The retinal rod outer segment (ROS) is maintained at a constant length through the formation of new discs and the phagocytosis of old discs by the pigment epithelium. The ROS is composed of approximately 50 mol% of phosphatidylethanolamine (PE), an unusually high PE content for biologic membranes. Because this lipid is highly fusogenic, due to its low head group hydration, the ability of ROS disc membranes to fuse with PE large unilamellar vesicles (LUV) was examined. The initial rates of fusion of discs with LUV were measured by following the relief of self-quenching of octadecylrhodamine B chloride (R18)-labeled disc membranes. Fusion was initiated by reducing the pH of the mixture to 7.2. The initial rates of fusion of disc membranes with transphosphatidylated PE (trans-PE LUV) were measured as a function of temperature. The ROS discs fused readily with these LUV. Fusion was confirmed by density-gradient centrifugation. The initial rates of fusion increased with increasing temperature. Subsequently, the initial rates of fusion between disc membranes and disc lipid vesicles were examined using the R18 mixing assay. Fusion of the two membranes was confirmed by sucrose density-gradient centrifugation. Calcium and EGTA had no significant effect on disc membrane-trans-PE LUV fusion or on disc membrane-disc lipid vesicle membrane fusion. Papain proteolysis of the disc membranes enhanced initial rates of fusion between disc membrane and PE LUV but inhibited disc membrane-disc lipid vesicle fusion. Invest Ophthalmol Vis Sci 33:484–493, 1992

The rod photoreceptor in vertebrate retinas responds to light and initiates the nerve impulse that is transmitted to the brain. The rod cells are divided morphologically into an inner and an outer segment. The outer segment of the rod cell (ROS) encloses a stack of densely packed, closed, flattened membranous discs, which are stacked along the length of the outer segment. The discs are formed from evaginations of the ROS plasma membrane at the base of the ROS and progressively move up the outer segment as new discs are formed. Old discs at the apical tip form packets that are phagocytized by the surrounding pigment epithelium. Thus, the outer segment is in a constant state of degradation and renewal. This renewal process takes approximately 10 days in mammals.

Much work has focused on the route from the inner to outer segment taken by rhodopsin and the lipid constituents that make up the disc membranes.1–3 Less is known, however, about the determinants of disc morphogenesis and the regulators of this process. Posttranslational modifications of rhodopsin have been implicated in disc morphogenesis. Inhibiting glycosylation of rhodopsin and/or other disc membrane proteins in Xenopus retinas with the drug tunicamycin, for example, inhibits normal disc morphogenesis.4 Taurine also has been reported to inhibit disc morphogenesis.5 The actual process by which closed discs form from open discs by fusion of the plasma membrane is not well characterized. This, however, may be the focal point of the sorting process that differentiates discs from plasma membrane.6

Disc shedding occurs at regular intervals and can be stimulated by light after a dark period.3 Calcium apparently is required for disc shedding.4 Melatonin enhanced the light-stimulated disc shedding in Xenopus.9 By contrast, cyclic adenosine monophosphate stimulated disc shedding in the dark.10 Disc shedding at the apical tip of the ROS morphologically requires membrane fusion. Recent work, using Lucifer yellow staining, indicated that the interior of some discs became continuous with the external medium during the process of packet formation, suggesting fusion.11 Membrane fusion is involved in the formation of new discs and the shedding of old discs. Although the morphologic dynamics of disc degradation and renewal have been known for some time, how these...
processes proceed and are regulated is not. The membrane fusion processes integral to disc formation and shedding are likely points for regulation, based on precedents in other cellular systems where fusion is a highly regulated process.

To our knowledge, membrane fusion processes in the ROS have not been studied as have such processes in other biologic systems. The self-quenching, lipophilic, fluorescent probe octadecylrhodamine B chloride (R$_{18}$, a probe used extensively to characterize virus-vesicle and cell-vesicle fusion) was used to measure the rate of membrane fusion. To begin studying these membrane fusion processes, the fusogenic properties of the disc membrane were examined. Our studies represent the first step in developing a suitable fusion assay of ROS constituents and lend insight into the dynamic processes surrounding ROS maintenance.

Materials and Methods

Preparation of Large Unilamellar Vesicles (LUVs)

Transphosphatidylated (from egg phosphatidylcholine [PC]) phosphatidylethanolamine (trans-PE) was obtained from Avanti Polar Lipids (Birmingham, AL). The trans-PE LUVs were prepared as described previously. The trans-PE was hydrated in 100 mM NaCl, 10 mM glycine, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 9.5 (extrusion buffer). The lipid suspension underwent five freeze–thaw cycles followed by ten extrusions through a polycarbonate membrane with 0.1-μm pores (Nucleopore, Pleasanton, CA). The phosphate content of the extruded material was determined, and the vesicles were formulated to contain 1 μmol/ml of phosphate before use. All vesicles were stored on ice under nitrogen gas and used in 2–3 days.

Preparation of Disc Lipid Vesicles

Disc lipid vesicles were prepared as described previously. The disc membranes were extracted with two volumes of 2:1 chloroform and methanol, essentially as described. The extracted lipids were dried under nitrogen and lyophilized to remove trace amounts of chloroform. The disc lipids were hydrated in extrusion buffer and underwent five freeze–thaw cycles. The formation of large vesicular species was confirmed by negative-stain transmission electron microscopy. The vesicles were stored on ice under nitrogen gas and used in 2–3 days. They were formulated to contain 1 μmol/ml of total phosphate in extrusion buffer before use. The fatty acid composition of the disc lipid vesicle preparation corresponded to previously published values.

Preparation and Labeling of Disc Membranes

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

The R$_{18}$ was obtained from Molecular Probes (Junction City, OR). Typically, it was added to approximately 3–5 mol% relative to the phospholipid. The disc membranes were labeled with R$_{18}$ as described. Briefly, 10 nmol of R$_{18}$ in 10 μl of ethanol was added for each milligram of rhodopsin in a total volume of 1 ml. The mixture was vortexed and allowed to incubate at room temperature for 1 hr. Labeled discs were separated from unincorporated R$_{18}$ by passing the mixture through a Sephadex G-75 column (Pharmacia, Piscataway, NJ) and eluting labeled material with 100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA, pH 7.5.

Papain Proteolysis of Disc Membranes

The ROS disc membranes were treated with papain as described previously after labeling with R$_{18}$. Briefly, R$_{18}$-labeled discs were resuspended in 100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA, with 5 mM cysteine, pH 7.2, to a final rhodopsin concentration of 1.5 mg/ml. The mixture was incubated with papain for 30 min in the dark. The reaction was stopped with the addition of iodoacetamide to a final concentration of 10 mM. The papain-treated discs were washed and resuspended in 100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA, pH 7.2.

The extent of papain proteolysis was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described. All gels contained acrylamide 12%. Protein was visualized using Coomassie blue staining. The protein concentrations were quantified using an LKB (Washington, DC) Ultrascan XL enhanced laser densitometer, using known concentrations of rhodopsin as a standard. This type of proteolysis of the disc membranes resulted in degradation of 85–90% of the rhodopsin with a distribution of protein fragments of apparent molecular weights of 34,000; 26,000; 19,000; and 10,000. In addition, 10–15% of the rhodopsin was found in a band of molecular weight of 40,000. The proteolysis pattern observed was similar to that reported previously. No effect of labeling was observed on the proteolysis of rhodopsin.

Fusion Assays

All fluorescence measurements were done on an SLM (Urbana, IL) 8000D spectrofluorometer. The
fusion assays were done, essentially as described previously.\textsuperscript{19} in a dimly lit room. The LUVs were diluted to 1 \( \mu \)mol/ml total phosphate in 100 mM NaCl and 10 mM glycine, pH 9.5. The LUVs (1 ml) first were allowed to equilibrate to the appropriate temperature for 5 min. To initiate fusion, 50 \( \mu \)l of 2 M sodium acetate–acetic acid buffer and 50 \( \mu \)l of \( \text{R}_{18} \)-labeled discs were added simultaneously to the vesicles. The pH of the solution was reduced to approximately pH 7.2. The final fusion assay volume was 1.10 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 the 2 M sodium acetate–acetic acid buffer was taken as a baseline. We determined the 100% fluorescence intensity by adding 100 \( \mu \)l of 10% Triton X-100 to the vesicles.

**Separation of Fused Species**

Fusion of the various vesicles with \( \text{R}_{18} \)-labeled discs was confirmed using sucrose density-gradient centrifugation. The LUVs, \( \text{R}_{18} \)-labeled discs, and fused mixtures of these two species underwent sucrose density-gradient centrifugation. We used gradients of 0–50 w/w sucrose in distilled water. The gradients were centrifuged at 27,000 rpm in an SW-28 rotor (Beckman Instruments, Fullerton, CA) for 2 hr and the bands collected. Before assay, the refractive index of the isolated species was measured using a refractometer. The fractions were assayed for phosphate and protein content.

**\( ^{31} \text{P} \text{NMR} **

Both trans-PE and disc lipid vesicles were prepared as described. The pH of the vesicles was reduced to 7.2 to mimic fusion conditions. We obtained \( ^{31} \text{P} \) nuclear magnetic resonance (NMR) spectra with a JEOL (Boston, MA) FX270 Fourier transform spectrometer with a broad band probe in 10-mm tubes at 30°C. Normally, approximately 2000 transients are collected with a repetition rate of 1 sec. A fully phased (32 pulse) Hahn echo sequence is used with a 40-\( \mu \)sec echo. Data was collected before refocusing the echo, and the free induction decay was transformed from the top of the echo. The \( ^{1} \text{H} \) decoupler was gated “on” during acquisition and “off” the rest of the time to prevent sample heating. The \( ^{31} \text{P} \) NMR spectra were obtained as a function of temperature.

The relative abundance of isotropic phase was determined using spectral subtraction techniques. In essence, the bilayer powder pattern was subtracted from the observed spectrum, and the area of the remaining isotropic resonance was determined. The result was expressed as \% isotropic [ie, (area isotropic/total resonance area) \times 100], using a graphics tablet package (MAC PLOT, Apple Computers).

**Additional Assays**

Phosphate was measured as described.\textsuperscript{20,21} Cholesterol was determined as reported previously,\textsuperscript{22} as was protein.\textsuperscript{23} All spectral measurements were done on an SLM Aminco (Urbana, IL) 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 40,000.

For fatty acid analysis, the fatty acid methyl esters were generated by mild alkaline methanolysis.\textsuperscript{24} The fatty acid methyl esters were analyzed on a Chrompak-Packard (Raritan, NJ) Model 439 gas chromatograph, equipped with a flame ionization detector and Varian (Boston, MA) 4270 integrator. The fatty acid methyl esters were separated isocratically on a Supelcowax-10 wide-bore column (Supelco, Bellefonte, PA). Column temperature was maintained at 240°C, injector temperature at 250°C, and detector temperature at 300°C. We compared peaks obtained with a reference methyl ester mixture (no. 4-7015, PUFA mix 2; Supelco) to identify most experimental peaks.

**Results**

**Fusion of \( \text{R}_{18} \)-Labeled Discs With Phospholipid Vesicles**

The initial rates of fusion of ROS discs with two different types of lipid vesicles were measured by following the relief of fluorescence self-quenching of \( \text{R}_{18} \)-labeled disc membranes resulting from fusion with unlabeled LUVs as described. Upon fusion of an \( \text{R}_{18} \)-labeled disc membrane with an unlabeled membrane, the probe diffuses into the unlabeled membrane, reducing its effective membrane density. This relieves some of the quenching and an increase in fluorescence is observed. The trans-PE LUVs were formed at pH 9.5, a pH at which they do not fuse. To initiate fusion, the pH of the mixture was reduced to 7.2, where the trans-PE LUVs can fuse, with the simultaneous addition of the labeled disc membranes. The change in fluorescence seen as a function of time is shown at 37°C (Fig. 1). This fluorescence change reflects uniquely the fusion of ROS disc membranes with the PE LUVs (not LUV–LUV fusion) because the \( \text{R}_{18} \) is in the discs.

The initial rates of fusion of trans-PE LUVs and disc membranes are shown as a function of temperature (Fig. 2). These data demonstrate that ROS disc membranes can fuse with other membranes, eg, trans-
Fig. 1. Change in fluorescence of R$_{18}$ upon fusion of disc membranes and transPE LUV. A representative tracing of the change in fluorescence observed with the R$_{18}$ mixing assay is shown. Fusion was initiated with the simultaneous addition of R$_{18}$-labeled disk membranes and sodium acetate buffer to drop the pH to 7.2; this addition is labeled la. One hundred percent fluorescence intensity was calculated with the addition of Triton X-100; this addition is labeled lb.

Fig. 2. Initial rates of disc membrane–transPE LUV fusion (squares) and DOC inhibition (diamonds) of this fusion as a function of temperature. The rates of disc membrane–transPE fusion was determined by the R$_{18}$ lipid-mixing assay using 20 µg of labeled discs, as described in Methods. Fusion was initiated with the simultaneous addition of R$_{18}$-labeled disc membranes and sodium acetate buffer to drop the pH to 7.2. DOC was added to the LUV before the initiation of fusion in a 1:1 (mole of phospholipid to mole of DOC) ratio. The data shown represent an average of three separate experiments.

Table 1. Isolation of fused membrane species using sucrose density gradient centrifugation

<table>
<thead>
<tr>
<th>Membrane species</th>
<th>Refractive index</th>
<th>Phosphate recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>TransPE LUV</td>
<td>1.3380</td>
<td>98%</td>
</tr>
<tr>
<td>R$_{18}$ discs</td>
<td>1.3778</td>
<td>90%</td>
</tr>
<tr>
<td>Fusion mixture</td>
<td>1.3420</td>
<td>93%</td>
</tr>
</tbody>
</table>

Through a series of studies on membrane fusion systems done previously, several useful inhibitors of membrane fusion were identified. These are compounds that, when added to the medium before a fusion assay, inhibit membrane fusion. Because deoxycholate (DOC) inhibits viral membrane fusion, but not vesicle–vesicle fusion involving N-methyl PE LUVs (Yeagle PL, unpublished results), the ability of DOC to inhibit disc membrane–LUV fusion was investigated. These experiments showed that fusion of disc membranes with trans-PE LUVs was inhibited by the addition of DOC in a 1:1 (mole of phospholipid to mole of DOC) ratio at all temperatures studied (Fig. 2), without solubilizing the membranes.

One of the artifacts that can arise from the R$_{18}$ lipid mixing assay is a spurious signal resulting from the spontaneous transfer of R$_{18}$ from the donor membrane to the target membrane. As a control, therefore, on the fidelity of the R$_{18}$ assay for monitoring disc membrane fusion, R$_{18}$-labeled discs were mixed with egg PC membranes at pH 7.2. These PC vesicles were not fusogenic (data not shown). Under these conditions, no change in fluorescence of R$_{18}$ was observed with time, suggesting that there was no transfer of the R$_{18}$ probe independent of fusion in this system. As a further control, labeled ROS disc membranes were mixed with unlabeled disc membranes. No change in fluorescence intensity of R$_{18}$, indicating spontaneous probe transfer, was observed. These data also show that ROS discs prepared as described do not fuse spontaneously with each other under these conditions.

To confirm further that the increase in fluorescence observed from this R$_{18}$ assay reflected membrane fusion between ROS discs and trans-PE LUVs, we subjected the trans-PE LUVs, R$_{18}$-labeled discs, and mixtures of these two species to sucrose density-gradient centrifugation. Unfused R$_{18}$-labeled disc membranes were expected to be recovered at a higher density because of disc membrane proteins. The fusion product of disc membranes and trans-PE LUVs was expected to be recovered at a lower density than the disc mem-

TransPE LUVs, R$_{18}$-labeled discs, and fused mixtures of these two species were subjected to sucrose density gradient centrifugation. The 0–50 w/w sucrose gradients were centrifuged and bands collected as described in Methods. The refractive index of the isolated species was measured using a refractometer. The amount of total phosphate recovered in the isolated bands is given as percent recovered.
branes because of incorporation of the lipid of the target vesicles after fusion. As seen in Table 1, controls containing pure trans-PE LUVs were recovered in a band at the top of the gradient corresponding to a refractive index of 1.3380. Controls containing only R18-labeled discs were recovered at approximately 28% sucrose. The apparently fused mixture of trans-PE LUVs and R18-labeled discs was recovered at approximately 10% sucrose; this was consistent with the incorporation of disc membrane protein into the trans-PE LUVs. This latter band of lipid and protein was pink, characteristic of unbleached rhodopsin from the ROS disc membranes. Thus, the position of the rhodopsin shifted from a high density to a lower density after experimentation, suggesting that fusion between ROS discs and trans-PE LUVs had occurred. In all cases, more than 90% of the total phosphate was recovered in the bands described.

Two types of lipid-mediated fusion pathways have been described in membrane fusion studies of lipid bilayers. One mechanism involved calcium and phosphatidylserine (PS, cardiolipin), which may involve a gel (dehydrated) phase formation by the calcium-PS complex. The second pathway may involve isotropic structures, identified as isotropic resonances in $^{31}$P NMR spectra of lipid dispersions as possible intermediates in membrane fusion. A number of elegant studies confirmed the correlation between the appearance of Is and membrane fusion in vesicle systems containing PE.

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**Table 2. Temperature-dependent change in isotropic phase (Is) of transPE LUV**

<table>
<thead>
<tr>
<th>Temp, °C</th>
<th>% Isotropic</th>
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</thead>
<tbody>
<tr>
<td>20</td>
<td>4.2</td>
</tr>
<tr>
<td>25</td>
<td>9.6</td>
</tr>
<tr>
<td>30</td>
<td>10.7</td>
</tr>
<tr>
<td>35</td>
<td>14.7</td>
</tr>
<tr>
<td>40</td>
<td>17.6</td>
</tr>
</tbody>
</table>

The phase behavior of transPE LUV was followed as described in the Methods. The extent of the isotropic phase is measured as described in Methods, and is represented as an area percent of total.

A powerful, nonperturbing probe, $^{31}$P NMR spectroscopy is used extensively to examine the structure and dynamics of membranes. Several distinct $^{31}$P NMR line shapes are distinguishable and represent the various phase structures assumed by phospholipids. These line shapes are predictable from the motional averaging associated with the membrane phases. The lipid bilayer, or lamellar phase, is the structure formed by the lipids of cell membranes. Because bilayers are highly anisotropic, a characteristic powder pattern can be identified with the phospholipid bilayer configuration. The observed bilayer powder pattern is distinguishable easily from spectral line shapes associated with hexagonal and isotropic phase. The Is that may be involved in membrane fusion are characterized by a single narrow symmetric resonance. Because $^{31}$P NMR spectroscopy is sensitive to all phospholipids in a sample, this technique can show the relative populations of each of the phase structures present.

To determine the possible mechanism by which disc membranes fused with trans-PE LUVs, the formation of Is in trans-PE LUVs was examined as a function of temperature using $^{31}$P NMR spectroscopy as described. A representative spectrum is shown in Figure 3, illustrating the extent of Is. The shaded area on the spectrum represents the isotropic resonance. The extent of formation of Is is quantified in Table 2 from the $^{31}$P NMR spectra. The increase in the prevalence of Is correlates with the increase in the initial rates of membrane fusion (Fig. 2). These results suggest that fusion between trans-PE LUVs and disc membranes may involve a packing defect in the lipid bilayer, characterized as an isotropic resonance in the $^{31}$P NMR spectra of the trans-PE LUVs and similar to previous observations.

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**Modulation of Disc Membrane-Phospholipid Vesicle Fusion**

The calcium ion has been shown to play a role in the phagocytosis of old discs by the pigment epithe-
Fig. 4. Modulators of disc membrane-transPE fusion. The R \textsubscript{14} mixing assay was used to follow (A) disc membrane-transPE LUV fusion, and the effects of (B) papain proteolysis, (C) calcium, and (D) EGTA on this fusion at 37°C. (B) The R \textsubscript{14}-labeled discs were papain-proteolyzed after labeling and were added to the transPE LUV simultaneously with sodium-acetate buffer. (C) Calcium was added to the transPE LUV simultaneously with R \textsubscript{14}-labeled disc membranes to a final calcium concentration of 0.9 mM, as described in Methods. (D) EGTA was added simultaneously with the labeled disc to a final EGTA concentration of 0.05 mM, as described in Methods. The data shown represent an average of three separate experiments.

Calcium also influences the phase behavior of the disc membrane lipids that ultimately may influence their ability to fuse.\textsuperscript{35} Thus, the role of calcium in disc membrane phospholipid LUV fusion was investigated. The effect of calcium on disc membrane phospholipid vesicle fusion was determined using the R \textsubscript{18} mixing assay as described. The initial rates of disc membrane-trans-PE vesicle fusion were determined at 37°C. Calcium was added in a 2 M sodium acetate-acetic acid buffer and the pH reduced to 7.2, with the simultaneous addition of labeled disc membranes. As shown in Figure 4, calcium ion, at a concentration of 0.9 mM, had no effect on disc membrane-trans-PE vesicle fusion.

A potent calcium chelator, EGTA, was added to the fusion mixtures to remove trace levels of calcium that might be associated with the discs. The chelator was added in a sodium acetate-acetic acid buffer, and the pH was reduced to 7.2, with the simultaneous addition of labeled disc membranes. As shown in Figure 4, EGTA had no effect on disc membrane-trans-PE vesicle fusion.

To investigate the role of the cytoplasmic surface rhodopsin in disc membrane fusion, the disc membranes were treated with papain as described. During fusion of ROS disc membranes with trans-PE LUVs (Fig. 4), papain proteolysis of the surface of the disc membranes resulted in an almost twofold increase in the initial rate of fusion observed at 37°C. In all papain experiments, more than 85% of the rhodopsin was proteolyzed, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteolysis pattern observed was similar to that reported previously.\textsuperscript{16}

Disc Membrane–Disc Lipid Vesicle Fusion

After the initial fusion parameters were established with the model systems described, the ability of disc membrane to fuse with membrane vesicles made of disc lipids was examined. Disc lipid vesicles were prepared as described. Fusion of disc membranes with disc lipid vesicles was induced with the addition of R \textsubscript{18}-labeled discs and a drop in pH to 7.2.

The initial rates of fusion observed between disc membranes and disc lipid vesicles are shown in Figure 5 as a function of temperature. Disc membranes fused with disc lipid vesicles. This contrasted with no observable disc membrane–disc membrane fusion. In this case, there was an increase in the rate of fusion as a function of temperature, with a maximum at approximately 37°C. Disc membrane–disc lipid vesicle fusion was inhibited by DOC added in a 1:1 molar ratio at all temperatures studied (data not shown).

To confirm that disc membranes were fusing with disc lipid vesicles, sucrose density-gradient experiments were done as described. Disc lipid vesicles were found to band at a position in the sucrose density gradient equal to a refractive index of 1.3380. When a mixture of disc membranes and disc lipid vesicles was allowed to fuse (as measured by an increase in fluorescence), the rates of disc membrane-disc lipid vesicle fusion were determined by the R \textsubscript{14} lipid-mixing assay using 20 \mu g of labeled discs, as described in Methods. Fusion was initiated with the simultaneous addition of R \textsubscript{14}-labeled disc membranes and sodium acetate buffer to drop the pH to 7.2. The data shown represent an average of three separate experiments. Error bars representative of ± 1 SD are given for all initial rates of fusion as a function of temperature are given.

Fig. 5. Initial rates of disc membrane–disc lipid vesicle fusion. The rates of disc membrane–disc lipid vesicle fusion were determined by the R \textsubscript{14} lipid-mixing assay using 20 \mu g of labeled discs, as described in Methods. Fusion was initiated with the simultaneous addition of R \textsubscript{14}-labeled disc membranes and sodium acetate buffer to drop the pH to 7.2. The data shown represent an average of three separate experiments. Error bars representative of ± 1 SD are given for all initial rates of fusion as a function of temperature are given.
Fig. 6. Modulators of disc membrane-disc-lipid vesicle fusion. The R18 mixing assay was used to follow (A) disc membrane-disc lipid vesicle fusion, and the effects of (B) calcium, (C) EGTA, and (D) papain proteolysis of the disc membranes on this fusion at 37°C. (B) Calcium was added to the disc lipid vesicles simultaneously with R18-labeled disc membranes to a final calcium concentration of 0.9 mM, as described in Methods. (C) EGTA was added simultaneously with the labeled disc to a final EGTA concentration of 5.0 mM, as described in Methods. (D) ROS disc membranes were proteolyzed, as described in Methods. In all experiments, greater than 85% of the rhodopsin was found to be proteolyzed. The data shown represent an average of three separate experiments.

Discussion

The first set of experiments tested the ability of disc membranes to fuse with PE LUVs. Membranes rich in PE or in derivatives of PE previously spontaneously fused with themselves. The fluorescence lipid-mixing assay we used selectively detected fusion of the discs with the LUVs and was blind to any fusion among the LUVs. Our data from these fusion experiments revealed that the ROS disc membranes can fuse with PE LUVs. The temperature dependence of this membrane fusion was similar, although not identical to, the temperature dependence of membrane fusion reported previously for N-methyl-PE-rich LUV fusion.31,32 The following, therefore, is a brief summary of previously published findings.

Membrane fusion has been suggested to include the

Table 3. Temperature-dependent change in isotropic phase (Is) of disc lipid vesicles

<table>
<thead>
<tr>
<th>Temp, °C</th>
<th>% Isotropic</th>
</tr>
</thead>
<tbody>
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<td>25</td>
<td>10.8</td>
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<td>40</td>
<td>10.2</td>
</tr>
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<td>45</td>
<td>9.3</td>
</tr>
</tbody>
</table>

The phase behavior of transPE LUV was followed as described in the Methods. The extent of the isotropic phase is measured as described in Methods, and is represented as an area percent of total.
following events: (1) aggregation or adhesion of the membranes that will fuse; (2) close approach of the lipid bilayers of the membranes, leading to removal of some of the water separating the membranes (partial dehydration); (3) destabilization of the bilayer at the point of fusion (two bilayers closely opposed will not fuse spontaneously by themselves); and (4) mixing of the bilayers and ultimate separation from the point of fusion into the new membrane structure(s). 32 33

It was suggested previously that punctate, nonlamellar structures in the lipid bilayer of PE-rich membranes under some conditions might provide an important point of bilayer destabilization that would facilitate membrane fusion. 37 These structures were shown in the 31P NMR spectra of the LUVs as isotropic resonances and in freeze-fracture electron micrographs of the LUV as lipid particles. 38,39 It was suggested that these structures might be intermediates in the membrane fusion of these PE-rich LUVs. 31,32 An increase in membrane fusion of these LUVs with temperature was correlated with an increase in the relative intensity of the isotropic 31P NMR resonance.

During fusion of disc membranes with PE LUVs, an increase in the relative intensity of the isotropic 31P resonance of the PE LUVs was observed concomitant with an increase in the rate of membrane fusion as detected by the lipid-mixing assay. The increase in both phenomena was less pronounced than in the case of N-methyl dioleoyl-PE LUVs reported previously. However, as in these LUVs, 31 the initial rate of membrane fusion of ROS discs with PE LUVs (as detected by the R18 assay) was directly proportional to the relative resonance intensity of the isotropic 31P NMR resonance from those PE LUVs. These data suggested that the pathway of membrane fusion for disc membranes and PE LUVs was similar to that for the N-methyl dioleoyl-PE LUVs reported previously.

The hypothesis of a lipid-mediated membrane fusion pathway for disc membrane fusion with PE LUVs was strengthened by the observation of an increase in the rate of membrane fusion after papain proteolysis of the disc membrane. Such proteolysis led to loss of a peptide on the carboxyl terminal of the photoreceptor and cleaved the loop region involved in transducin binding. 16 Although most of the rhodopsin remained associated with the disc membrane, papain proteolysis removed a substantial portion of the extramembranous portions of the membrane proteins and the peripheral membrane proteins of the disc membrane. The lipid-mediated fusion mechanism we described relied on close contact of the lipid bilayers to facilitate the formation of the nonlamellar structures. 37,40 Therefore, any modification that would enhance the close apposition of the lipid bilayers might be expected to enhance the fusion observed. Removal of any protein capable of inhibiting bilayer contact between disc membranes and PE LUVs would be expected, therefore, to enhance membrane fusion. This was observed.

Using phospholipid vesicles made of the total lipid extract of ROS discs was the next step toward biologic fusion. Our data were consistent with membrane fusion between the ROS discs labeled with R18 and disc lipid vesicles. The temperature dependence of the initial rate of membrane fusion was different from that observed with ROS disc membrane–PE LUV fusion. There was no correlation between the relative intensity of an isotropic 31P NMR resonance and the initial rate of fusion. The isotropic resonance observed in most of the 31P NMR spectra was at the threshold of detectability and represented less than 1% of the total lipid. This low level of I0 in the N-methyl dioleoyl-PE LUV fusion system would have led to only modest initial rates of fusion. The dramatic increase in the initial rate of fusion between 25 and 37°C therefore was not explained readily by the formation of significant nonlamellar structures in the lipid bilayer of the target vesicles.

High temperatures (> 37°C) and papain treatment of the disc membranes inhibited ROS disc fusion with disc lipid vesicles. High temperature could have altered the conformation of the extramembranous portions of some of the disc membrane proteins. This would not necessarily have affected the absorption spectrum of rhodopsin because intramembranous portions of membrane proteins appear to be more stable than extramembranous ones. 41 Proteolysis removed some of the exposed (extramembranous) portions of the rhodopsin (carboxyl terminus) and other disc membrane proteins and inhibited this fusion. These observations suggest that an extramembranous portion of some disc protein(s) may have been involved in promoting membrane fusion between disc membranes and vesicles of disc membrane lipids. Removal (by proteolysis) or change in conformation (by increased temperature) of disc membrane proteins therefore might render the discs incapable of fusion with membranes that were not themselves fusogenic. Removal of the extramembranous portions of the disc membrane proteins also may have changed the net charge on the lipid bilayer which might have altered the ability of the discs to fuse.

These considerations led to the conclusion that disc membrane–disc lipid vesicle fusion proceeded by a different mechanism than disc membrane–PE LUV fusion. Our findings suggest that disc membrane–disc lipid vesicle membrane fusion may be a protein-mediated event; disc membrane–PE LUV membrane fu-
sion may be a lipid-mediated event. Therefore, discs may be able to fuse by at least two different mechanisms.

The continuous renewal of ROS disc membranes probably involves multiple fusion events. The coordinated processes of disc renewal and shedding maintain a relatively constant length of the outer segment in normal, healthy rod cell. Although it is clear that fusion occurs at the tip and base of the outer segment, fusion events must not occur along the length of the outer segment. Thus, the control of membrane fusion is critical to the normal functioning of the ROS. An understanding of the mechanism governing fusion in the ROS will provide insight, not only into the normal formation and shedding of discs, but also in understanding ROS loss in degenerative diseases. Understanding fusion mechanisms during ROS formation also may provide clues to the lack of discrete discs (separate from the plasma membrane) in the cone outer segment, which may have a relationship to the differential sensitivity to light of these two photoreceptors.

In our experiments, we have begun to understand some of the factors that stimulate ROS discs to fuse with other membranes. Under defined conditions, disc membranes have been shown to fuse with PE or disc lipid vesicles. Studies currently are ongoing to characterize disc membrane–plasma membrane fusion.

Key words: membrane fusion, ROS discs

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References

29. Silvius JR and Gagne J: Lipid phase behavior and calcium-in-