Cholesterol Heterogeneity in Bovine Rod Outer Segment Disk Membranes

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Abstract

Rod outer segment disk membranes have been used to study visual transduction events. Numerous studies have also focused on protein-lipid interactions in these membranes. The possible heterogeneity of the disk membrane composition has not been addressed in such studies. Freeze fracture studies (Andrews, L. D., and Cohn, A. I. (1979) J. Cell Biol. 81,215–220; Caldwell, R., and McLaughlin, B. (1985) J. Comp. Neurol. 236, 523–537) suggest a difference in cholesterol content between newly formed and old disks. This potential heterogeneity in disk membrane composition was investigated using digitonin. Osmotically intact bovine rod outer segment disk membranes prepared by Ficoll flotation were separated based on the cholesterol content of the disks. The addition of digitonin to disk membrane suspensions in a one-to-one molar ratio with respect to cholesterol produced an increase in the density of the membranes in proportion to the amount of cholesterol present. The digitonin-treated disks were separated into subpopulations using a sucrose density gradient. Disks were shown to vary in cholesterol to phospholipid ratio from 0.30 to 0.05. The ratio of phospholipid to protein remained constant in all disk subpopulations at approximately 65 phospholipids per protein. No significant change in the fatty acid composition of the disks was observed as a function of change in cholesterol content. This work demonstrates compositional heterogeneity in disk membranes which may ultimately affect function.

The initial events of visual transduction take place in the disk membranes located in the outer segment of rod cells. The disks are flattened vesicles stacked along the length of the rod outer segment (ROS). They are formed from evaginations of the plasma membrane. However, after formation of the disk is completed, its lipid bilayer is no longer continuous with the plasma membrane. As the disks age, they move up the length of the rod cell, with older disks being phagositized at the apical end by the pigment epithelium. Rhodopsin, the light receptor protein, remains in a given disk as that disk travels up the rod, while the membrane phospholipids undergo metabolic turnover (Schichi, 1973).

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†The abbreviations used are: ROS, rod outer segment; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SUV, small unilamellar vesicles.
The average ROS disk membrane phospholipid composition is well documented (Fliesler and Anderson, 1983). It consists of approximately 35% phosphatidylcholine, 40% phosphatidylethanolamine, 13% phosphatidylserine, and less than 2% phosphatidylinositol. The disk membrane contains a high proportion of unsaturated fatty acids (Stone et al., 1979). The specific role of the individual phospholipids and the fatty acid unsaturation have not been well established. Cholesterol is present at about 10 mol % (Fliesler and Schröepfer, 1982). The role of cholesterol in visual transduction is also not known. This compositional information is an average for all disks. Thus, the composition of disks at the tip of the rod outer segment may be different from that at the base. Freeze fracture electron microscopic evidence suggests that the cholesterol content of the newly formed disks at the base is higher than in disks at the apical tip of the ROS (Andrews and Cohn, 1983; Caldwell and McLaughlin, 1985).

We have investigated this reported cholesterol variation by exploiting the membrane density change induced by a digitonin-cholesterol interaction (Lange and Steck, 1985). We have demonstrated that disks can be separated into subpopulations which vary in lipid composition. This variation in disk composition may reflect the relative position of the disk in the ROS.

MATERIALS AND METHODS

Disk Membrane Preparation

Retinal rod outer segment disk membranes were prepared from frozen bovine retinas (J. Lawson Inc., Lincoln, NE) as described by Smith et al. (1975). All manipulations of the rod outer segment and disk membranes were performed under a Kodak 1A red filter. The isolated disks were washed and resuspended in 10 mM Hepes, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.0 (buffer A) to a final rhodopsin concentration of 4–6 mg/ml. The buffers used were made 1 mM in EDTA, 0.5 mM in dithiothreitol and perfused with nitrogen or argon to reduce lipid oxidation (Stone et al., 1979).

Digitonin was purchased from Sigma and recrystallized according to the method of Bridges (1977). Stock solutions of digitonin were made 50–100 mg/ml in 90% EtOH. To completely solubilize the digitonin, this solution was gently heated. The stock digitonin solution was stored at 10 °C. The digitonin was added in a 1:1 mol of digitonin/mol of cholesterol ratio from the stock solution to freshly prepared disks. Control disks were treated with an equal volume of 90% EtOH. Both disk preparations were incubated at 5 °C for 1 h. Both digitonin-treated and control disks were layered onto continuous sucrose density gradients. The gradients were spun in a SW-28 swinging bucket rotor at 22,500 rpm for 7 h. Additional centrifugation for up to 10 h produced no difference in the banding pattern. The sucrose density gradients used were isoosmotic, 20–40% w/w or 0–50% w/w sucrose in 50 mM sodium acetate buffer, pH 6.0.

Separation of Disk Subpopulations

The sucrose gradients were fractionated from the bottom using a Gilson minipuls 2 pump. The elution was monitored using a Gilson Model 111 LC detector set at 254 nm, and the
absorbance was recorded on a Linear 1200 Graphic Controls chart recorder. Fractions were collected under dim red light to avoid rhodopsin bleaching and light-induced lipid oxidation. The samples were either assayed immediately or pelleted in 50 mM sodium acetate buffer, pH 6.0 (45,000 rpm for 30 min) and stored frozen.

**Preparation of Modified Disks**

Disks containing a uniform cholesterol distribution were produced by allowing the disks to incubate at least 8 h in buffer A, after isolation. These disks will be referred to as cholesterol-equilibrated disks.

ROS disk membranes were depleted of approximately 50% of their original cholesterol content by incubating the disks with SUVs (small unilamellar vesicles) as described by Yeagle and Young (1986) (SUV preparation is described below). This technique was shown to apply to disk membranes in this laboratory. Briefly, the disk membranes were incubated with phosphatidylcholine SUVs containing an equimolar amount of phosphate, at room temperature overnight. The disks were washed twice in 50 mM sodium acetate, pH 6.0, and resuspended in this same buffer. The cholesterol-depleted disks were treated with digitonin as described above.

To prepare SUVs, egg phosphatidylcholine obtained from Avanti Polar Lipids was hydrated with buffer A to a final phosphate concentration of 10 mg/ml. The lipid dispersion was sonicated to clarity (three times for 3 min each) in a Branson 350 sonifier. The SUVs were separated from the larger species by centrifugation (40,000 × g for 80 min). The SUVs remain in the supernatant while the larger species pellet (Barenholtz et al., 1977).

Disk membrane cholesterol was converted to cholestanone by treatment with cholesterol oxidase as described by Lange and Steck (1985). These disks are treated with digitonin based on their original cholesterol content as described above.

**Phospholipid and Fatty Acid Composition**

The disk lipids were isolated from the sucrose density gradient fractions using a modification of the extraction method of Bligh and Dyer (1959). Lipid samples were extracted with the addition of 2.5 ml of MeOH and 1.5 ml of CHCl₃. Samples were vortexed for 5 min and centrifuged. The resulting pellet was re-extracted. The two supernatants were pooled and further extracted with 1.0 ml of H₂O and 1.0 ml of CHCl₃. The lipids were isolated from the lower organic phase, dried down under nitrogen, and assayed for fatty acid.

For fatty acid analysis, the fatty acid methyl esters were generated from the dried samples by either mild alkaline methanolysis or strong acid methanolysis. In the case of strong acid hydrolysis, the lipid samples were resuspended in 5 ml of MeOH:H₂O:HCl (4.1:0.47:0.43) and incubated under nitrogen at 70 °C overnight. The fatty acid components were extracted three times with 1 ml of hexane. Prior to injection onto the GC, the samples were dried down and resuspended in 50–100 μl of hexane.

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2K. House, D. Badgett, and A. Albert, submitted for publication.
For mild alkaline metholysis, the dried samples were resuspended in 2 ml of chloroform, and then 2 ml of 0.6 M NaOH in methanol was added. The samples were incubated at room temperature for 1 h. The reaction was stopped by the addition of 1.3 ml of 1 M HCl and 0.5 ml of H₂O. The lower chloroform layer, containing the fatty acid methyl esters and intact sphingolipids, was removed and dried down for further analysis.

The fatty acid methyl esters were analyzed using a Model 3700 Varian gas chromatograph equipped with a flame ionization detector and a Varian 4270 integrator. Separation was carried out on a column (6 feet × ¼ inch) containing 10% SP-2330 on 80/100 Supelcoport (Supelco, Inc.). The column was maintained at 195 °C. The temperature of the injector was 200 °C, and the detector was maintained at 300 °C. In later runs, a temperature program was used in order to lower the retention time of the 22:6 fatty acid to under 20 min. The temperature program began with an initial column temperature of 160 °C, and the temperature was increased at a rate of 6 °C/min to a final temperature of 220 °C. Identification of most peaks was by comparison with reference methyl ester mixtures, PUFA No. 189-15, 189-3, 189-1, from Sigma. Assignments were firmly established by gas chromatography-mass spectrometry of disk membranes treated with digitonin.

**Sodium Dodecyl Sulfate Gel Electrophoresis**

Gel electrophoresis was performed using the methods of Laemmli (1970), as modified by Smith et al. (1975). Protein concentrations were quantitated using an LKB UltraScan XL enhanced laser densitometer, using known concentrations of rhodopsin as a standard.

**Assays**

Phosphate was determined as described by Bartlett (1959) and modified by Litman (1973). Cholesterol was determined as described by Allain et al. (1974). Protein was determined as described by Lowry et al. (1951). 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 40,000. Meta I to total rhodopsin was calculated as described by De Grip et al. (1983) using the buffer system of Baldwin and Hubbell (1985).

**RESULTS**

**Digitonin Modifications the Buoyant Density of Disks due to Their Cholesterol Content**

Digitonin will interact with cholesterol in cellular membranes in a manner which results in an increase in membrane density (Lange and Steck, 1985). Therefore, this technique was used to investigate the reported variation in cholesterol content among disk membranes. Freshly isolated, osmotically intact ROS disk membranes were incubated at 0 °C with 1 mol of digitonin per mol of cholesterol. Both digitonin-treated disks and untreated control disks were centrifuged on a sucrose density gradient, pumped through a UV monitor, and fractions were collected. The profiles of the density gradients were monitored at 254 nm. Fig. 1A presents typical profiles for both the digitonin-treated disk membranes and the control disk.
membranes. Both control and digitonin-treated disks band with a maximum at approximately 35% sucrose, according to the UV measurements.

A portion of the digitonin-treated disks band at a higher density than observed with the control disks. This increase in density results from digitonin cholesterol complexation (Lange and Steck, 1985). If all disks were identical, the effect of digitonin would be uniform, and the density of all the disks would be altered proportionally to the cholesterol content. This would cause the disks to band at a higher percent sucrose, but digitonin treatment would not broaden the band on the gradient. Therefore, the observed broadening of the band is indicative of a heterogeneity in cholesterol composition of the disks. Those disks with higher cholesterol content move to higher densities upon digitonin treatment, while those with low cholesterol contents show little change in position on the sucrose density gradient upon treatment with digitonin.

The percent of initial cholesterol recovered in each fraction of digitonin-treated and control disks is shown in Fig. 1B. Sixty percent of the cholesterol is recovered at a single sucrose density position for the control disk membranes. The cholesterol recovery from the digitonin-treated membranes is spread uniformly over five fractions, reflecting the broadening in Fig. 1A.

**Digitonin Induces a Uniform Buoyant Change in a Disk Preparation with a Uniform Cholesterol Content**

Prolonged incubation of disk membranes permitted migration of cholesterol among disks until a more uniform cholesterol content was reached. This is consistent with previous studies of cholesterol migration into model and native membranes.2 When incubations of 8 h or more were permitted, digitonin treatment of the disks resulted in a density gradient profile shape like that of the control, but shifted to higher density. There was no apparent broadening. This result is consistent with a uniform cholesterol distribution among disk membranes. This result contrasts with the broadening observed from the freshly isolated native disk membranes.

**Removal of Cholesterol or Oxidation of Cholesterol to Cholestanone Eliminates the Digitonin-induced Change in Buoyant Density**

To lower disk membrane cholesterol content, ROS disks were incubated with phosphatidylcholine SUV which contained no cholesterol. As shown previously,2 cholesterol will migrate out of the disks and into the SUV, thereby depleting the disks of cholesterol. Using this approach, disk membrane cholesterol/phospholipid mole ratios can be reduced from 0.12 to 0.05. When treated with digitonin and subjected to sucrose density gradient centrifugation, these cholesterol-depleted disks show only a marginal shift to higher density. No broadening of the band was observed, since equilibration of the remaining cholesterol among all the disks occurred during the incubation time as described above.

Using cholesterol oxidase, cholesterol can be oxidized to cholestanone which no longer binds digitonin (Lange and Steck, 1985). This method requires only a 30-min incubation, which is insufficient for cholesterol redistribution. Digitonin treatment did not change the density profiles of disks treated with cholesterol oxidase relative to untreated controls.
The Cholesterol to Phospholipid Ratio of Digitonin-treated ROS Disk Subpopulations Correlates with Their Position on the Sucrose Gradient

The various sucrose density gradient fractions from digitonin-treated and control ROS disks were analyzed with respect to phospholipid and cholesterol. Fig. 2A shows the cholesterol/phospholipid mole ratios of the control disks (no digitonin added) as a function of position on the sucrose gradient. The figure includes data from five independent experiments. No change in this ratio is detected across the major portion of the band in the sucrose density gradient. The average cholesterol/phospholipid mole ratio is approximately 0.12, in agreement with previous reports of the cholesterol content of disk membranes (Fliesler and Anderson, 1983).

Digitonin-treated disks range in cholesterol/phospholipid mole ratio from 0.05 to 0.32 as a function of increasing density in the sucrose gradient. Fig. 2B represents the results from seven independent experiments with an exponential curve as a best fit function through these points. These experiments demonstrate that it is possible to separate ROS disk membranes based upon their cholesterol content.

Disk Subpopulations Have the Same Rhodopsin and Total Protein Content

The ratio of phospholipid to protein was determined for each fraction obtained from the sucrose density gradients for both control and digitonin-treated disks. No significant variation in the phospholipid/protein ratio as a function of density was observed in either control (data not shown) or digitonin-treated disks (Fig. 3). Therefore, the variation in density induced by treatment with digitonin reflects a variation in cholesterol, not protein or phospholipid content. The protein content was further examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyacrylamide gel tracings of the various fractions showed that opsin was the dominant protein, comprising greater than 90% of the total protein in all fractions (not shown).

Having established that the protein content in the disks does not vary with cholesterol/phospholipid ratio, two rhodopsin properties were investigated. The ratio of rhodopsin (as determined by the change in absorption at 550 nm upon bleaching) to total protein remained constant as a function of density in the digitonin-treated disks. The ratio of Meta I to total rhodopsin in low cholesterol disks was compared to an average ratio for all disks. Only low cholesterol disks were used to avoid using samples with high amounts of digitonin. An increase in this ratio would indicate an environment which blocks Meta I formation. No difference between the average control disks and low cholesterol disks was observed.

The Fatty Acid Composition of the ROS Disk Subpopulations Is Similar

Since a difference in the cholesterol content of the disks was demonstrated, the membrane fatty acid composition was investigated. Gas chromatography was used to determine the fatty acid composition of the extracted disk lipids. Total lipids extracted from digitonin-treated and control disks before separation on a sucrose density gradient were compared. Fatty acid methyl esters were prepared using strong acid methanolysis. There was no difference in the fatty acid retention times in the presence or absence of digitonin. The fatty
acid composition agreed well with previously published fatty acid compositions (Fliesler and Anderson, 1983).

The average fatty acid composition of the lower, middle, and upper portions of the sucrose gradient band of digitonin-treated disks is presented in Table I. The lower, middle, and upper portions have average cholesterol to phospholipid ratios of 0.27, 0.17, and 0.07, respectively. Together, they account for the total phospholipid of the gradient as indicated in Table I. Each fatty acid composition presented is an average of six points. These fatty acid composition values are within the range of reported values. However, the docosahexanoic acid (22:6) content determined probably represents a lower limit of this value due to the required time for the experimental manipulations. From Table I, it is apparent that there are no dramatic changes in lipid composition of disks with high and low cholesterol/phospholipid ratios.

**DISCUSSION**

In this study, the buoyant density of disk membranes was altered in direct relation to the amount of cholesterol in the membrane by addition of digitonin, 1:1 with cholesterol. It has been shown that digitonin will form a 1:1 complex with cholesterol in a membrane resulting in an increase in membrane density (Lange and Steck, 1985). The density profile of freshly isolated disks treated with digitonin exhibits a distinct broadening to a higher density than is observed in untreated disks. Disks with a uniform cholesterol distribution do not exhibit a broadened density profile. Oxidation of cholesterol to cholestanone, which cannot bind digitonin, or depletion of cholesterol in the disk membrane also yields preparations which do not exhibit broadening relative to control disks. These results suggest that the digitonin-induced buoyant density change is due to the concentration of intact cholesterol in the disk membrane.

This conclusion was confirmed by analysis of the cholesterol:phospholipid ratio of digitonin disk fractions isolated from the sucrose gradient. Digitonin-treated disks were separated into fractions which range in cholesterol:phospholipid ratios from 0.30 to 0.05. Untreated disks exhibited a constant cholesterol:phospholipid ratio with respect to density on a sucrose gradient. This result establishes that disk lipid composition is not uniform with respect to cholesterol content.

The distribution of rhodopsin, with respect to membrane cholesterol content, was also determined in these studies. Rhodopsin was shown to account for 90% of the total protein in the total disk membranes in agreement with previous studies (Schichi, 1973). Here it is shown that rhodopsin also accounts for 90% of the disk membrane protein in each of the disk subpopulations. The ratio of phospholipid to protein remains constant in the subpopulations of the disks, as a function of cholesterol content, at approximately 65 phospholipids per protein. Due to an increase in the cholesterol content among the disks, the total lipid:protein ratios increases across the gradient. These findings, which demonstrate compositional differences between disks, have an important bearing on the study of lipid rhodopsin interactions and on the behavior of extracted disk lipids. It has been tacitly assumed that the average disk lipid composition reflects the composition of individual disks.
This work shows that there are differences in disk membrane compositions which may have functional consequences.

This work also has important potential consequences for disk order and function in the ROS. Electron microscopy studies of filipin binding suggested cholesterol is present at higher levels in the newly formed disks at the base of the ROS than in the old disks at the apical tip (Andrews and Cohn, 1979 and Caldwell and McLaughlin, 1983). This study provides a biochemical basis for those findings. By correlating the electron microscopy studies with the findings presented here, a model of disks stacking in the ROS can be proposed. At this point, the most likely interpretation of the data presented here is that disks which have been separated with respect to cholesterol content have also been separated with respect to their age/position in the ROS. Thus, disks with cholesterol:phospholipid of 0.30 may represent relatively new disks, while those with cholesterol:phospholipid of 0.05 are older disks. A model of the ROS then emerges in which cholesterol decreases in concentration along the length of the rod from a concentration nearly equivalent to a plasma membrane to essentially no cholesterol at the tip. This loss in cholesterol reflects a net lipid loss but not a loss of phospholipids.

The compositional change in disk lipids may be important to understanding several disk properties. 1) The formation of new disks depends on mechanisms which must involve membrane fusion. The tendency to undergo fusion is dependent upon bilayer composition. 2) Rods of dystrophic RCS rats, which do not undergo normal phagocytosis, also do not exhibit cholesterol inhomogeneity as detected by filipin binding (Caldwell and McLaughlin, 1983). The cholesterol homogeneity may reflect a lipid defect in these rats. 3) The amplitude of the single photon response decreases from base to the tip of rods (Schnapf, 1983). As no difference in protein composition was detected in the separated disks studied here, the decreased response amplitude may be an effect of altered lipid composition on membrane protein function. 4) The ability of rhodopsin to regenerate was found to be dependent on disk position in the ROS (Williams, 1985). Thus, the regenerability of rhodopsin may also depend on its lipid environment.

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Fig. 1.
A, sucrose density gradient elution profile of control and digitonin-treated disks. Both control and digitonin-treated disks were spun on 0–50% sucrose density gradients and fractionated. Relative absorbance at 254 nm of control (—) and digitonin-treated disks (——) versus percent sucrose. B, cholesterol recovery in control and digitonin-treated disks. Both control and digitonin-treated disks were assayed for percent of initial cholesterol recovered in the various isolated fractions. ◆, control; □, digitonin-treated disks. Data represent averages of seven individual experiments.
Fig. 2.

A, the cholesterol to phospholipid ratio of untreated disks remains constant relative to position on sucrose gradient. Cholesterol:phospholipid composition of control disks isolated from sucrose density gradients. Data represent five individual experiments. B, digitonin induces a buoyant density change in disks based on their cholesterol content. Cholesterol:phospholipid composition of digitonin-treated disks isolated from sucrose density gradients. Data represent seven individual experiments.
Fig. 3. Digitonin-treated disks have the same phospholipid to protein ratio as a function of density
Phospholipid:protein composition of digitonin-treated disks as a function of percent sucrose on continuous sucrose density gradients. Data represent seven individual experiments.
Table I

Fatty acids show no differences in their relative amounts in high, middle, and low density disks

Fatty acid analysis of digitonin-treated disks with high, medium and low cholesterol content and of fractionated control disks are represented as a weight percent of the total. Fatty acid methyl esters were formed using 0.6 M methanolic:NaOH as described under “Materials and Methods.” 22:nn represents 22 carbon fatty acid species with an unconfirmed number of double bonds.

|                                 | Digitonin-treated disks | Control disks |
|---------------------------------|-------------------------|---------------|
|                                 | High        | Medium       | Low          |               |
| Cholesterol:phospholipid        | 0.27        | 0.17         | 0.07         | 0.11         |
| % total phosphate               | 31.1        | 39.0         | 29.9         | 98.0         |
| Fatty acid species              |             |              |              |              |
| 16:0                            | 23.1 (1.2)a  | 19.8 (1.5)   | 19.8 (1.6)   | 18.6 (1.6)   |
| 18:0                            | 21.7 (1.3)   | 18.2 (1.3)   | 18.7 (1.9)   | 20.3 (1.8)   |
| 18:1                            | 14.5 (1.3)   | 12.1 (0.9)   | 8.10 (2.6)   | 6.75 (0.8)   |
| 18:2                            | 1.56 (0.5)   | 2.50 (1.5)   | 2.00 (1.7)   | 1.10 (0.4)   |
| 18:3                            | 0.32 (0.2)   | NDb          | ND           | 0.43 (0.2)   |
| 18:4                            | 0.70 (0.1)   | ND           | ND           | 0.35 (0.1)   |
| 20:4                            | 9.78 (1.5)   | 9.90 (1.7)   | 9.20 (1.8)   | 10.8 (1.5)   |
| 22:nn                           | 6.20 (1.7)   | 5.50 (0.2)   | 1.90 (1.5)   | ND           |
| 22:6                            | 25.9 (4.2)   | 19.2 (0.9)   | 29.5 (5.0)   | 25.2 (2.1)   |

a Numbers in parentheses represent ± S.D.

b ND = not detected.