ANALYSIS OF MEMBRANE BILAYER ASYMMETRY USING PARINARIC ACID FLUORESCENT PROBES

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1. Introduction

Asymmetry in the lipid composition of the two opposing monolayers of biological membranes is documented in a number of cells and organelles [1] and may be of fundamental importance in understanding membrane function. In this report the asymmetry of composition and physical properties of the two sides of the native retinal rod outer segment membrane is assessed. We use a simple procedure to reconstruct the thermal behavior of the native membrane from the fluorescence properties of parinaric acid probes [2–6] in lipid dispersions which mimic the composition of the inner and outer monolayers. This approach should also be applicable to other membranes.

The asymmetry of the phospholipids in the retinal rod disc membrane has been described in [7–11]. The phospholipids with free amino groups (phosphatidylethanolamine and phosphatidylserine) are localized predominantly in the outer monolayer of the membrane while the phosphatidylcholine is thought to be predominantly localized in the inner monolayer [7–10]. These three classes of phospholipids comprise ~98% of the phospholipids in the membrane.

The parinaric acid fluorescent probes (*cis*-PnA = 9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid; and *trans*-PnA = 9,11,13,15-all *trans*-octadecatetraenoic acid) are natural product fluorescent fatty acids [2]. *cis*-PnA partitions nearly equally between coexisting fluid and solid phospholipid phases, while *trans*-PnA partitions preferentially into solid lipid phases where its quantum yield is strongly enhanced [2,7]. These features render *trans*-PnA especially sensitive to the formation of solid phospholipid.

2. Materials and methods

Rod outer segment (ROS) disk membranes and phospholipids [7], parinaric acid probes [2–4], and samples for fluorescence measurements [4,5] were prepared as described.

Fluorescence measurements involved determining the polarization ratio ($I_{\parallel}/I_{\perp}$) for *trans*-PnA which has been shown to be sensitive to the proportion of solid and fluid lipids in rod outer segments and their phospholipid dispersions [4–7]. The fluorescence polarization ratio is analyzed in terms of 'partial' quantum yields [4], $Q_{\parallel}$ and $Q_{\perp}$, where the total quantum yield, Q, is given by:

$$Q = Q_{\parallel} + 2Q_{\perp} \tag{1}$$

and the polarization ratio is simply:

$$i_{\parallel}/i_{\perp} = Q_{\parallel}/Q_{\perp} \tag{2}$$

For more than one coexisting lipid or domain (i), the observed polarization may be written:

$$i_{\parallel}/i_{\perp} = \sum_i x(i)Q_{\parallel}(i)/\sum_i x(i)Q_{\perp}(i) \tag{3}$$

where $x(i)$ are weighting factors which describe the distribution of probe molecules between domains and $\sum_i x(i) = 1$.

3. Results and discussion

The temperature dependence of the quantum

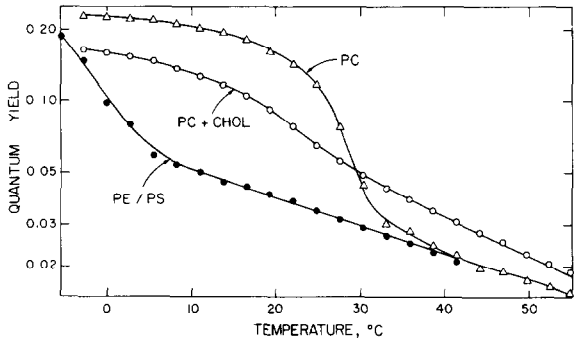

Fig.1. The temperature dependence of the fluorescence quantum yield of *trans*-PnA probes in rod outer segment (ROS) disc membrane phospholipids. The rod outer segment monolayer lipids (PC or PC + CHOL) are mimicked by a synthetic mixture containing 22% dipalmitoylphosphatidylcholine and 78% 1-palmitoyl,2-docosahexenoyl phosphatidylcholine with or without 10 mol% cholesterol. The thermal properties and composition of this synthetic PC mixture are similar to disk phosphatidylcholine [4,5,7]. This mixture exhibited a thermal phase separation beginning ~5°C higher than a typical preparation of disc phosphatidylcholine [5,7]. The outer monolayer lipids are mimicked by a dispersion containing 2.7 ROS PE/1 ROS PS [7]. *trans*-PnA fatty acid was used in the inner monolayer model measurements, while *trans*-PnA methyl ester is used in the outer monolayer measurements. These probes have essentially identical spectroscopic behavior [4,5].

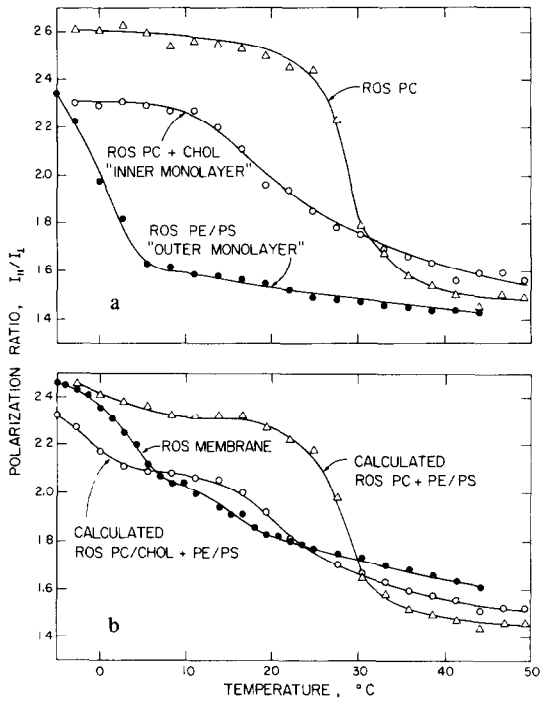

Fig.2. Temperature dependence of parinaric acid fluorescence polarization in: (a) ROS phospholipids; and (b) intact ROS membranes and calculated values. The lipid samples are as in fig.1. Calculated curves in (b) are calculated according to eq. (3) using the quantum yield data of fig.1 and the polarization data in (2a) to obtain the partial quantum yields $Q_{\parallel}$ and $Q_{\perp}$ of each monolayer.

yields and polarization of *trans*-PnA probes in bilayer membranes which mimic the phospholipid composition of the rod outer segment 'inner' and 'outer' monolayers are shown in fig.1 and 2a. The phospholipids which are thought to be in the outer monolayer undergo thermal reorganization below 5°C, that we attribute to a thermal phase separation of ~50% of the phosphatidylserine and phosphatidylethanolamine species [4,5]. The phosphatidylcholine, thought to be the major phospholipid component of the inner monolayer, undergoes a lateral phase separation below 30°C. We attribute the phosphatidylcholine phase separation to a substantial subfraction of this lipid that contains two saturated fatty acids [4,5,7]. The addition of cholesterol to the phosphatidylcholine is found to depress the magnitude of the membrane reorganization and broaden the temperature range of the reorganization without greatly shifting the midpoint [5].

The temperature dependence of PnA-probe polarization in native membranes is compared in fig.2b to the polarization calculated according to eq. (3) from the data of fig.1 and fig.2a. The calculations, shown in fig.2b, assume that the parinaric acid probes partition equally between the two monolayers [i.e., x(inner) = x(outer) = 0.5] and that the thermal properties of one side of the bilayer is not greatly perturbed by different thermal properties of the other side. If the calculations are based on an inner monolayer of pure phosphatidylcholine and an outer monolayer of phosphatidylserine and phosphatidylethanolamine, then the calculated temperature dependence of the polarization (fig.2b, △) tends to be dominated by the high quantum yield and polarization values of the phosphatidylcholine solid phase which forms below 30°C. In this case the calculated curve does not resemble the polarization data of the intact ROS membrane (fig.2b, •). The PnA polarization in a dispersion of the total membrane lipids (not shown) has a temperature range which is midway between the dispersions that are models for the 'outer' and 'inner' layers and does not resemble the native membrane [5].

If however, the phosphatidylcholine-rich inner monolayer of the ROS is assumed to contain most of the cholesterol which is found in the ROS membrane (~10 mol%), then the calculated PnA polarization curve (fig.2b, ○) shows thermal reorganization over two temperature ranges that match rather closely the native ROS (fig.2b, •). This result suggests that the phosphatidylcholine monolayer in the ROS may be rich in cholesterol and that the physical properties of the two monolayers of the ROS membrane can be modeled by a superposition of two component bilayers. The lower temperature range in the native membrane is to be associated with the outer monolayer. In the native membrane cholesterol apparently suppresses the anticipated thermal phase separation of the disaturated phosphatidylcholine species which is detected in the pure ROS phosphatidylcholine (fig.1) [4,5,7]. Cholesterol may be concentrated in the phosphatidylcholine-rich monolayer in the red blood cell [12].

The differences between the calculated and observed thermal dependence of PnA fluorescence polarization (fig.2b, cf. ○ vs •) are likely to reflect slight compositional differences between the actual membrane monolayers and our model dispersions (see legend to fig.1) and the influence of protein in the membrane. The magnitude and exact temperature range of the two calculated components is very sensitive to the precise composition of the 'inner' monolayer model dispersion. Protein can, in principle, affect both the temperature range of the lipid phase separation and significantly elevate the polarization at all temperatures.

The present work provides a useful point of departure for studying membrane asymmetry and organization. Using this approach, the effects of changing the compositions of the membrane monolayers (including protein) could be related back to the measured properties of the native membrane. Furthermore, the observations and approach presented here seem to provide new insight into the detection, interpretation and quantitation of phase separations in biological membranes. Most native membranes show rather broad, featureless thermal behavior. Our analysis shows that the superposition of rather sharp thermal components at different temperatures can mask the presence of these components in experimental membrane probe data. The superposition tends to make it appear that only broad featureless thermal changes are present (fig.2b). We find that the *trans*-PnA probes are appropriate for detecting rather small amounts of solid phase lipid, but proper analysis of membrane organization using these probes must take into account their nonlinear partitioning and quantum yield behavior in solid and fluid lipid [4]. In contrast, a fluorescent probe like diphenylhexatriene which shows only a small quantum yield difference in solid and fluid phases is expected to be rather insensitive to the phase separations in the ROS [13] since the analysis in fig.2b and [5,7] indicates that the amount of solid phase in bovine retinal membranes even at 0°C is likely to be ≤10–20% of the total phospholipids. Comparison of the fluorescent properties of *cis*- and *trans*-PnA in the same membrane preparation provides independent estimates of the magnitude, extent, and temperature of membrane phase separations [4–7].

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