subsequent total bleach (1 min; 300 W tungsten lamp; same filter) followed by a final recording, then permits calculation of the total amount of rhodopsin originally present.

Results

Photoreceptor membrane lipids

As a control for rhodopsin reconstituted in pure PS, we use preparations reconstituted with endogenous photoreceptor membrane lipids, varying in lipid/rhodopsin ratio. Such preparations are ob-

tained by dissolving photoreceptor membranes in detergent buffer and addition of the required amount of purified rhodopsin, followed by reconstitution via dialysis or dilution as described in the Materials and Methods section. Three phospholipid/protein ratios have been investigated: (1) 62 : 1, i.e., the 'native' ratio, obtained without addition of exogenous rhodopsin; (2) 30 : 1, i.e., slightly more then 20–25 required for one complete lipid annulus per rhodopsin [15]; (3) 10 : 1,

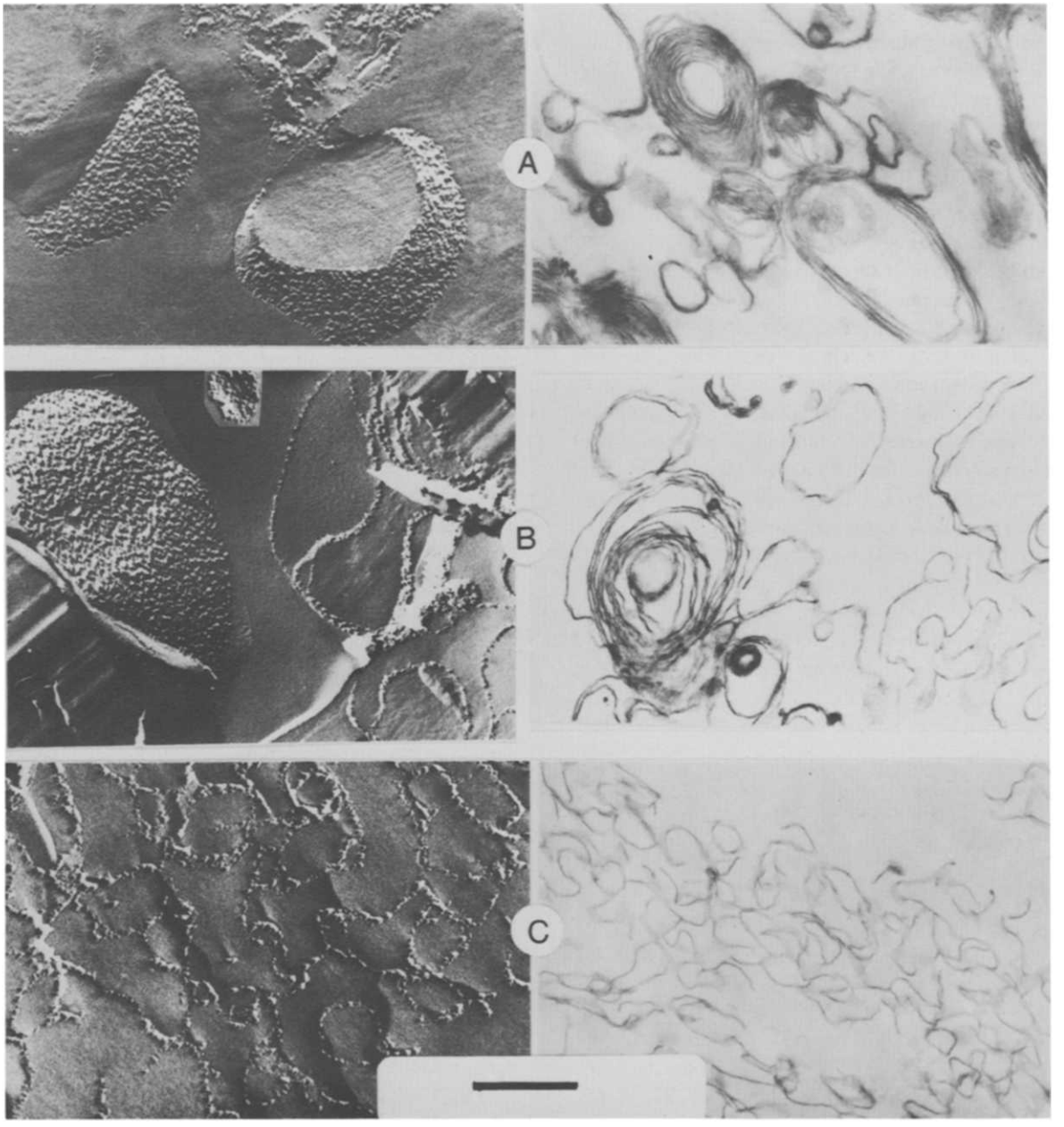

Fig. 1. Electromicrographs of rhodopsin reconstituted in endogenous photoreceptor membrane lipids. Left, freeze-fracture pictures; right, thin sections stained as described in the Materials and Methods section. Phospholipid/rhodopsin ratios: 62 : 1 ("native", A); 30 : 1 (B); 10 : 1 (C). Bar represents 0.5 μm.

i.e., a lipid-poor membrane with just enough lipid for solvation of rhodopsin. The ultrastructural and photolytic properties of these three types of preparations are presented in Figs. 1 and 2. Neither of these properties is affected by changing the Ca^{2+} concentration from the micromolar range up to 20 mM.

Ultrastructure. Reconstituted preparations, obtained from the native condition (62 : 1) by dialysis, form a large-sized multilamellar array of bilayer membranes (liposomes), with a symmetric particle distribution (Fig. 1A). The particle density averages $2700/\mu m^2$ and the particle diameter averages 9.5 nm. Sometimes, short rows of particles are observable. A lower lipid/protein ratio (30 : 1) yields smaller, but still largely oligolamellar structures with little change in average particle size (8.5 nm). The particle density is difficult to estimate. At low lipid/protein ratios (10 : 1), different configurations are observed: sheet-like structures together with monolamellar vesicles, which sometimes seem to fuse (Fig. 1C). Due to their tendency to yield predominantly oblique fracture-faces, particle-density again is difficult to estimate, but the particle size does not differ very much from the two former conditions (8 nm).

Photolytic behaviour. The photolytic behaviour of the 'native-like' preparations (Fig. 2A) is identical to that of the original membrane (not shown, cf. Ref. 4). Metarhodopsin II (λ_{max} 380 nm; spectrum 2) is generated within the 'dead' time of the measurement (approx. 100 ms under special conditions). Metarhodopsin II slowly decays either under liberation of all-*trans*-retinal ($\pm 30\%$) or under conversion into metarhodopsin III (λ_{max} 455 nm; $\pm 70\%$). The latter is formed with first-order kinetics and a half-time of 4.9 ± 0.4 min, again comparable to isolated membranes (4.8 ± 0.3 min). Addition of hydroxylamine converts all photoproducts into retinal oxime ($\lambda_{max} = 365$ nm) and opsin. The remaining band around 490 nm derives from residual rhodopsin and isorhodopsin generated by photoregeneration. From the difference in absorbance at 480 nm between the latter spectrum, corrected for 5% dilution due to addition of hydroxylamine, and spectrum 2, the amount of 'blocked' metarhodopsin I is calculated. The amounts of metarhodopsin I remaining in different preparations relative to the amount of rhodopsin bleached are presented in Table I. Similar to isolated membranes under the pertinent conditions (20°C; pH 6.5), no significant amounts

TABLE I

PHOTOLYTIC BEHAVIOUR OF RHODOPSIN (Rh) IN VARIOUS PREPARATIONS

Average of 2–5 experiments on different preparations ± S.D.

Lipid	Lipid/Rh ratio	M_I/Rh [a]	$\Delta M_{III}/\Delta M_{II}$ [b]	$t_{1/2}(M_{III})$ [d] (min)
Endo-	62	< 0.10	0.79 ± 0.03	4.9 ± 0.4
genous	30	0.21 ± 0.02	– [c]	5.0 ± 0.3
	10	0.50 ± 0.08	– [c]	– [c]
	0	0.90 ± 0.09	–	–
PS	100 (EDTA)	0.06 ± 0.05	0.96 ± 0.07	4.9 ± 0.5
	100 (Ca^{2+})	0.42 ± 0.04	– [c]	– [c]
	30 (EDTA)	0.04 ± 0.02	0.99 ± 0.09	3.8 ± 0.2
	30 (Ca^{2+})	0.08 ± 0.04	0.90 ± 0.04	3.6 ± 0.2
	10 (EDTA)	0.15 ± 0.05	0.80 ± 0.06	3.3 ± 0.2
	10 (Ca^{2+})	0.16 ± 0.04	0.77 ± 0.03	3.3 ± 0.4
Photoreceptor membrane	62	0.03 ± 0.03	0.74 ± 0.06	4.8 ± 0.3

[a] Amount of metarhodopsin I remaining 30 s after bleaching, relative to total amount of rhodopsin bleached.
[b] Amount of metarhodopsin III formed relative to amount of metarhodopsin II decayed.
[c] Not accurately measurable due to slowly decaying metarhodopsin I.
[d] Half-time of the metarhodopsin II → III transition. Note the slight rate increase at lower PS/rhodopsin ratios.

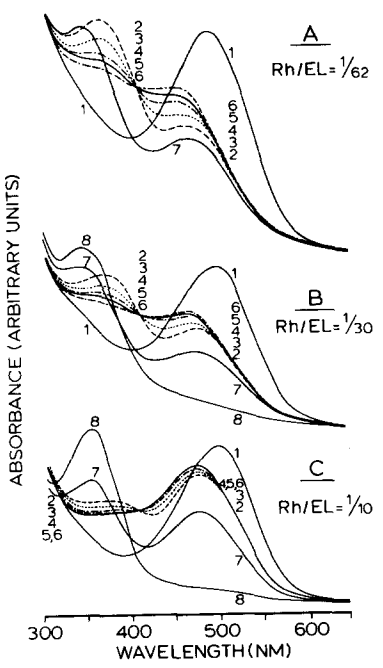

Fig. 2. Photolytic behaviour of rhodopsin reconstituted in endogenous photoreceptor membrane lipids. Phospholipid/rhodopsin ratios: 62:1 (A); 30:1 (B); 10:1 (C). Curve 1 shows the dark spectrum. Spectra 2 (— — —), 3 (· · · · · ·), 4 (– · – · –), 5 (———) and 6 (· – – · – – ·) are recorded respectively 1.1; 5; 10; 15 and 25 min after illumination. Spectrum 7 is recorded after addition of hydroxylamine (final concn. = 50 mM) to spectrum 6. Spectrum 8 is then obtained after a final total bleach. All spectra are normalized at 650 nm.

of blocked metarhodopsin I are observed in 'native-like' reconstituted preparations, However, in the preparation with a 30:1 lipid/rhodopsin ratio part of the rhodopsin becomes blocked already at the metarhodopsin I stage (Fig. 2B, Table I). The remainder passes normally into metarhodopsin II, which in its turn converts with normal kinetics into metarhodopsin III. The blocked metarhodopsin I decays only very slowy (less than 10% in 30 min). Going to the 10:1 ratio, the observed trend is continued: now a large part of the rhodopsin is blocked at the metarhodopsin I stage (Fig. 2C; Table I). This behaviour approaches that of lipid-free rhodopsin, where over 90% of the rhodopsin decays only very slowly beyond metarhodopsin I (not shown, cf. Refs. 4, 7).

Rhodopsin reconstituted in phosphatidylserine

Since the phospholipid/rhodopsin ratios taken for endogenous lipids clearly represent two extremes and an intermediate condition in the photolytic behaviour of rhodopsin, similar ratios are used for reconstitution of rhodopsin in pure phosphatidylserine (30:1 and 10:1), except for the highest one, where no difference was observed between 60- and 100-fold excess of lipid and a ratio of 100:1 has been routinely employed. For Ca^{2+} to be present during reconstitution the normal buffer is used. In subsequent experiments, requiring removal of calcium, EDTA is added in order to keep the free calcium concentration in the micromolar range. When Ca^{2+} has to be absent during reconstitution, 3 mM EDTA is added to the reconstitution buffer, and 3 mM EDTA replaces Ca^{2+} and Mg^{2+} in the dialysis buffer and dilution buffer. Subsequent effects of calcium were tested at different free concentrations: 1 and 10 mM produce similar effects. Magnesium (tested up to 15 mM) could not reproduce any of the calcium effects. No other cations have been tested yet with respect to structural effects.

Ultrastructure. In the presence of EDTA, reconstituted preparations of comparable structure to those obtained with endogenous lipids are formed (Fig. 3). In all cases, the particle size against shows little variability, and is also independent of the dark-light history. The 100:1 ratio results in very large multilamellar liposomes, with a random particle distribution (average: $1600/\mu m^2$). The 30:1 ratio yields a heterogeneous picture, both fairly large oligolamellar vesicles and small monolamellar vesicles being present. At a 10:1 ratio, mainly small monolamellar vesicles are obtained together with sheet-like material. The electromicrographs suggest a homogeneous particle distribution. In all cases, particle size is comparable to preparations reconstituted in endogenous lipids (9.5 nm average).

Under all these conditions addition of Ca^{2+} gives results which are similar to those observed upon reconstitution in the presence of Ca^{2+}. Again, we do not observe any significant effect on particle size. Marked structural changes occur only in the preparations with a 100:1 ratio: the opaque 'cochleate cylinders', known to be induced in pure PS by addition of Ca^{2+} [9], also appear here but are sometimes covered with particles and seem to be interfaced with particle-rich vesicles and patches

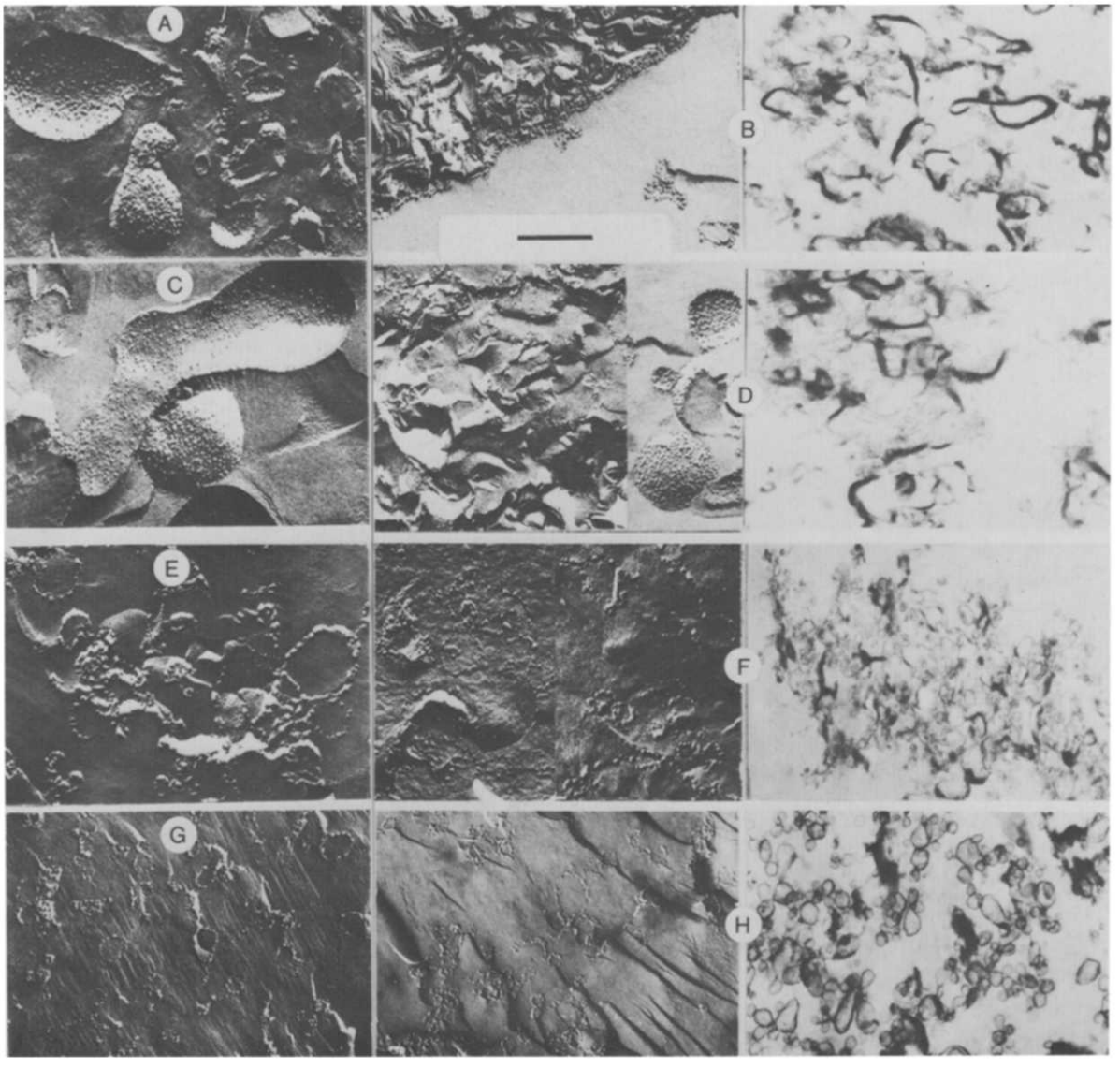

Fig. 3. Electromicrographs of rhodopsin in phosphatidylserine. Thin sections are only shown for the high free-Ca^{2+} conditions. Phospholipid/rhodopsin ratios: 100 : 1 (A, B, C, D); 30 : 1 (E, F); 10 : 1 (G, H). Low free Ca^{2+} conditions are shown in A, C, E and G; Ca^{2+}-rich in B, D, F and H. Conditions A, E and G are obtained by reconstituting rhodopsin in the presence of 3 mM EDTA. Addition of 12 mM Ca^{2+} to these reconstitutes yields B, F and H, respectively. Condition D is obtained by reconstituting rhodopsin in the presence of 3 mM Ca^{2+}. Addition of 10 mM EDTA to this reconstitute yields C. Bar represents 0.5 μm, except for freeze-fracture pictures E and F where it represents 1 μm.

(Fig. 3B). In the 30 : 1 preparation, small opaque structures do appear but only to a minor extent, mainly vesicular structures being present without evidence for an extensive phase separation. In the case of the 10 : 1 preparation, the Ca^{2+}-rich and Ca^{2+}-poor conditions do not yield any clear differences (Fig. 3G, H).

These observations predict that removal of calcium by addition of EDTA to the calcium-rich situation will produce profound changes in the preparation only with the 100 : 1 lipid/rhodopsin ratio. This is borne out experimentally. Under such conditions, the cochleate cylinders of pure PS reorient into large monolamellar vesicles [9,10],

and the 100 : 1 preparation behaves similarly (Fig. 3C). Remarkably, all newly formed vesicles again contain particles, but now the particle density varies for different vesicles.

Photolytic behaviour. The photolytic behaviour of the PS-preparations completely corroborates the structural data. In the absence of Ca^{2+} either during reconstitution, or by addition of EDTA afterwards, the 100 : 1 preparation behaves normally (Fig. 4A, C; Table I). No blocked metarhodopsin I is observed and metarhodopsin III formation has normal kinetics. Only the amount of metarhodopsin III rhodopsin formed relative to the amount of metarhodopsin II decaying (90–100%) is somewhat increased as compared to preparations reconstituted with endogenous lipids (70–80%). Remarkably, decrease of the lipid/protein ratio now influences the photolytic behaviour of rhodopsin to only a minor extent, and much less so than observed for the endogenous lipids (Fig. 4; Table I). The relative amount of rhodopsin, becoming blocked into the metarhodopsin I stage is less than 5% for the 30 : 1 and only 10–15% for the 10 : 1 PS/rhodopsin ratio.

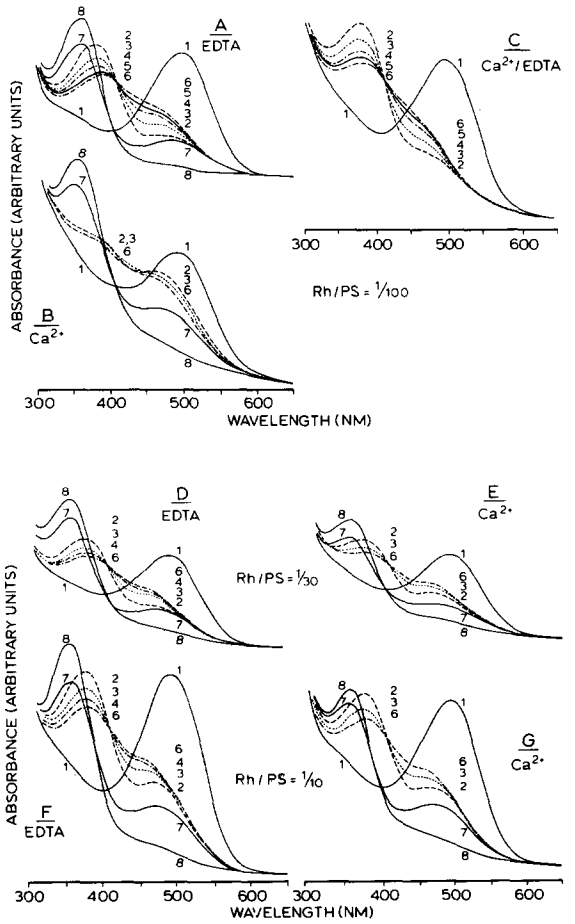

Fig. 4. Photolytic behaviour of rhodopsin reconstituted in phosphatidylserine. Phospholipid/rhodopsin ratios: 100 : 1 (A, B, C); 30 : 1 (D, E); 10 : 1 (F, G). Low free Ca^{2+} conditions: A, C, D, F. High free Ca^{2+} conditions (10 mM): B, E, G. C shows the same sample as B, but now before photolysis excess EDTA has been added in order to convert it to the low free Ca^{2+} condition. Experimental details as in Fig. 2.

The presence of Ca^{2+} again induces only marked changed in the behaviour of the 100 : 1 ratio (Fig. 4B, Table I). The resulting phase separation and rigidification causes a considerable increase in blocked metarhodopsin I intermediate (42 ± 4%). This behaviour, however, does not completely parallel the changes observed upon decrease of the lipid/protein ratio. In the latter case, the block of metarhodopsin I intermediates is less extensive (not more than 16%), but more complete, since the blocked metarhodopsin I decays much more slowly than in the case of the calcium-transformed 100 : 1 preparation (Fig. 4). Here, the relatively rapid decay of the 'blocked' metarhodopsin I interferes with the Metarhodopsin II → III transition. With respect to the 30 : 1 and 10 : 1 preparations, the presence of calcium has only minor effects, just slightly increasing the amounts of blocked metarhodopsin I in the first one. None of the effects of calcium observed at the high lipid/rhodopsin ratio (100 : 1) can be produced by Mn^{2+} and La^{3+} (tested at a free concentration of 15 mM) or by Mg^{2+} (tested up to 100 mM). However, at lower lipid/rhodopsin ratios, slightly more blocked metarhodopsin I is observed in the presence of 15 mM Mn^{2+} (0.11 resp. 0.21) and significantly more in the presence of 15 mM La^{3+} (0.30 resp. 0.34) at ratios of 30 : 1 and 10 : 1, respectively.

Discussion

Of the known properties of rhodopsin (photolytic behaviour; regeneration with 11-*cis* retinal; activation of a GTP-binding protein/cGMP-phosphodiesterase sequence) the second one is only moderately dependent on the microenvironment [7], without clear correlation with lipid species or

unsaturation. However, the photolytic behaviour of rhodopsin is strongly dependent on its microenvironment, as indicated by the enormous decrease in the rate of metarhodopsin I → II transition in more rigid systems [3,4,8]. Since photolytic behaviour and enzyme activation by illuminated rhodopsin are probably interrelated, we investigated the photolytic behaviour of rhodopsin in a system, the order parameter of which can be simply manipulated by a change in conditions without the necessity of a change in the lipid composition. Two combinations have been tested: (1) phosphatidylserine bilayers, which undergo dramatic rigidification upon binding of Ca^{2+} [9,10]; (2) lowering of lipid/protein ratio, which generally leads to stronger immobilisation of lipid [16,34]. Endogenous photoreceptor membrane lipids are used as a control.

The following phenomena observed will be discussed: (1) particle size is not strongly affected by varying lipid/rhodopsin ratio; (2) rhodopsin tends to form small sheets at low lipid/protein ratios; (3) the metarhodopsin I → II transition is affected by lowering the lipid/protein ratio but to a much larger extent in case of endogenous lipids than with phosphatidylserine; (4) when rhodopsin is reconstituted in phosphatidylserine, the metarhodopsin I → II transition is markedly influenced by the presence of Ca^{2+} but only at higher lipid/rhodopsin ratios.

(1) Particle size

The observed particle size is comparable in the various reconstituted preparations and is not influenced by illumination (8.5–11 nm). It is also comparable to the particle size we observe in photoreceptor membranes (7–10 nm). This confirms the low tendency of both rhodopsin and opsin to form larger aggregates, even at low lipid volume. The increase in cross-linking reported for opsin [17] might very well be due to a lower resistance of opsin against modification-induced denaturation, resulting in 'artificial' aggregation. The observed particle size is, however, difficult to correlate with biochemical evidence, which suggests a particle diameter of approx. 4 nm (see Ref. 18). While size estimations by freeze-fracture electronmicroscopy tend to overestimate particle size somewhat, we cannot exclude the possibility that, under our conditions of fixation, dimers or tetramers are generated. This would correspond with the tendency of rhodopsin to form dimers or tetramers in two-dimensional crystalline aggregates under certain conditions of pH and ionic strength (Ref. 20; Dratz, Van Breemen, Kamps and Van Bruggen, unpublished data).

Particle distribution is homogeneous over both fracture faces (Figs. 1, 3A), confirming that reconstitution yields a homogeneous and symmetric particle distribution. This is further supported by the particle density of the 'native-like' reconstituted preparation ($2700/\mu m^2$), which is about half of that in photoreceptor membranes ($5000/\mu m^2$). Furthermore, the particle density of the 100 : 1 PS-preparation ($1700/\mu m^2$) is about 60% of the preparation reconstituted with a normal ratio of endogenous phospholipid to rhodopsin (62 : 1). Hence, the procedure followed indeed allows investigation of rhodopsin properties at different lipid/protein ratios.

A non-homogeneous particle distribution is observed in phase-separated samples (high PS + Ca^{2+}), as is to be expected, but also after transformation of such samples by calcium chelation into large monolamellar vesicles (Fig. 3C). The latter phenomenon suggests that the particle-rich patches present in the high PS + Ca^{2+} samples are continuous with the 'cochleate cylinder-like' structures. The large monolamellar vesicles formed at first upon removal of Ca^{2+} will then taken up the majority of the particles which are concentrated near the surface of the cylinders. Vesicles formed later will consequently catch less particles.

(2) Sheet-formation at low lipid/protein ratios

Figs. 1C and 3G show clearly that rhodopsin tends to form small sheet-like structures at a low lipid/protein ratio. This becomes even more prominent at still lower ratios. At no more than 5 lipids/rhodopsin, only small sheets form until at under 1 lipid/rhodopsin larger, rather structureless, three-dimensional aggregates are formed (De Grip and Olive, unpublished data). Two-dimensional sheet-like structures might offer a suitable substrate for structure analysis by electron diffraction [20], but so far we have not been able to produce conditions under which sufficient order could be detected (Van Breemen and De Grip, unpublished data).

(3) Metarhodopsin I → II transition

Lowering the lipid/rhodopsin ratio with respect to endogenous lipids, achieved either by reconstitution or by treatment with phospholipase C [4], has comparable effects on the metarhodopsin I → II transition. This transition becomes partially blocked at lipid/rhodopsin ratios of about half the normal value (approx. 30), and lower ratios show increased blockage. This parallel behaviour shows that the reconstitution procedure in itself has not produced artifacts in this respect and further that rhodopsin photolysis is little affected by a symmetric distribution over the membrane. The kinetics of the metarhodopsin I → II transition strongly depend on the rigidity of the microenvironment [3,4,8]. A decrease in lipid/rhodopsin ratio and hence in lipid volume will rigidify the lipid matrix [16,22,34], as well as enhance the probability of protein-protein contacts. The latter have a strong immobilizing effect [34], as also clearly demonstrated in lipid-free rhodopsin. Remarkably, however, these effects express themselves much less strongly with phosphatidylserine. Here, only minor blockage of the metarhodopsin I → II transition is observed even at a lipid/rhodopsin ratio as low as 10. This exceptional preservation or rhodopsin functionality can be explained in two ways (a, b):

(a) Decrease in lipid/protein ratio imposes less restrictions on the dynamics of phosphatidylserine molecules than on those of endogenous lipids. This seems unlikely, since the latter are much higher unsaturated than the phosphatidylserine sample used here. However, the endogeneous lipids contain a small amount (approx. 15%) of other lipids, including 4–5 mol% cholesterol. The possibility cannot be excluded that at low lipid-to-protein ratios, these lipids exert an additional rigidifying effect, or create small lipid pools, thereby intensifying protein-protein interaction. The extent of this possible effect is presently investigated by isolating the total phospholipid fraction of photoreceptor membranes and using this for reconstitution.

(b) Solvation of rhodopsin is better achieved by pure phosphatidylserine than by the complex mixture of endogenous lipids, i.e., phosphatidylserine minimizes protein-protein contacts also at low lipid/protein ratios. This might be due both to electrostatic repulsion between the negatively charged phosphatidylserine molecules, and to strong electrostatic and possibly hydrophobic interaction between rhodopsin and phosphatidylserine. Both phenomena may effectively disperse the lipids between the protein. In the first case, Ca^{2+} would be expected to exert a strong influence. This is not observed (see below), suggesting that the second phenomenon predominates. We conclude that phosphatidylserine interacts more strongly with rhodopsin than the average endogenous phospholipid, which consists of about 45 mol% PE, 36 mol% PC and only 15 mol% PS [1,2,11]. Preference of rhodopsin for phosphatidylserine over other photoreceptor lipids has been reported [15,23,24], but is still a matter of dispute. Considering the lipid contributing to metarhodopsin III, PE might be closely associated with rhodopsin and may not be replacable by PS [25]. However, in our preparations reconstituted with PS, over 90% of metarhodopsin II decays into metarhodopsin III (Fig. 4A, C), comparable to photoreceptor membranes (70–80%) and preparations reconstituted with pure PE (85–90%), but significantly more than reported for PS (50–55%, [25]). This discrepancy may be due to the use of DTAB as a detergent in this earlier work, which is positively charged and not as easily removed as nonylglucose. Small amounts of detergent left, in particular with the negatively charged PS, could interefere with lipid structure and/or lipid-protein interaction. Under our conditions, PS behaves equivalent to PE. Pure PC generally does not support rhodopsin's photolytic integrity as well as PE or PS (Refs. 3, 4; De Grip, unpublished data). Altogether, pure PS will be more suitable for this purpose than the average photoreceptor membrane phospholipid.

Assuming that protein-protein interactions are largely responsible for slowing down the metarhodopsin I → II transition, we tentatively conclude that, on the average, lipid-lipid interaction in the photoreceptor membrane is stronger than lipid-protein interaction, effecting a phase separation at low lipid/protein ratios. The reverse situation would exist for PS. This also predicts that PE will behave similarly to PS, while rhodopsin's photolytic integrity should be less well preserved at low lipid protein ratios by PC than by

endogenous lipids. Preliminary experiments support this hypothesis. Hence, the effect of different lipid/rhodopsin ratios on the metarhodopsin I → II transition presents a promising way to measure the relative affinity of rhodopsin for lipids differing in headgroup or in acyl composition. The sensitivity of such a method could be further increased by also measuring the kinetics of the rapid phase of this transition.

(4) Calcium effects in preparations reconstituted with PS

The metarhodopsin I → II transition in preparations reconstituted with PS is strongly affected by the presence of Ca^{2+} only at higher lipid/rhodopsin ratios. Ca^{2+} apparently binds to PS molecules which do not strongly interact with rhodopsin, resulting in phase separation and transformation of the lipid-rich phase into cochleate structures [9]. Protein is left in vesicles or patches with a higher particle density (probably representing rhodopsin showing normal metarhodopsin I → II kinetics) or may be trapped within lipid-rich structures or lipid-poor domains (possibly representing rhodopsin showing anomalous metarhodopsin I → II kinetics). At these high lipid/protein ratios, the effects of calcium cannot be reproduced by La^{3+}, Mn^{2+} or Mg^{2+}, probably since, unlike Ca^{2+}, these ions do not form crystalline-like aggregates with PS [9,27,28].

The small effect of Ca^{2+} observed at lower ratios (no more than 30) supports our earlier conclusion that the rhodopsin-PS interaction has a strong electrostatic contribution. Otherwise, one would expect a phase separation also at low lipid concentration [26]. The stronger effects observed now with Mn^{2+} and La^{3+}, which have higher affinity for PS than Ca^{2+} [27–29], lend further support. Whether such an electrostatic interaction is specific for rhodopsin and has functional aspects is presently not clear. Several other membrane proteins contain positively charged groups located at the boundary layer between lipid and water phase, postulated to interact with negatively charged lipid groups [30,31]. Also the interaction is not restricted to a small area of rhodopsin, since already at 30 lipids/rhodopsin (i.e., little over one complete 'annulus'), the influence of Ca^{2+} is small. As a matter of fact, the proposed arrangement of the rhodopsin peptide-chain through the membrane [32,33], places at least eight positively charged residue and at least ten residues with hydrogen-bonding capacity in the membrane interface.

With excess lipid, Ca^{2+} produces a phase separation. If our foregoing assumptions are correct, lipids strongly interacting with rhodopsin should not contribute to the separated lipid phase, and consequently rhodopsin would show a photolytic pattern comparable to a lower lipid/rhodopsin ratio. Fig. 4B, F shows that this is not completely the case. In the phase-separated samples, 58% of the rhodopsin shows normal photolytic behaviour, confirming that no extensive protein aggregation has occurred, but the amount of 'blocked metarhodopsin I' is larger (42%) than in the low-lipid samples (no more than 16%). In further contrast, the 'blocked' metarhodopsin I in the phase-separated samples decays relatively rapidly. Both phenomena are explicable if rhodopsin-rich phases are trapped in between tightly packed layers of pure lipid, which will sufficiently restrict the mobility so as to increase the percentage of blocked metarhodopsin I relative to the low-lipid conditions, but without slowing down the metarhodopsin I decay as dramatically. Separation of the lipid and protein-rich phases would permit separate analyses to prove this point, but this has not been achieved so far. An alternative possibility is that Ca^{2+} induces separate phases with a very low lipid/protein ratio. This is less likely in view of the fact that: (1) such a phenomenon is then also expected at lower lipid/rhodopsin ratio, but is not observed there; (2) in such phases, the metarhodopsin I → II transition rate would be much more affected; (3) this condition is not so easily reversed by treatment with EDTA.

Conclusion

Spinal cord phosphatidylserine much better preserves the photolytic integrity of bovine rhodopsin at low lipid/protein ratio than the endogenous photoreceptor membrane lipids, although it is much less unsaturated (0.9 versus 3.1 double bonds per average acyl chain). Upon illumination, only a relatively small percentage of rhodopsin becomes blocked in the metarhodopsin

I stage, while the rate of the metarhodopsin II → III transition is slightly increased (Table I).

In view of the differential effects of various cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, La^{3+}) we suggest that the phosphatidylserine-rhodopsin interaction has a strong electrostatic component, which minimizes protein-protein interaction and keeps the protein dispersed, even at low lipid volumes.

Whether such interactions are essential for the functioning of the photoreceptor membranes has to be established. Changes in surface charge occurring during the photolytic transitions respectively upon phosphorylation of opsin might influence protein-lipid interaction, and could consequently modulate lipid surface charge, cation binding or interaction with extrinsic proteins. The highly unsaturated character of the photoreceptor membrane lipids might, among other things, be a compensation for their lower ability to maintain rhodopsin monodispersed.

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