Phase behavior of isolated photoreceptor membrane lipids is modulated by bivalent cations

Leo C.P.J. Mollevanger and Willem J. De Grip

Department of Biochemistry, University of Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands

Received 13 February 1984

The phase behavior of isolated photoreceptor membrane lipids is further investigated by ^{31}P-NMR, in view of earlier discrepant results [(1979) Biochim. Biophys. Acta 558, 330–337; (1982) FEBS Lett. 124, 93–99]. We present evidence that the discrepancy is due to bivalent cations. When resuspended in aqueous media at neutral pH in the absence of bivalent cations, the isolated photoreceptor membrane lipids largely adopt the bilayer configuration. However, upon addition of such cations ($Ca^{2+} > Mg^{2+}$) or when resuspended in their presence, the formation of other phases (hexagonal H_{II}, lipidic particles) results. The rate of this transition depends on cation concentration and temperature. The transition is not easily reversed by addition of EDTA. Implications with regard to photoreceptor membrane structure and function need further study.

^{31}P-NMR *Cation* *Photoreceptor membrane* *Lipid phase behavior*

1. INTRODUCTION

Several years ago we demonstrated by means of ^{31}P-NMR and electron microscopy that the photoreceptor membrane shows normal bilayer features under various conditions, but the isolated membrane lipids, resuspended under identical conditions, adopt the hexagonal H_{II} phase together with a lamellar phase interspersed with lipidic particles ('inverted micelles') [1]. Subsequently, authors in [2] confirmed our results with regard to the native membrane, but claimed that the isolated lipids also prefer the lamellar phase. They suggested that this discrepancy with our results might be due to lipid oxidation or incomplete extraction of bilayer-stabilizing species like PC or PS. Analysis of their experimental conditions, however, reveals one major difference with ours: all spectra were obtained in media containing bivalent cations, while the buffers we used contained physiological concentrations of Ca^{2+} and Mg^{2+} (2 and 3 mM, respectively). We have now further investigated this matter and hereby present results which resolve this apparent discrepancy.

Abbreviations: FID, free induction decay; Mops, 3-(*N*-morpholino)propanesulfonic acid; PE, 3-*sn*-phosphatidylethanolamine; PC, 3-*sn*-phosphatidylcholine; PS, 3-*sn*-phosphatidylserine; SM, sphingomyelin; ROS, rod outer segment

2. MATERIALS AND METHODS

Bovine rod outer segments (ROS) are isolated by sucrose density centrifugation as in [3]. Following hypotonic lysis (0.15 mM Ca–EDTA solution, pH 7.0; 2×) they yield an A_{280}/A_{500} ratio of 2.0–2.2. To obtain lipid extracts free of retinal or retinoxime [1] ROS are isolated in the light [3], which yields a membrane preparation virtually free of retinal and reginol. The SDS-gel pattern, phospholipid and fatty acid composition are checked routinely and found to agree with literature data [4,5]. 'Light' or 'dark' preparations do not show significant differences in these respects.

Total lipid extracts are obtained by a modified Folch procedure [4]. All manipulations are performed under strict nitrogen-protection with N_2-

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

saturated solutions, to minimize oxidative degradation of the highly unsaturated photoreceptor membrane lipids. The final extracts are evaporated in a small round-bottom flask by a stream of nitrogen and subsequently dried at high vacuum on a rotary evaporator for at least 1 h at room temperature to remove the last traces of organic solvent. The lipid residue is rehydrated by adding the required volume of aqueous medium and some glass beads under nitrogen and rotating for 15–30 min (final lipid concentration: ~40 μmol/ml).

Lipid phosphate (modified Fisko-Subarov [6]) and fatty acid (GLC of methyl esters) are assayed as in [4]. The phospholipid composition is determined both by two-dimensional TLC of the lipid extract [4] and by ^{31}P-NMR in detergent solution [7]. The latter method allows direct assay of the entire membrane and therefore does not depend on lipid isolation.

^{31}P-NMR spectra were recorded at 81 MHz, on a Bruker WM-200 spectrometer using broad-b and bi-level high-power proton decoupling [0.3 W between pulses (0.8 s), 3 W during acquisition (0.25 s)]. Routinely 2000–3000 FIDs were accumulated using 60° pulses, an interpulse time of 0.8 s and a 16 kHz sweep width. Before Fourier-transformation an exponential multiplication was applied to the accumulated FIDs, resulting in a 50 Hz line broadening. Sample volume was 1.4 ml.

The following two buffer solutions are used. (A) Bivalent cation-free buffer: 20 mM Mops, 0.1 mM EDTA (pH 7.2); (B) Mg/Ca buffer: 20 mM Mops, 130 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA (pH 7.2). Both buffers are dissolved in H_2O/D_2O (85:15, v/v).

3. RESULTS

Fig.1 presents a typical ^{31}P-NMR spectrum of washed ROS membranes, showing a characteristic bilayer spectrum [1,2,8]. Small percentages of non-bilayer phases (<1%) may however go undetected in the broad bilayer band. Upon solubilizing the membrane in 5% sodium cholate (pH 8.0), isotropic signals are generated which allows direct calculation of the composition of the major membrane phospholipids [7]. The results compare well with analysis of lipid extracts by TLC (table 1). Table 1 futher shows that, with regard to phospholipid and fatty acid composition, our preparations

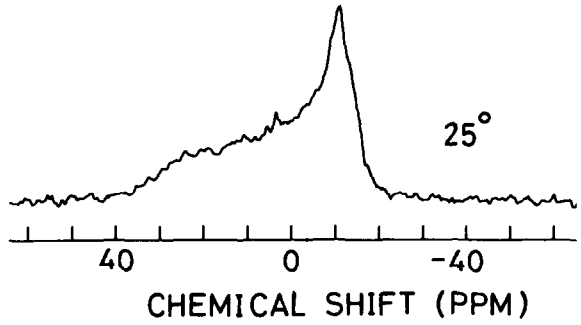

Fig.1. ^{31}P-NMR spectrum of washed ROS membrane suspended in Ca^{2+} + Mg^{2+}-containing buffer B. In the temperature range studied (5–45°C) the spectra show little temperature dependence · Ca^{2+} (tested up to 10 mM) has no influence on the spectrum.

are very similar to those analyzed in [2].

Total lipid extracts suspended in the Mg^{2+}-+Ca^{2+} containing medium (B) yield spectra as shown in fig.2, which present similar features to those in [1]: an isotropic component, representing a lamellar phase with lipidic particles, superimposed on an anisotropic component with properties of a bilayer below 15°C and of a hexagonal H_{II} phase at temperatures over 15°C. Apparently, these components exchange not at all or only very slowly on the NMR time scale microseconds. However, suspension of total lipid extract in the buffer solution without free bivalent cations (A) results in spectra as shown in fig.3A, which are very similar to those in [2]: predominantly lamellar phase (>85%), with only small amounts of isotropic phase. The latter may be due to smaller vesicles or a separate independent of the temperature between 5 and 55°C, except that the isotropic peak becomes sharper at higher temperatures (fig.3A).

This reconciles our earlier results [1] with those in [2]. The following experiment shows that the presence of cations like Ca^{2+} or Mg^{2+} is decisive (fig.3B). Upon addition of Ca^{2+} to a suspension of photoreceptor membrane lipids in medium A at 25°C, no significant effects are observed until the total Ca^{2+}:PS molar ratios of 0.1–0.2. Then the suspension becomes more turbid and eventually large flaky structures appear. The ^{31}P-NMR spectra show a slow transition to the status observed for total lipid extract suspended directly in solution B. The rate of this transition can be considerably increased by increasing Ca^{2+} concentration or by

Table 1

Phospholipid[a] and major fatty acid composition of washed photoreceptor membranes and lipid extracts

Reference	Lipid origin	Molar percentage (±SD) of			Molar percentage of major fatty acid classes		
		PE	PC	PS	16:0 18:0	20:4, 22:4 22:5, 22:6	24:4 24:5
Here	membrane[b]	43 ± 1	38 ± 2	19 ± 2			
	lipid extract[b]	47 ± 3	40 ± 3	14 ± 2	41	52	1.5
[4]	lipid extract[c]	46.3 ± 1.7	37.8 ± 1.6	15.9 ± 1.0	42	53	1.2
[2]	lipid extract[c]	45.7 ± 0.2	39.8 ± 0.3	14.5 ± 0.2	35	56	3.0

[a] The sum of PE, PC and PS is set at 100%. Minor amounts of other phospholipids (3–5%) are not taken into account
[b] Determined by ^{31}P-NMR of solutions in 5% cholate. Average of 3 experiments
[c] Determined by two-dimensional thin-layer chromatography

raising the temperature (fig.3B). At 25°C the effects become maximal at 5–6 mM Ca^{2+}, corresponding to a molar ratio of Ca^{2+}:PS of about 1. Very similar effects are obtained with Mg^{2+} al-

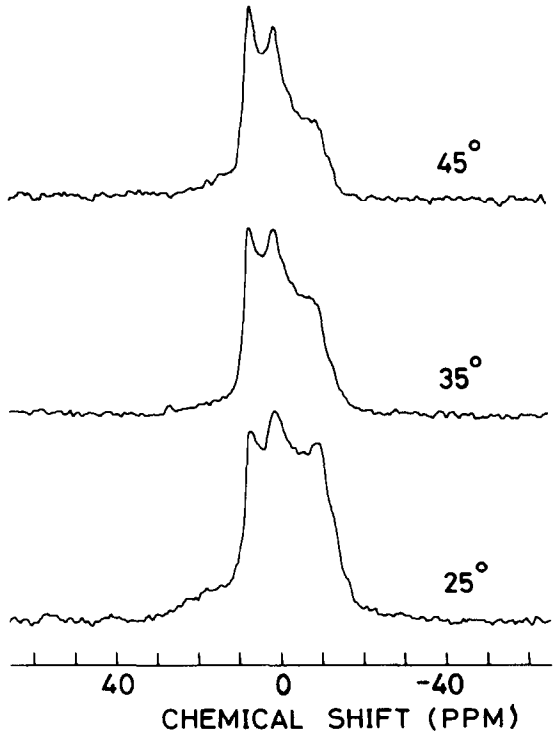

Fig.2. ^{31}P-NMR spectrum of total ROS lipids, resuspended in Ca^{2+} + Mg^{2+}-containing buffer B, as a function of temperature.

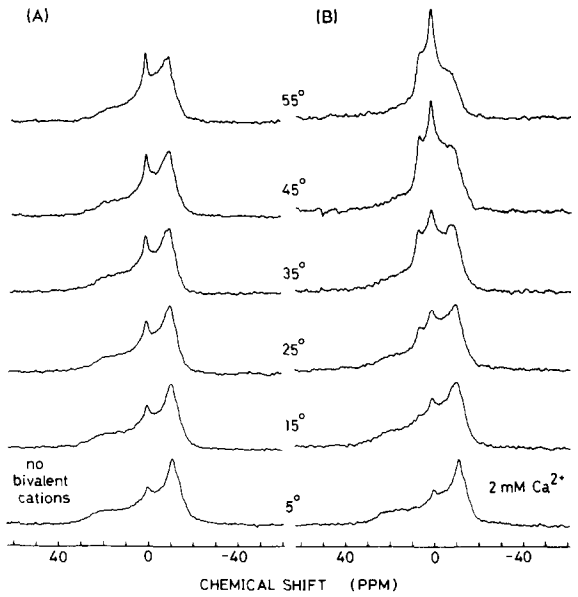

Fig.3. (A) ^{31}P-NMR spectra of total ROS lipids, resuspended in Ca^{2+}-, Mg^{2+}-free buffer A, as a function of temperature. The sharpening of the isotropic peak is due to a decrase in rotational correlation time (τ) at increasing temperature. The temperature effects are rapidly and completely reversible without any indication of hysteresis. (B) Similar spectra of total ROS lipids resuspended in buffer A, obtained upon addition of $CaCl_2$ at 5°C to a final concentration of 2 mM. This corresponds here to a molar ratio of Ca^{2+}:PS of about 1:3. Starting at 5°C, spectra were obtained after 1 h accumulation, whereupon the sample was warmed up to the next higher temperature, equilibrated for 10 min, and the next accumulation started. The observed effects are not or only slowly reversible by addition of excess EGTA.

though higher concentrations are required than for Ca^{2+}. Na^{2+} and K^{+} have no effect. Subsequent incubation with excess EGTA only very partially reverses the calcium effect. Even after prolonged incubation (2 weeks) in the presence of EGTA at 4°C to stabilize the lamellar configuration, the isotropic phase remains predominant.

The various manipulations do not significantly influence the fatty acid composition of the lipids, indicating absence of oxidative degradation.

4. DISCUSSION

Evidently, the extracted photoreceptor membrane lipids do largely adopt the lamellar configuration when resuspended at physiological pH in aqueous solution containing no bivalent cations. This confirms the results in [2]. However, addition of bivalent cations like Ca^{2+} or Mg^{2+} in millimolar concentration induces dramatic phase changes which proceed rapidly at physiological temperatures. This confirms our earlier results [1] and reconciles those in [1,2].

The question remains how this phase change is induced on a molecular level. It is well documented that in aqueous media at neutral pH unsaturated PE prefers the hexagonal phase at physiological temperatures, while under these conditions both PC and PS only adopt the lamellar phase [8]. To generate PE-containing bilayer membranes at temperatures above its transition to the H_{II} phase, an equal amount or more of a 'bilayer-stabilizing' phospholipid like PC or PS has to be incorporated [8,9]. Furthermore, there is ample evidence that PS has a high affinity for cations (bivalent ≫ monovalent; cf. [10]). In bilayers of pure PS, cations like Mn^{2+}, Ca^{2+} and Mg^{2+} induce a strong rigidification [11], while in mixtures of PS with other lipids (PE or PC) such cations induce a lateral segregation (cf. [8,12,13]), leading to two separate lipid domains containing largely PS, and largely PE or PC. In the case of PS/PE mixtures, the PE domain then may adopt the hexagonal phase [8]. Mixtures of the more saturated major erythrocyte inner-monolayer phospholipids (PE,PC,SM,PS) with equimolar amounts of cholesterol show a phase change from bilayer to hexagonal upon addition of Ca^{2+} [14].

Although alternative explanations may be devised, we consider, in view of the findings summarized above, the following one the most likely for the observed cation effects on the phase behavior of the highly unsaturated photoreceptor membrane lipids. In the absence of bivalent cations the lipids largely adopt the bilayer configuration, since the amount of PC and PS together (40% + 14%) is sufficient to stabilize a bilayer with 47% PE present. However, addition of Ca^{2+} or Mg^{2+} induces a lateral segregation creating separate PS domains, thereby withdrawing PS from the delicate equilibrium between PC + PS on the one hand and PE on the other. In the rest of the bilayer, largely containing PC and PE now, the ratio PC:PE is too low to stabilize the bilayer configuration. As a result, part of the PE will enter the hexagonal H_{II} phase. The remainder of the PE and PC might create a lamellar phase with lipidic particles and intermembrane contacts [1,8], permitting rapid equilibrium of the lipids over the two bilayer leaflets, which will generate an isotropic ^{31}P-NMR signal. This process is accelerated by raising the temperature, since (1) at temperatures below 15°C the lamellar organization is the most stable one also for PE, (2) lipid mobility and consequently the rate of phase separation and phase transition increases at higher temperatures. The rate of the phase change increases with increasing Ca^{2+} concentration up to a molar ratio of Ca^{2+}:PS of about 1, which indicates that formation of a Ca–PS complex is an essential element in the process and may be rate-determining. The fact that the transition is not easily reversible has also been observed in other systems, such as PS/PE mixtures [8]. Presently, we are testing the above hypothesis by purification of the phospholipid classes to recombine them in different ratios and to investigate cation effects on such systems.

Our observations have several implications for photoreceptor membrane structure and function. The Ca^{2+} content of the disk is relatively high [15], while intracellular [Mg^{2+}] very likely is in the millimolar range. Hence, irrespective of the transmembrane distribution of phospholipid species, under such conditions the extracted lipids would not prefer the lamellar configuration observed for the intact membrane. This implies that a bilayer-stabilizing factor has to be present in the membrane, for which the major (>90%) membrane protein rhodopsin is a likely candidate. As an intriguing consequence, protein-configuration changes or

local changes in cation concentration during visual excitation might allow functional, and transient local changes in lipid organization (e.g., the lipidic particles may considerably increase ion permeability). Such changes, which may effect only a small part of the total lipid population and hence may easily escape detection, clearly require further study.

ACKNOWLEDGEMENTS

We acknowledge helpful discussions with Dr B. de Kruyff, Professor C.W. Hilbers, Professor E.A. Dratz and Professor F.J.M. Daemen. This investigation was supported by the Netherlands Organization for the advancement of Basic Research (ZWO) through the foundation for Chemical Research in the Netherlands (SON). We thank Ing. P.A.W. van Dael and Dr C.A.G. Haasnoot of the ZWO/SON 500/200 hf-NMR facility Nijmegen, where the NMR measurements were performed, for their excellent assistance.

REFERENCES

[1] De Grip, W.J., Drenthe, E.H.S., Van Echteld, C.J.A., De Kruyff, B. and Verkley, A.J. (1979) Biochim. Biophys. Acta 558, 330–337.
[2] Deese, A.J., Dratz, E.A. and Brown, M.F. (1981) FEBS Lett. 124, 93–99.
[3] De Grip, W.J., Daemen, F.J.M. and Bonting, S.L. (1980) Methods Enzymol. 67, 301–320.
[4] Drenthe, E.H.S., Klompmakers, A.A., Bonting, S.L. and Daemen, F.J.M. (1980) Biochim. Biophys. Acta 641, 377–385.
[5] Miljanich, G.P., Sklar, L.A., White, D.L. and Dratz, E.A. (1979) Biochim. Biophys. Acta 552, 294–306.
[6] Broeckhuyse, R.M. (1968) Biochim. Biophys. Acta 152, 307–315.
[7] London, E. and Feigenson, G.W. (1979) J. Lipid Res. 20, 408–412.
[8] De Kruijff, B., Cullis, P.R. and Verkley, A.J. (1982) in: Membrane and Transport (Martonosi, A.N. ed.) vol. 1, pp. 43–49, Plenum, New York.
[9] De Kruijff, B., Cullis, P.R. and Verkley, A.J. (1980) Trends Biochem. Sci. 5, 79–81.
[10] Hammoudah, M.M., Nir, S., Bentz, J., Mayhew, E., Stewart, T.P., Hui, S.W. and Kurland, R.J. (1981) Biochim. Biophys. Acta 645, 102–114.
[11] Hauser, H., Finer, E.G. and Darke, A. (1977) Biochem. Biophys. Res. Commun. 76, 267–274.
[12] Ohnishi, S. and Ito, T. (1974) Biochemistry 13, 881–887.
[13] Hui, S.W., Boni, L.T., Stewart, T.P. and Isac, T. (1983) Biochemistry 22, 3511–3516.
[14] Hope, M.J. and Cullis, P.R. (1979) FEBS Lett. 107, 323–326.
[15] Kaupp, U.B. and Schnetkamp, P.P.M. (1982) Cell Calcium 3, 83–112.