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Retinal and retinol promote membrane fusion

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Disk membranes from the bovine retinal rod outer segments (ROS) were found to fuse with vesicles made of lipids extracted from unbleached ROS disk membranes, using a lipid mixing assay for membrane fusion (relief of self-quenching of R_{18}, octadecylrhodamine B chloride). If the retinal chromophore of rhodopsin was reductively linked to opsin before lipid extraction, the vesicles made of the extracted lipids were not suitable targets for fusion of the disk membranes. The addition of retinal and retinol to these vesicles restored their ability to fuse. Therefore, the presence of all-*trans* retinal was implicated in promoting membrane fusion in this system. To test this possibility, the ability of retinal and retinol to influence the phase behavior and the fusion capability of large unilamellar vesicles (LUV) of *N*-methyl dioleoylphosphatidylethanolamine (*N*-methyl-DOPE) was examined. Both retinal and retinol stimulated the fusion of vesicles of *N*-methyl-DOPE (contents mixing with ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; DPX, *p*-xylylene bis(pyridinium bromide)). Both compounds reduced the onset temperature for isotropic resonances in the ^{31}P-NMR spectra of *N*-methyl-DOPE dispersions and the onset temperature, T_{II}, for formation of hexagonal II phase. These results were consistent with previous studies in which the onset temperature for the ^{31}P-NMR isotropic resonances were correlated with stimulation of membrane fusion. These data suggested that both retinal and retinol may stimulate membrane fusion by destabilizing the bilayers of membranes.

Introduction

All-*trans* retinal and opsin are the products of the bleaching reaction following photon absorption by rhodopsin, the visual pigment of the rod photoreceptor. All-*trans* retinal is reduced by a ROS-associated dehydrogenase to retinol prior to transport to the retinal pigment epithelium, where it becomes esterified [1]. The enzymatic conversion of this retinyl ester to 11-*cis* retinal, with subsequent regeneration of the photopigment, completes the visual cycle [2]. Compounds of this sort have previously been linked with destabilization of the lamellar phase of lipid bilayers [3]. It is known that both retinal and retinol are available in the outer segment. It has also been suggested that the disk membranes are potentially unstable [4]. Thus, the question arises whether retinal and retinol influence membrane stability.

The outer segment of the rod cell is in a constant state of degradation and renewal. These processes are hypothesized to involve membrane fusion events, both in the formation of new disks from the plasma membrane and the phagocytosis of old disks by the pigment epithelium. Since disk assembly and synchronous disk shedding in various species are light-modulated processes [5,6] and retinal/retinol is produced upon rhodopsin bleaching, the question arises whether retinal and retinol may also influence membrane fusion processes.

Previous studies have investigated the effects of retinol on the gel-to-liquid crystalline phase-transition of dipalmitoylphosphatidylcholine [7,8]. At high concentrations, the main phase transition temperature was depressed by retinol. At concentrations less than 5 mol% the effects were negligible. Retinol has also been found to promote the formation of hexagonal II phase [3]. Other studies have identified a correlation

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Abbreviations: *N*-methyl-DOPE, *N*-methyl dioleoylphosphatidylethanolamine; LUV, large unilamellar vesicles; ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; DOC, deoxycholate; DPX, *p*-xylylene bis(pyridinium bromide); R_{18}, octadecylrhodamine B chloride; ^{31}P-NMR, ^{31}P nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; PC, phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; retinal, all-*trans*-retinal; retinol, all-*trans*-retinol; ROS, rod outer segment.

between non-lamellar structures in dispersions of *N*-methyl dioleoylphosphatidylethanolamine (*N*-methyl-DOPE) and fusion of LUV of this lipid [9]. Studies have also revealed that amphipathic compounds can destabilize *N*-methyl-DOPE bilayers and promote membrane fusion [10].

In the present study, the *N*-methyl-DOPE system was used as a sensitive measure of the ability of the retinal and retinol to affect phospholipid phase behavior and membrane fusion. At concentrations of 1 to 2 mol% retinal and retinol both destabilized membranes and promoted membrane fusion.

Materials and Methods

N-Methyl dioleoylphosphatidylethanolamine (*N*-methyl-DOPE) was obtained from Avanti Polar Lipids, Birmingham, AL. Octadecylrhodamine B chloride (R_{18}), 1-aminonaphthalene 3,6,8-trisulfonic acid (disodium salt) (ANTS) and *p*-xylene bis(pyridinium bromide) (DPX) were from Molecular Probes, Junction City, OR.

Vesicle preparation. Large unilamellar vesicles encapsulating ANTS or DPX, were prepared according to methods described in Ref. 11 with further details described in Ref. 9. *N*-Methyl-DOPE in chloroform/methanol (or *N*-methyl-DOPE + retinal/retinol) was dried under a stream of nitrogen and then under vacuum. The phospholipid (or phospholipid + retinal/retinol) was hydrated for 3 h on ice under N_2, in either 25 mM ANTS, 45 mM NaCl, 10 mM glycine (pH 9.5), or 90 mM DPX, 10 mM glycine (pH 9.5). The lipid suspension was next subjected to five freeze-thaw cycles followed by 10 extrusions through a polycarbonate membrane with 0.1 μm pores (Nucleopore, Pleasanton, CA). Encapsulated material was separated from unencapsulated material on a Sephadex G-50 column (Pharmacia) with 100 mM NaCl, 10 mM glycine, 0.1 mM EDTA (pH 9.5) used as the elution buffer. Vesicles were stored on ice, under N_2 and were used within 2 to 3 days.

Preparation of disk lipid vesicles (+/− retinals). Disk lipid vesicles labelled (+), containing retinal, were prepared as described previously [12]. The disk membranes were extracted with two volumes of 2:1 chloroform:methanol essentially as described in Ref. 13 under dim red light. The extracted lipids were dried under nitrogen and lyophilized to remove trace amounts of chloroform, the disk lipids were hydrated in extrusion buffer and subjected to five freeze-thaw cycles. The formation of large vesicular species was confirmed by negative stain transmission electron microscopy. Vesicles were stored on ice under N_2 and were used within 2 to 3 days. Vesicles were made 1 μmol/ml total phosphate in extrusion buffer prior to use.

Disk lipid vesicles labelled (−) (without retinal) were prepared by reducing the retinal-Schiff base linkage of rhodopsin prior to lipid extraction. The retinal was reduced using $NaCNBH_3$ as described previously [14]. Briefly, a solution of 2 M $NaCNBH_3$ in 1 M acetic acid was prepared immediately before use. The $NaCNBH_3$ solution was added to the disk suspension in a ratio of 2:1 under dim red light. The reaction was allowed to proceed at least 45 min in the dark. The disk membranes were then washed and resuspended in 10 mM Hepes (pH 7.4), prior to extraction. The disk membranes were extracted with two volumes of 2:1 chloroform:methanol essentially as described by Folch et al. [13]. The extracted lipids were then dried under nitrogen, lyophilized to remove trace amounts of chloroform, hydrated in extrusion buffer and subjected to five freeze-thaw cycles as described above. The fatty acid composition of the disk lipid vesicle preparations corresponded to previously published values [15].

Disk lipid vesicles labelled (added retinal/ol) were prepared by reducing the retinal onto the rhodopsin prior to extraction as described above. The disk lipids were extracted as described above and dried down under nitrogen. Retinal/retinol in a 1:1 mol:mol ratio was added to the disk lipids in ratio equal to the amount of rhodopsin mol:mol present in the original disk membrane prior to extraction. The retinal/ol and disk lipids were co-solubilized in $CHCL_3$, dried under nitrogen and lyophilized to remove trace levels of chloroform. The disk lipids were hydrated in extrusion buffer and subject to five freeze thaw cycles as described above. All manipulations of the disk membranes and subsequent disk lipid vesicles were preformed under dim red light and N_2 whenever possible.

Determination of the retinal/retinol content of extracted disk lipids. The retinal and retinol content of the extracted disk lipids both (+ and − retinal) was determined using high-performance liquid chromatography (HPLC). The retinoid derivatives were separated on a Li-Chrosorb Si-60 column with a gradient elution of 1–15% THF in hexane. The elution was monitored at 360 nm and the flow rate was 2 ml/min. The retinal and retinol peaks were identified and quantitated in comparison with authentic standards (Sigma, St. Louis, MO).

In order to determine the stability of the retinal and retinol during the course of the experiments, both disk lipid vesicles (+) and LUVs containing retinal or retinol were extracted as described above. The retinal and retinol content of the extracted samples was measured using HPLC initially, after the fusion assays and 24 h after preparation of the retinal/retinol LUVs. The change in the absorbance of retinal or retinol during was less than 4% during the course of the experiments and 24 h after preparation. A 25–50% decrease in absorbance of the retinal and retinol was

observed when the retinal/ol was in buffer or in hexane over the 24 h time period.

Preparation and labelling of disk membranes. ROS disk membranes were prepared from frozen bovine retinas (Lawson, Lincoln, NE) by floatation on Ficoll (Sigma, St. Louis, MO) as described previously [16]. The buffers used in these preparations were made 1 mM in EDTA, 0.5 mM in DTT and perfused with argon or nitrogen in order to reduce lipid oxidation. All manipulations of the disk membranes were done under dim red light.

Octadecylrhodamine B chloride (R_{18}), was obtained from Molecular Probes, (Junction City, OR). Typically, R_{18} was added to about 3–5 mol% relative to the phospholipid. The disk membranes were labelled with R_{18} as described in Ref. 17. Briefly, 10 nmol of R_{18} in 10 μl of ethanol was added for each mg of rhodopsin in a total volume of 1 ml. The mixture was vortexed and allowed to incubate at room temperature for 1 h. R_{18}-labelled disks were separated from unincorporated R_{18} by passing the mixture through a Sephadex G-75 column and eluting labelled material with 100 mM NaCl, 10 mM glycine, 0.1 mM EDTA (pH 7.5).

Vesicle fusion and leakage assays. All fluorescence measurements were made on an SLM 8000D fluorimeter. The ANTS/DPX fusion and leakage assays were carried out as described in Ref. 18. Vesicles contained either 25 mM ANTS and 45 mM NaCl or 90 mM DPX. Fluorescence intensity was monitored with an excitation wavelength of 380 nm and an emission wavelength of 510 nm. All assays were carried out in a total volume of 1 ml. The final lipid concentration was 400 μM for both fusion and leakage assays. Fusion or leakage was initiated by lowering the pH from 9.5 to 4.5 with 25 μl of 2 M sodium acetate/acetic acid buffer. For fusion assays a 9:1 molar ratio of DPX-containing LUV to ANTS-containing LUV was used. Fluorescence quenching as a function of time due to contents mixing (resulting in an ANTS-DPX complex with reduced quantum yield) reflected the rate of LUV fusion. For vesicle-vesicle fusion assays, 100% fluorescence was taken to be the initial fluorescence intensity before lowering the pH. The initial rate of fusion was then calculated from the slope of the fluorescence decay curve during the period (1–2 min) immediately following the initiation of fusion and the rate expressed in terms of % change per minute.

Fusion assays with ROS disk membranes. All fluorescence measurements were performed on an SLM 8000D spectrofluorometer. The fusion assays were carried out essentially as described in Ref. 12. The fusion assays were performed in a dimly lit room. The LUVs were diluted to 1 μmol/ml total phosphate in 100 mM NaCl, 10 mM glycine (pH 7.4). The LUV (1 ml) were first allowed to equilibrate to the appropriate temperature for 5 min. In order to initiate fusion, 50 μl of R_{18} labelled disks were added to the vesicles. The final fusion assay volume was 1.05 ml. Fluorescence was monitored with an excitation wavelength of 560 nm and an emission wavelength of 586 nm. The fluorescence intensity obtained without the addition of labelled disk membranes was taken as a baseline. 100% fluorescence intensity was determined by adding 100 μl of 10% Triton X-100 to the vesicles.

Separation of fused species. Fusion of the disk lipid vesicles with R_{18}-labelled disks was confirmed using sucrose density gradient centrifugation. LUVs, R_{18}-labeled disks and fused mixtures of these two species were subjected to sucrose density gradient centrifugation. The sucrose density gradients were 0–50% (w/w) sucrose in distilled water. The gradients were centrifuged at 27 K for 2 h and the bands collected. Prior to assay the refractive index of the isolated species was measured using a refractometer. The fractions were assayed for phosphate and protein content.

Additional assays. Phosphate was determined as described in Ref. 19 and modified according to Ref. 20. All spectral measurements were performed on an SLM Aminco DW-2 spectrophotometer. Rhodopsin concentration was determined by measuring the difference in absorbance at 500 nm before and after illumination in the presence of 50 mM neutralized hydroxylamine using an extinction coefficient of 40000.

For fatty-acid analysis, the fatty-acid methyl esters were generated by mild alkaline methanolysis as described previously [15]. The fatty-acid methyl esters were analyzed on a Chrompak-Packard Model 439 gas chromatograph, equipped with a flame ionization detector and Varian 4270 integrator. The fatty acid methyl esters were separated isocratically on a Supelcowax-10 wide-bore column. Column temperature was maintained at 240°C, injector at 250°C and detector at 300°C. Identification of most peaks was by comparison with reference methyl ester mixture, No 4-7015 (PUFA mix 2) from Supelco.

Nuclear magnetic resonance. ^{31}P nuclear magnetic resonance (NMR) spectra were obtained with a JEOL FX270 Fourier transform spectrometer on a broad band probe in 10 mm tubes at the indicated temperatures. A fully phased cycle (32 pulse) chemical shift anisotropy (CSA) echo was used with a 40 μs echo [21]. Gated proton decoupling (on only during acquisition) at a decoupling field of 9 kHz was employed to eliminate sample heating. A 50 kHz spectral width was used, with 50 Hz of linebroadening in the Fourier transformation. A delay time of 1 s was used between pulses. The only ^{31}P nuclei in the preparation were in the phospholipid component of these membranes. ^{31}P-NMR spectra were obtained as a function of temperature and composition of *N*-methyl-DOPE dispersions, using 0.8 ml of sample volume, all within the receiver coil. Spectra were obtained only with sequential in-

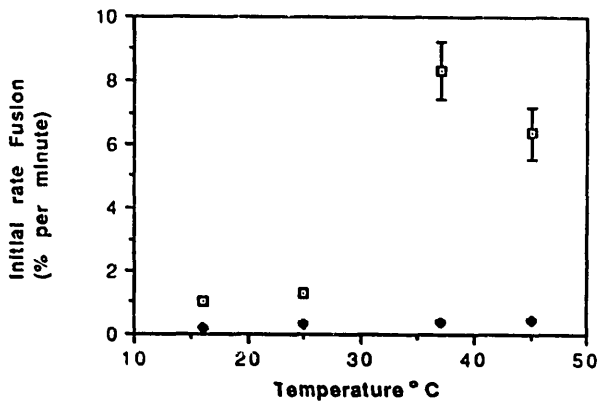

Fig. 1. Fusion of ROS disks with membranes of disk lipids. The initial rates of fusion of disk lipid (+) and disk lipid (−) vesicles with R_{18}-labelled disk membranes is shown as a function of temperature. Disk lipid (+) vesicles [□] and disk lipid (−) vesicles [●] were prepared as described in the Materials and Methods. Fusion of these disk lipid vesicles with disks was induced by the addition of R_{18}-labelled ROS disks to the disk lipid vesicle preparations as described in detail in Methods. The results shown are an average of three independent experiments. Error bars representative of +/− standard deviation are given for all values ($n = 9$).

creases in temperature, from a sample which had been freeze-thawed prior to incubation at the starting temperature. Thus, each temperature represented the first exposure of the sample to that temperature.

Results

Fusion of ROS disks with membranes of disk lipids with and without retinal

ROS disk lipid (+) vesicles were prepared as described in Materials and Methods. Fusion of disk membranes with disk lipid vesicles was induced with the addition of R_{18}-labelled disks as described in Materials and Methods.

The initial rates of fusion observed between disk membranes and disk lipid (+) vesicles are shown in Fig. 1 as a function of temperature. Disk membranes were found to fuse with disk lipid vesicles (+). As a control, no observable fusion was observed when labeled disk membranes were incubated with unlabeled disk membranes in excess (data not shown). For disk membrane-disk lipid vesicle (+) fusion, there is an increase in the rate of fusion as a function of temperature, with a maximum at approx. 37°C. Disk membrane-disk lipid vesicle (+) fusion was inhibited by DOC (added in a 1:1 mol ratio with the phospholipids) at all temperatures studied (data not shown).

As seen in Fig. 1, no dequenching of the fluorescence of R_{18} was observed when R_{18}-labelled disks were incubated with disk lipid (−) vesicles made from an ROS disk preparation in which the retinal chromophore of rhodopsin was covalently reduced onto the opsin by cyanoborohydride reduction to prevent its extraction along with the disk lipids. It was, therefore, concluded that ROS disks did not fuse with disk lipid (−) vesicles at all temperatures studied.

In order to confirm that disk membranes were fusing with disk lipid vesicles, sucrose density gradient experiments were carried out as described in Materials and Methods. Disk lipid (+/−) vesicles were found to band at approx. 5% sucrose on the sucrose density gradient. R_{18}-labelled disk membranes alone were found to band at 29% sucrose on the sucrose density gradient. When disk membranes and disk lipid (+) vesicles were allowed to fuse (as measured by an increase in fluorescence) and then analyzed on a sucrose density gradient, a single band was recovered corresponding to approx. 10% sucrose. This band of lipid and protein was pink, characteristic of rhodopsin from the disk membranes. Only a single species could be identified, suggesting fusion of the two original species.

In order to show that the presence of retinal/ol in the disk lipid suspension does promote fusion, disk lipid (−) vesicles were prepared to which retinal/ol was added back as described in Materials and Methods. The initial rate of fusion of these vesicles with R_{18}-labeled disk membranes was determined at 37°C. Under these conditions, the addition of retinal/ol to the disk membranes (−) resulted in an increase in the initial rate of fusion, to a degree comparable to that seen in disk lipid (+) fusion. Thus, the retinal/ol was able to promote fusion when added to a non-fusogenic target membrane.

These data suggested that retinal was capable of inducing a fusion-permissive state in a target membrane containing the total lipid extract of ROS disks. Whether retinal was indeed fusogenic needed to be more extensively examined. To investigate the ability of retinal to stimulate membrane fusion required a well-characterized, simple membrane fusion system that depended upon the lipids of the membranes involved. The fusion of LUV of *N*-methyl-DOPE offered the needed, well-characterized membrane fusion system. These vesicles were previously reported to fuse spontaneously at elevated temperatures and acidic pH. Furthermore, previous studies indicated that the fusion pathway involved non-lamellar lipid intermediates that could be detected readily with ^{31}P-NMR spectroscopy. Therefore, this fusion system offered the ability to test whether retinal could stimulate membrane fusion in a system where one could also determine the effects of retinal on the pathway of fusion. In the following studies, it is not assumed that the pathway of fusion of the *N*-methyl-DOPE LUV and the pathway of fusion of ROS disks with disk lipid vesicles (+) are the same. The goal was simply to determine whether retinal could be stimulatory to fusion in another membrane fusion system.

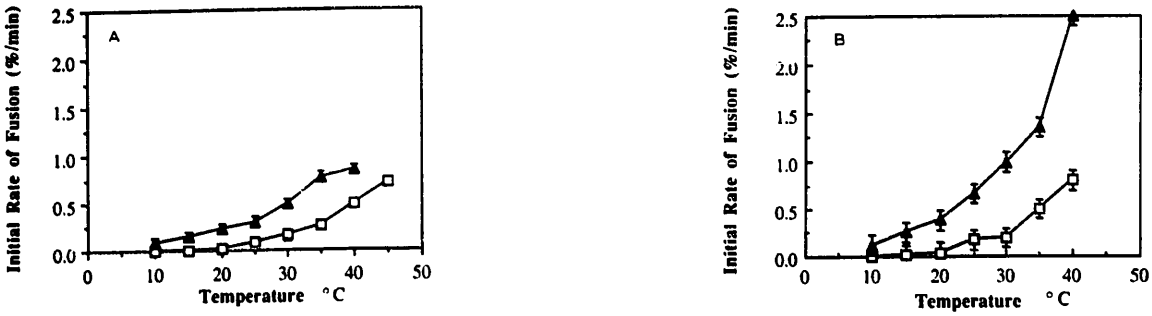

Fig. 2. Fusion of *N*-methyl-DOPE LUV in the presence and absence of retinal and retinol. (A) The initial rates of fusion of LUV (of *N*-methyl-DOPE) with or without retinal are presented as a function of temperature. *N*-Methyl-DOPE LUV were prepared as described in Methods. The initial rates of *N*-methyl-DOPE fusion were measured using a contents mixing fluorescence assay (ANT/DPX) as described in Materials and Methods. Fusion was initiated with a drop in pH from 9.5 to pH 4.5 as described. The results represent an average of two independent experiments each of which was assayed in triplicate ($n = 6$). ▲, Fusion in the presence of 2 mol% retinal; □, fusion in the absence of retinal. (B) The initial rates of fusion of LUV (of *N*-methyl-DOPE) with or without retinol are presented as a function of temperature. Conditions as in panel A. ▲, Fusion in the presence of 2 mol% retinol; □, fusion in the absence of retinol. The results represent an average of two independent experiments, each of which was assayed in triplicate ($n = 6$).

Fusion of N-methyl-DOPE LUV with and without retinal

LUV of *N*-methyl-DOPE spontaneously fused at neutral and low pH, with a rate that increased as a function of temperature [9,22]. This observation was reproduced with a contents mixing fluorescence assay in this study. The data are presented as a control in Fig. 2A where the initial rate of fusion is graphed as a function of temperature. LUV of *N*-methyl-DOPE were made with 2 mol% retinal as described in Methods. The initial rates of fusion (contents mixing) were measured as a function of temperature and the results presented in Fig. 2A as the average of two independent experiments, in each of which the individual points were themselves the average of three determinations. The results show an increase with temperature, analogous to the control. However, at each temperature there was a 2-fold or greater increase in the initial rate of fusion in the presence of the retinal. At temperatures above 40°C, leakage of dye from the vesicles became too great to successfully measure fusion rates.

Fusion of N-methyl-DOPE LUV with and without retinol

Since retinal in the ROS can, under physiological conditions, be converted to retinol via a dehydrogenase, a set of membrane fusion experiments with *N*-methyl-DOPE LUV, analogous to those described above with retinal, were carried out with retinol. These results are shown in Fig. 2B. The presence of 2 mol% retinol in the membranes of these LUV enhanced the initial rate of fusion at every temperature relative to control experiments performed with the same lot of *N*-methyl-DOPE and in the same time frame. The data represent an average of two independent experiments, in each of which the individual points were themselves the average of three determinations.

Effect of retinal on the ^{31}P-NMR powder patterns of N-methyl-DOPE

Previous experiments had revealed that compounds that increased membrane fusion in this *N*-methyl-

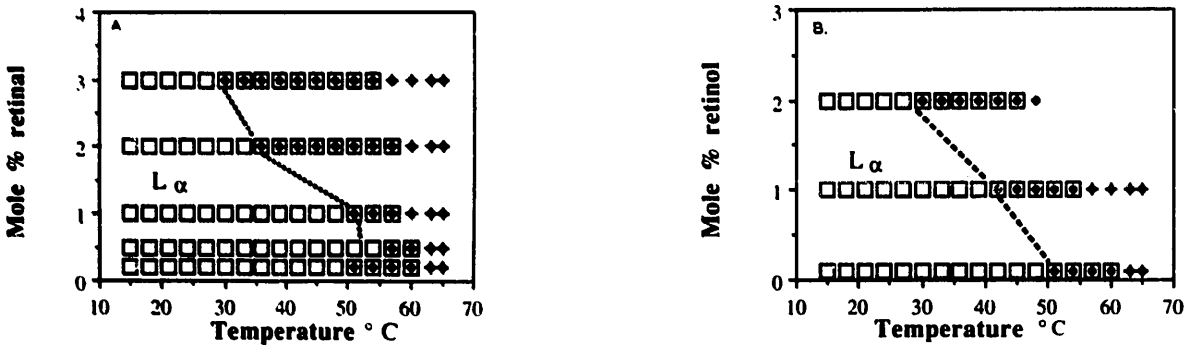

Fig. 3. Partial phase diagram of *N*-methyl-DOPE mixtures with retinal and retinol as detected using ^{31}P-NMR. The symbols represent: □, lamellar, ◆, isotropic structures; ✦, hexagonal II structures. To the left of the solid line is pure lamellar phase. (A) *N*-methyl-DOPE with retinal; (B) *N*-methyl-DOPE with retinol.

DOPE LUV system had a corresponding effect on the phase behavior of *N*-methyl-DOPE, as determined from the ^{31}P-NMR powder patterns [23]. Therefore, the phase behavior of *N*-methyl-DOPE membranes containing retinal was studied with ^{31}P-NMR. Multilamellar dispersions were used for this study, since it was previously reported that multilamellar dispersions and LUV of *N*-methyl-DOPE behaved in an indistinguishable manner with respect to their ^{31}P-NMR powder patterns [9].

To carry out this study, ^{31}P-NMR spectra of dispersions of *N*-methyl-DOPE were obtained as a function of retinal content and temperature. The resulting spectra were examined for the presence of powder patterns characteristic of bilayer (L_α) and hexagonal II phases and what was previously characterized as an isotropic structure with an isotropic ^{31}P-NMR resonance (I_s) [9,24]. Fig. 3A shows the results obtained. To the left of the solid line is the pure lamellar region. The solid line itself indicates the onset temperature for the visible development of I_s. To the right of the solid line, lamellar and hexagonal II phases may co-exist over a limited temperature range, while the isotropic structures are found over a broad temperature and composition range. Fig. 3A shows clearly that an increase in retinal concentration in the *N*-methyl-DOPE membranes produced a corresponding decrease in the onset temperature for the formation of the I_s structures. This observation was significant, because it was previously suggested that these structures (or companion structures that formed with the same temperature-dependence) were involved as intermediates in the fusion of *N*-methyl-DOPE LUV [9,23]. Thus, an increase in retinal concentration enhanced the presence of putative intermediates in the fusion process and increased the rate of membrane fusion.

Effect of retinol on the ^{31}P-NMR powder patterns of N-methyl-DOPE

A set of ^{31}P-NMR experiments, analogous to the experiments described above for retinal, were performed on dispersions of *N*-methyl-DOPE with various concentrations of retinol. measurements were made as a function of temperature and as a function of retinol concentration between 0 and 2 mol%. The spectra were analyzed as before for the presence of lamellar and hexagonal II phases and for the presence of I_s. The results are presented in Fig. 3B. Once again, the presence of retinol decreased the onset temperature for the formation of I_s.

Discussion

Recent work in this laboratory has focused on the fusion of disk membranes with various target membranes [12]. In the course of these investigations, the apparent influence of all-*trans* retinal on the ability of disks to fuse with vesicles made of ROS disk lipids was noted. The present study was designed to shed some light on the role, if any, of these retinoids in the fusion process.

Initially, the experimentation was directed towards clearly delineating whether retinal was the lipid component that stimulated fusion between ROS disks and the disk lipid (+) vesicles. Upon confirmation that retinal was capable of creating a fusion-permissive state in the disk lipid target vesicles, it became of interest to determine whether retinal (and retinol) could stimulate fusion in other membrane fusion systems.

To investigate this question, use was made of a vesicle fusion system whose properties had been reported previously. *N*-methyl-DOPE LUV were shown to spontaneously fuse at low and neutral pH with an initial rate of fusion that was directly proportional to the incidence of non-lamellar structures. The presence of these putative intermediate structures was indicated by isotropic ^{31}P-NMR resonances and lipidic particles in freeze-fracture electron micrographs of the samples [9]. This fusion system was used previously to investigate the influence of diacylglycerol on the phase structure of *N*-methyl-DOPE and the correlation between changes in the phase structure and membrane fusion [23]. This system seemed sufficiently sensitive to examine the influence of retinal on membrane fusion.

In this study, retinal was found to significantly increase the initial rate of membrane fusion (contents mixing assay) at all temperatures when present at 2 mol% in the membrane. The increase in fusion was about 50% increase/mol% retinal.

^{31}P-NMR studies revealed that retinal reduced the onset temperature of the formation of structures (I_s) giving rise to isotropic ^{31}P-NMR resonances. The temperature range over which the isotropic resonances were observed was previously referred to as ΔT_1 [23]. The onset of ΔT_1 was reduced by retinal in the membrane. T_{II}, the temperature at which hexagonal II phase first appears as detected by ^{31}P-NMR, decreased with retinal in the membrane (see Table I). These decreases in onset temperatures were similar to the reductions produced by diacylglycerol in the same *N*-methyl-DOPE system [23]. Retinol induced similar alterations in membrane phase behavior and membrane fusion (see Table I).

TABLE I

Compound	Onset of ΔT_1 (°C decrease/ mol% cmpd)	T_{II} (°C decrease/ mol% cmpd)	Increase in fusion (%/mol% cmpd)
Retinal	8	6	50
Retinol	10	7	150

Because of the similarities in the correlated influence of retinal and retinol on membrane fusion and membrane instability of LUV of *N*-methyl-DOPE with the previously reported influence of diacylglycerol on the same system, the same interpretation for mechanism seems most likely. Retinal and retinol, like diacylglycerol, appear to promote the destabilization of lipid bilayers necessary for a membrane-fusion event. However, whether the mechanism of action of retinal is the same as for diacylglycerol, that is reduction in the intrinsic radius of curvature [23], is not known.

Returning to the consideration of the original subject of this study, the data discussed above would seem to confirm that retinal and retinol could stimulate fusion as was observed in the fusion of ROS disks with disk lipid (+) vesicles. Previously, it was noted that protein appeared to play a role in the fusion of ROS disks with disk lipid (+) vesicles [12]. In particular, proteolysis of the surface of the disk inhibited the fusion reaction. This fusion was, therefore, referred to as 'protein-mediated' membrane fusion to refer to the apparent role of disk membrane protein in facilitating this fusion event. On the basis of the data in the present report, the above description should be modified. The retinal content of the target disk lipid (+) vesicles also plays a role in the rate at which membrane fusion occurs between these vesicles and the ROS disks.

The mechanism by which the retinal renders the disk lipid (+) vesicles capable of fusion with ROS disks is not yet clear. Experiments were performed in which ^{31}P-NMR was utilized to determine whether non-lamellar structures formed in disk lipid (+) vesicles under the influence of retinal, analogous to the observations in the *N*-methyl-DOPE LUV fusion system. The results were negative at all temperatures at which fusion had been measured (data not shown). Therefore, one cannot conclude at this time that the mechanism by which retinal stimulates fusion in the fusion of disks with disk lipid (+) vesicles is the same as the mechanism by which retinal stimulates fusion of *N*-methyl-DOPE LUV.

The ability of retinal to stimulate fusion is interesting in light of the architecture and dynamics of the retinal rod outer segment. New disks are added at the base of the ROS and old disks are shed in packets from the distal tip and phagocytosed by the overlying pigment epithelium. This latter process must involve membrane fusion, perhaps between the disks and the ROS plasma membrane. Disk shedding has been shown to be modulated by light [25] which would lead to release of retinal and the formation of retinol. A disease state (retinal dystrophy) in the RCS rats in which the accumulation of membrane debris may be due to an aberrant fusion event is characterized by a decrease in the conversion of retinal to retinol and a decrease in the rate of retinol transfer to the pigment epithelium [26]. Thus, a scenario is produced in which an accumulation of retinal/ol in the disk membranes may affect normal fusion processes.

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References

1 Knowles, A. and Dartnell, H.J.A. (1977) in The Eye (Davson, H., ed.), p. 499, Academic Press, New York.
2 Rando, R.R., Bernstein, P.S. and Barry, R.J. (1991) in Progress in Retinal Research (Osbrone, N. and Chader, G., eds.), pp. 161–178, Pergamon Press, New York.
3 Epand, R.M. (1987) Chem.-Biol. Interact. 63, 239–247.
4 Albert, A.D., Sen, A. and Yeagle, P.L. (1984) Biochim. Biophys. Acta 771, 28–34.
5 Besharse, J. (1982) in Progress in Retinal Research (Osborne, N. and Chader, G., eds.), pp. 81–124, Pergamon Press, New York
6 Besharse, J. (1986) in The Retina: A Model for Cell Biology Studies (Adler, R. and Farber, D., eds.), pp. 297–352, Academic Press, New York.
7 Boeck, H.D. and Zidovetzki, R. (1988) Biochim. Biophys. Acta 946, 244–252.
8 Wassel, S.R., Phelps, T.M., Albrecht, M.R., Langsford, C.A. and Stillwell, W. (1988) Biochim. Biophys. Acta 939, 393–402.
9 Ellens, H., Siegel, D.P., Alford, D., Yeagle, P.L., Boni, L., Lis, L.J., Quinn, P.J. and Bentz, J. (1989) Biochemistry 28, 3692–3703.
10 Siegel, D.P., Banschbach, J., Alford, D., Ellens, H., Lis, L., Quinn, P.J., Yeagle, P.L. and Bentz, J. (1989) Biochemistry 28, 3703–3709.
11 Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E. and Papahadjopoulos, D. (1980) Biochim. Biophys. Acta 601, 559–571.
12 Boesze-Battaglia, K. and Yeagle, P.L. (1992) Invest. Ophthalmol. Visual. Sci. 33, 484–493.
13 Folch, J., Lees, M. and Sloane-Stanley, G.A. (1957) J. Biol. Chem., 226, 497–509.
14 Fager, R.S., Sejnowski, P. and Abrahamson, E.W. (1972) Biochem. Biophys. Res. Comm. 47, 1244–1247.
15 Boesze-Battaglia, K., Hennessey, T. and Albert, A.D. (1989) J. Biol. Chem. 264, 8151–8155.
16 Smith, H.G., Stubbs, G.W. and Litman, B.J. (1975) Exp. Eye Res. 20, 211–217.
17 Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) Biochemistry 23, 5675–5681.
18 Ellens, H., Bentz, J. and Szoka, F.C. (1985) Proc. Natl. Acad. Sci. USA 82, 5742.
19 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–473.
20 Litman, B.J. (1973) Biochemistry 13, 2545–2554.
21 Rance, M. and Byrd, A. (1983) J. Magn. Reson. 52, 221–240.
22 Kelsey, D.R., Flanagan, T.D., Young, J. and Yeagle, P.L. (1990) J. Biol. Chem. 265, 12178–12183.
23 Siegel, D.P., Banschbach, J. and Yeagle, P.L. (1989) Biochemistry 28, 5010–5018.
24 Gagne, J., Stamatatos, L., Diacovo, T., Hui, S.W., Yeagle, P.L. and Silvius, J. (1985) Biochemistry 24, 4400–4408.
25 Besharse, J.C. and Dunis, D.A. (1982) in The Structure of The Eye (Hollyfield, J.G., ed.) Elsevier/North Holland, New York.
26 Delmelle, M., Noell, W. and Organisciak, D. (1975) Exp. Eye Res. 21, 369–380.