Fluorescence Anisotropy Measurements of Lipid Order in Plasma Membranes and Lipid Rafts from RBL-2H3 Mast Cells†

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ABSTRACT: Specialized plasma membrane domains known as lipid rafts participate in signal transduction and other cellular processes, and their liquid ordered (L_o) phase appears to be important for their function. To quantify ordered lipids in biological membranes, we investigated steady-state fluorescence anisotropy of two lipid probes, 2-[3-(diphenylhexatrienyl)propanoyl]-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (DPH-PC) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE). We show using model membranes with varying amounts of cholesterol that steady-state fluorescence anisotropy is a sensitive measure of cholesterol-dependent ordering. The results suggest that DPH-PC is a more sensitive probe than NBD-PE. In the presence of cholesterol, ordering also depends on the degree of saturation of the phospholipid acyl chains. Using DPH-PC, we find that the plasma membrane of RBL-2H3 mast cells is substantially ordered, roughly 40%, as determined by comparison with anisotropy values for model membranes entirely in a liquid ordered (L_o) phase and in a liquid disordered (L_α) phase. This result is consistent with the finding that ~30% of plasma membrane phospholipids are insoluble in 0.5% Triton X-100. Furthermore, detergent-resistant membranes isolated by sucrose gradient fractionation of Triton X-100 cell lysates are more ordered than plasma membrane vesicles, suggesting that they represent a more ordered subset of the plasma membrane. Treatment of plasma membrane vesicles with methyl-β-cyclodextrin resulting in 75% cholesterol depletion leads to commensurate decreases in lipid order as measured by anisotropy of DPH-PC and NBD-PE. These results demonstrate that steady-state fluorescence anisotropy of DPH-PC is a useful way to measure the amount of lipid order in biological membranes.

There is increasing evidence that specialized regions in the plasma membrane of eukaryotic cells, known as lipid rafts, play important roles in signal transduction and other cellular processes (*1*). These regions are resistant to solubilization by certain nonionic detergents, including Triton X-100, and are sometimes called detergent-resistant membranes (DRMs).[1] These are enriched in sphingolipids, cholesterol, and glycerophospholipids with saturated acyl chains, as well as glycosylphosphatidylinositol- (GPI-) anchored proteins and Src family protein tyrosine kinases anchored via dually saturated acyl chains. Model membranes with similar lipid compositions are also detergent insoluble and are found to be in the liquid ordered (L_o) phase (*2*) that is characterized by high lateral mobility as in the liquid disordered (or liquid crystalline, L_α) phase and high orientational order as in the gel phase (*3*, *4*).

Our laboratory previously characterized the participation of lipid rafts in coupling FcϵRI, the high-affinity receptor for IgE, with the tyrosine kinase Lyn to initiate signaling by this receptor (*5*−*7*). ESR studies on isolated lipid rafts show that they have a substantial amount of L_o character (*8*). These rafts are postulated to form L_o phase regions distinct from the L_α phase in the plasma membrane (*9*). Recent studies in model membranes provide evidence for the coexistence of separate L_o and L_α phases in cholesterol-containing three-component bilayers (*10*, *11*). However, the amount and stability of the L_o phase domains in plasma membranes are uncertain and remain issues of considerable debate (*12*, *13*).

To quantify the amount of lipid order associated with the L_o phase in biological membranes, we investigated the use of two different fluorescence anisotropy probes, DPH-PC and NBD-PE, that previously have been shown to be sensitive to acyl chain order in model membrane studies (*14*, *15*). A simple theoretical framework for interpreting the data in terms of orientational order of lipid acyl chains makes this a very attractive approach (*14*). Here we report fluorescence anisotropy measurements on model membranes and on biological membrane preparations from RBL-2H3 mast cells that show high sensitivity to cholesterol-dependent ordering. The results provide a practical assessment of the amount of lipid order in the plasma membrane of these cells.

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[1] Abbreviations: BSS, buffered saline solution; DMSO, dimethyl sulfoxide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPH-PC, 2-[3-(diphenylhexatrienyl)propanoyl]-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; DRM, detergent-resistant membranes; GPI, glycosylphosphatidylinositol; L_α, liquid crystalline or liquid disordered; L_o, liquid ordered; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; T_m, gel−L_α phase transition temperature.

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MATERIALS AND METHODS

Lipids. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and cholesterol solutions in chloroform were from Avanti Polar Lipids (Alabaster, AL). 2-[3-(Diphenylhexatrienyl)propanoyl]-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (DPH-PC) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (NBD-PE) were purchased from Molecular Probes (Eugene, OR) and dissolved in methanol and dimethyl sulfoxide (DMSO), respectively, at 1 mg/mL (DPH-PC) and 0.5 mg/mL (NBD-PE).

Preparation of Model Membrane Vesicles. Aqueous phospholipid dispersions were prepared by mixing the required amounts of phospholipid, cholesterol, and the fluorescent lipid probe (molar ratio of probe:phospholipid was 1:200) as solutions in chloroform, and the mixture was dried under a stream of nitrogen at 50 °C. The residual thin film was resuspended in buffered saline solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 20 mM HEPES, pH 7.4), vortexed vigorously, and probe-sonicated (Vibra Cell ASI, Sonics & Materials, Danbury, CT) at highest power for 30 min at 45 °C. For some experiments, the probe was added after vesicle formation as described below for plasma membrane vesicles. Centrifugation of model membranes to remove unincorporated probe was carried out at 160000*g* for 30 min at 4 °C in a Beckman Airfuge (Palo Alto, CA). Vesicles made with probe incorporated before or after sonication yielded the same results.

Plasma Membrane Vesicle Isolation and Labeling. Plasma membrane vesicles were isolated from RBL-2H3 cells by chemically induced "cell blebbing" as described previously (*16*, *17*). This method results in the recovery of ~20−25% of the plasma membrane IgE receptors, with little or no contamination of intracellular membranes (*18*). Vesicles (~5 × 10^7 cell equivalents/mL) were labeled with the fluorescent lipid probes by adding the probe in methanol or DMSO (<1% of final volume) at a molar ratio of <1:100 probe:phospholipid and incubating at 37 °C for 5 min. Labeled vesicles were centrifuged in a Sorvall SL-50T rotor at 27000*g* for 45 min at 4 °C, washed in BSS by recentrifugation at least once to remove unincorporated probe, and finally resuspended in BSS for fluorescence measurements.

For some experiments, lipids were extracted from the plasma membrane vesicles and reconstituted as protein-depleted vesicles for fluorescence anisotropy measurements. For this preparation, vesicles were centrifuged, and the pellet was resuspended in 1 mL of methanol and transferred to a glass vial. Chloroform (1 mL) was added, and the suspension was probe-sonicated for 5 min at 45 °C. After being rocked for at least 2 h at room temperature, this suspension was centrifuged at 400*g* for 10 min and the supernatant removed carefully. The pellet was reextracted in 1 mL of chloroform:methanol (1:1 v/v) and recentrifuged. The collected supernatants were pooled, organic solvents were evaporated, and the lipids were resuspended in 1 mL of BSS, followed by probe sonication for 30 min at 37 °C. Lipid probes were incorporated as described above for the intact plasma membrane vesicles.

For some experiments, cholesterol was depleted from plasma membrane vesicles by incubation with methyl-β-cyclodextrin (MβCD) at 37 °C for 1 h. Control vesicles were incubated with buffer without MβCD under the same conditions. The vesicles were then centrifuged in a Sorvall SL-50T rotor at 27000*g* for 45 min at 4 °C, washed once, and finally resuspended in BSS. Fluorescent probes were incorporated as above for anisotropy measurements. The amount of cholesterol depleted was quantified by measuring the amount of cholesterol with the Amplex red fluorometric method (Molecular Probes, Eugene, OR) (*19*). Percent decrease in lipid order due to cholesterol depletion was calculated as

$$\frac{(r_{\mathrm{unttd}} - r_{\mathrm{ttd}})}{(r_{\mathrm{unttd}} - r_{\mathrm{L}\alpha})} \times 100 \qquad (1)$$

where r_{unttd} = fluorescence anisotropy in untreated vesicles, r_{ttd} = fluorescence anisotropy in MβCD treated vesicles, and $r_{\mathrm{L}\alpha}$ = fluorescence anisotropy in the model membrane in the L_α phase (POPC at 37 °C for DPH-PC; DPPC at 45 °C for NBD-PE).

Preparation of DRMs. RBL-2H3 cells were harvested in 1.5 mM EDTA, 135 mM NaCl, and 20 mM HEPES, pH 7.4, pelleted by centrifugation (200*g*, 8 min), and washed twice in BSS as described previously (*20*). Cells in BSS (typically 15 mL of 8 × 10^6 cells/mL in each sample) were lysed by mixing 1:1 with 2 × ice-cold lysis buffer containing 0.08% Triton X-100 (v/v) as previously described (*5*). The cell lysates were then diluted with an equal volume of 80% sucrose (w/v) in HEPES/saline buffer (25 mM HEPES, 150 mM NaCl, 2 mM EDTA, pH 7.5) at 4 °C; 20 mL of this lysate was added to each polycarbonate centrifuge tube (Beckman Corp., Palo Alto, CA), and then 5 mL of 35% sucrose and 5 mL of 5% sucrose solutions in buffer were sequentially layered on the top of the 40% sucrose lysate. These tubes were centrifuged in a Beckman SW28 rotor at 100000*g* for 12−18 h at 4 °C in a Beckman L8-70M ultracentrifuge. After centrifugation, two opaque bands were visible near the top of the centrifuge tubes. The upper band was located at the interface between the 5% and 35% sucrose layers and contained the DRM. This band material was collected, diluted 2−3-fold in PBS/EDTA, and centrifuged at 27000*g* in a Sorvall SL-50T rotor for 1 h at 6 °C. The pelleted DRM vesicles were resuspended in 1 mL of BSS, and the lipid probe was incorporated as described above for plasma membrane vesicles.

Fluorescence Anisotropy Measurements. Fluorescence anisotropy measurements were performed in a SLM 8000C spectrofluorometer with Glan-Thompson polarizers placed in L-geometry. For DPH-PC, excitation was at 360 nm and emission was measured at 430 nm; for NBD-PE, excitation was at 463 nm and emission at 534 nm. For each experiment, 2 mL of the membrane suspension (~100−150 μM in phospholipid) in BSS was placed in a 10 × 10 × 40 mm acrylic cuvette, and the mixture was stirred continuously in a thermostatic sample chamber. For all of the samples, background fluorescence due to unlabeled membranes and buffers was negligible. Steady-state fluorescence anisotropy, *r*, was calculated as

$$\frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (2)$$

where I_{VV} and I_{VH} are the parallel and perpendicular polarized fluorescence intensities measured with the vertically polarized excitation light, I_{HV} and I_{HH} are the same fluorescence intensities measured with the excitation light horizontally polarized, and G is the monochromator grating correction factor given by $G = I_{HV}/I_{HH}$.

Measurement of an Apparent Partition Coefficient for DPH-PC. The excitation spectrum of DPH-PC in DPPC liposomes at 45 °C (emission 430 nm) exhibits a shoulder at 350 nm and peaks at 362 and 382 nm (Figure 1A), similar to the spectrum reported previously (*21*). DPPC vesicles with increasing mole percent cholesterol exhibit a red shift in the excitation spectrum of DPH-PC that increases up to 33 mol % cholesterol (Figure 1A). This red shift provides a convenient means to estimate the apparent partition coefficient for DPH-PC between cholesterol-rich ordered and cholesterol-poor disordered regions of these model membranes. In Figure 1B, the long-wavelength excitation maximum (λ_{max}^{ex}) is plotted as a function of mole percent cholesterol in DPPC liposomes, and this is compared to plots predicted for various partition coefficients assuming 100% ordered at 33 mol % cholesterol and a linear combination of disordered and ordered regions for DPPC liposomes with cholesterol between 0 and 33 mol %. The best-fit value for the apparent partition coefficient is 1.1 on the basis of root mean square deviation analysis, similar to results obtained for DPH partitioning between ordered and disordered regions (*22*).

Organic Phosphate Assay To Determine the Detergent-Insoluble Fraction of the Plasma Membrane. Plasma membrane vesicles from RBL-2H3 cells at $\sim 5 \times 10^7$ cell equivalents/mL were incubated with 0.5% Triton X-100 in BSS for 10 min at 4 °C. Control vesicles were treated with BSS without Triton X-100 under the same conditions. Both samples were centrifuged in a Beckman SW60 rotor at 400000*g*, the pellets were washed once by recentrifugation, and then lipids were extracted into organic solvents as described above for plasma membrane vesicles and finally resuspended in BSS after organic solvents were evaporated. Aliquots were taken for quantification of phospholipid according to Kingsley and Feigenson (*23*). The percentage of plasma membrane that is detergent insoluble was determined by calculating the percentage of phospholipid recovered in the pellet of Triton X-100 treated vesicles relative to those recovered in the control (no Triton X-100) sample.

RESULTS

Figure 2 shows the structures of two different lipid analogues that we used to probe lipid order in biological membranes. Unlike the L_α phase, both gel and L_o phases have a high degree of lipid order, which depends on cholesterol for the L_o phase. DPH-PC contains the diphenylhexatriene fluorescent moiety attached to the *sn*-2 acyl chain of the PC such that the transition dipole moment is aligned along the length of the conjugated double bonds and is roughly collinear with the long molecular axis (*14*). Thus, DPH-PC fluorescence anisotropy should be particularly sensitive to the wagging motion of the acyl chains that takes place in fluid bilayer environments. The second lipid analogue, NBD-PE, has the NBD fluorophore attached to its phospholipid headgroup where it can interact directly with the membrane. A recent study showed that the fluorescence anisotropy of this probe is sensitive to membrane fluidity in the presence or absence of cholesterol (*15*).

To evaluate the sensitivities of steady-state fluorescence anisotropy measurements with respect to lipid order, we made measurements on liposome preparations containing variable amounts of cholesterol. Figure 3A summarizes these results for DPH-PC incorporated at ~0.5 mol %. For DPPC at 37 °C, below its gel−L_α phase transition temperature (T_m) of 41 °C, the steady-state anisotropy is high (0.30−0.32) and nearly constant from 0 to 50 mol % cholesterol, consistent with a high degree of acyl chain order that is independent of the amount of cholesterol. For DPPC at 45 °C, above its T_m, the steady-state anisotropy of DPH-PC is much lower in the absence of cholesterol (~0.17), as expected for lipids in a disordered phase. Increasing mole percent cholesterol results in a proportional increase in anisotropy up to a maximum value at ~33 mol % cholesterol that is only slightly less than the value for the same liposomes below the T_m. These results are consistent with previous NMR (*24*) and ESR (*25*) studies and indicate that maximum acyl chain order is achieved at an ~2:1 molar ratio of DPPC:cholesterol. We also compared the steady-state anisotropy of DPH-PC in membranes of monounsaturated POPC ($T_m = -2$ °C) and diunsaturated DOPC ($T_m = -20$ °C) as a function of cholesterol content. For these phospholipids at 45 °C, DPH-PC anisotropy values in the absence of cholesterol are a little lower than that for DPPC, consistent with slightly more freedom for wagging motion of DPH-PC in the unsaturated acyl chain environment. Increasing mole percent cholesterol for both POPC and DOPC results in roughly linear increases in DPH-PC anisotropy up to the highest amount of cholesterol tested, 50 mol %, with progressively smaller slopes compared with DPPC. The trends in anisotropy values are consistent with the respective T_m values of these lipids. These results indicate that DPH-PC steady-state anisotropy is sensitive both to the phospholipid:cholesterol ratio and to the degree of unsaturation in the phospholipid acyl chains. Previous studies indicated that the fluorescence lifetime of DPH-PC is only slightly affected by the mole percent cholesterol in model membranes (*26*, *27*), and our spectroscopic measurements described in Materials and Methods indicate that DPH-PC partitions similarly between cholesterol-rich and cholesterol-poor regions (Figure 1). Therefore, it is unlikely that the substantial changes in steady-state anisotropy observed can be explained by changes in excited state lifetimes of the probe or by differential partitioning into one or the other environment.

Steady-state fluorescence anisotropy of NBD-PE in DPPC vs DPPC/cholesterol was similarly compared. Figure 3B shows that, at 37 °C, the anisotropy of NBD-PE is similar between 0 and 50 mol % cholesterol (0.15−0.17), albeit considerably smaller than the corresponding values for DPH-PC (Figure 3A). At 45 °C, the anisotropy values increase steadily from 0.075 to 0.14 with increasing cholesterol to 30 mol %, qualitatively consistent with the trends for DPH-PC. However, this range of values is substantially smaller than that for DPH-PC in the same liposomes, indicating that the latter probe provides greater sensitivity to the amount of

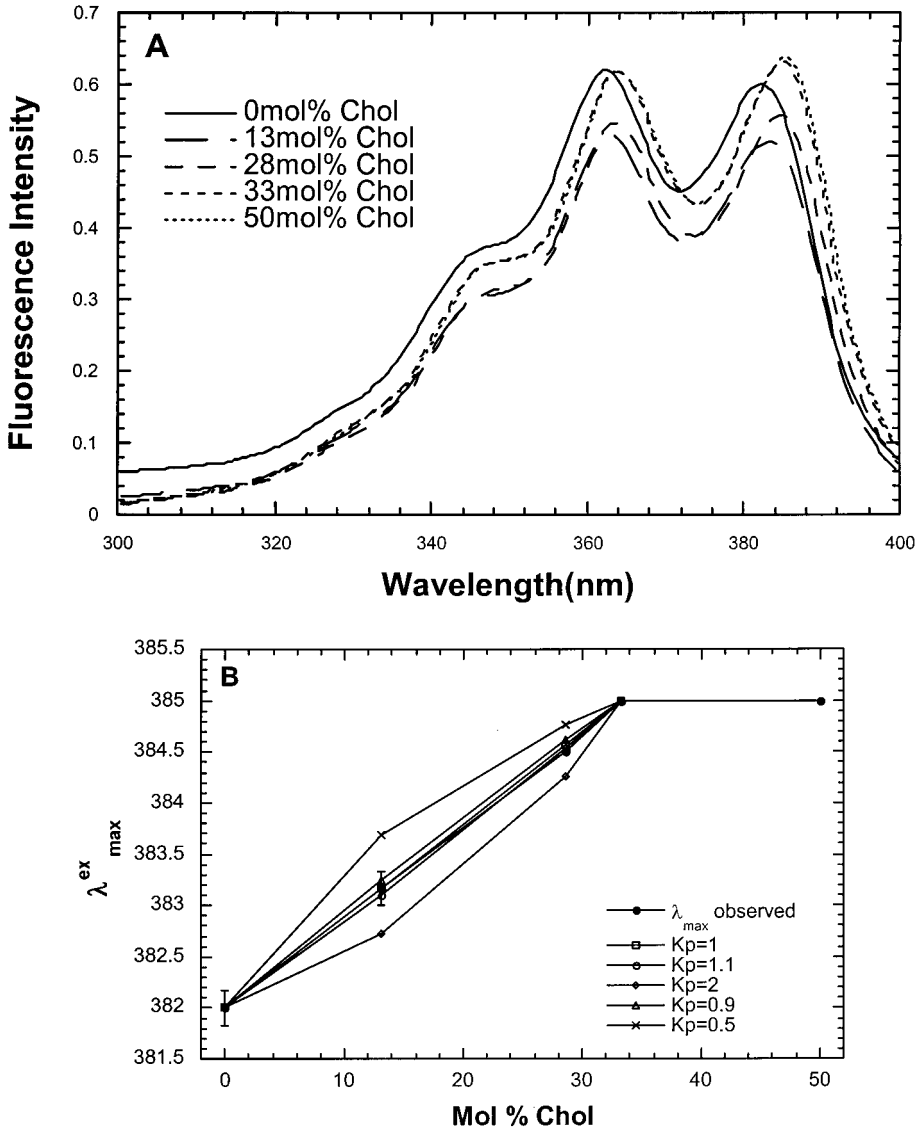

FIGURE 1: (A) Fluorescence excitation spectra for DPH-PC at 45 °C in DPPC liposomes containing varying amounts of cholesterol. The uncorrected spectra (excitation slit width 0.5 nm, emission at 430 nm) were smoothed using the trinomial smoothing algorithm of the SLM 8100 Series 2 Software. Small differences in intensities are due to different amounts of probe in the different samples. (B) Measured λ^{ex}_{max} values (●) and values predicted for various apparent partition coefficients (K_p) of DPH-PC between L_α (DPPC) and L_o (DPPC + 33 mol % cholesterol) phases: (□) $K_p = 1$; (○) $K_p = 1.1$; (◇) $K_p = 2$; (△) $K_p = 0.9$; (×) $K_p = 0.5$. Error bars indicate standard deviations from three experiments where these are greater than the width of the data points. Root mean square deviations from data are 0.051 for $K_p = 1.1$, 0.054 for $K_p = 1$, and 0.100 for $K_p = 0.9$.

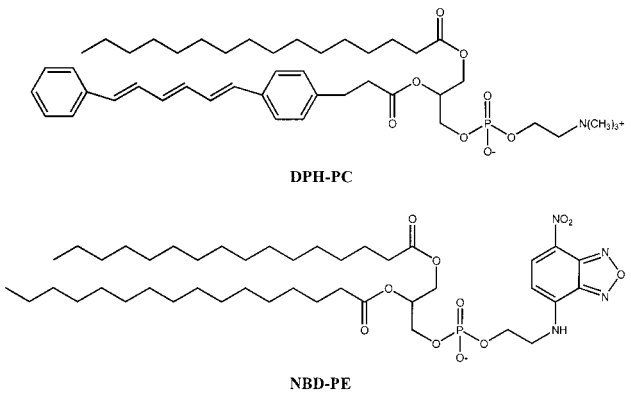

FIGURE 2: Structures of the lipid probes used for fluorescence anisotropy measurements.

lipid order. Furthermore, the anisotropy value for NBD-PE between 30 and 50 mol % cholesterol differs significantly at 37 and 45 °C, suggesting a larger temperature coefficient for the depolarization of this probe. Thus, NBD-PE anisotropy is less sensitive than DPH-PC as a direct measure of lipid order. The physical basis for the dependence of NBD-PE anisotropy on variable cholesterol content is unclear, but it appears that the NBD moiety inserts into the acyl chain region of the bilayer to a limited extent (*28*, *29*).

Having established the sensitivity of these probes to lipid order in model membranes, we used them to determine relative ordering in biological membrane preparations from RBL-2H3 cells. Figure 4 shows averaged DPH-PC anisotropy dependence on temperature and composition from at least three separate experiments for each membrane preparation. For model membranes in the L_o phase (DPPC/33 mol % cholesterol, □), anisotropy values are high (~0.31 at 37 °C) and fairly constant between 5 and 37 °C, only gradually declining with increasing temperatures in this range. In contrast, POPC membranes in the L_α phase show moderately low anisotropy values (~0.15 at 37 °C). DRMs isolated from

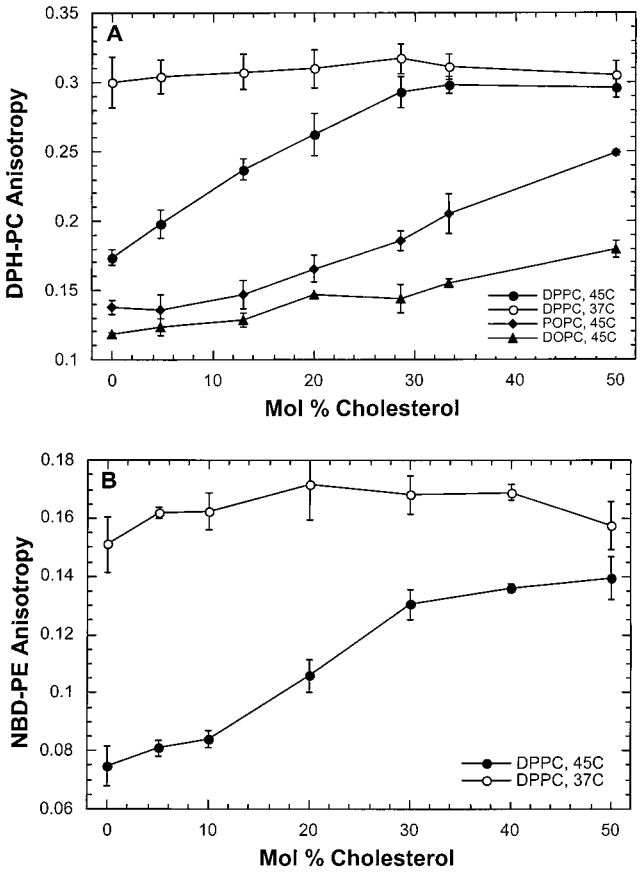

FIGURE 3: Effect of cholesterol and unsaturation on lipid order in model membranes as measured by DPH-PC (A) or NBD-PE (B) anisotropy. Results shown as average values of four independent experiments (two experiments with DOPC); error bars show the standard deviations. Plots: (●) DPPC at 45 °C; (○) DPPC at 37 °C; (◆) POPC at 45 °C; (▲) DOPC at 45 °C.

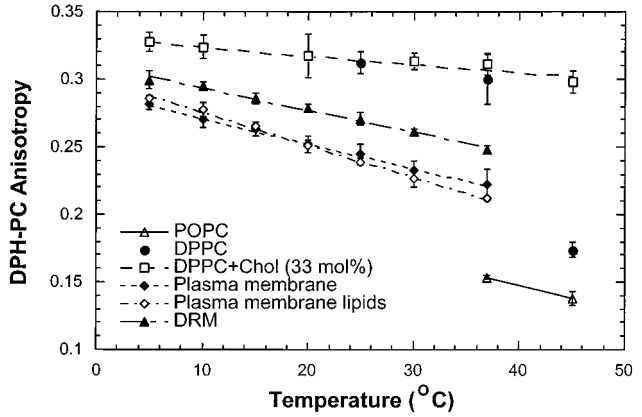

FIGURE 4: Measurement of lipid order in model membranes and biological membranes from RBL-2H3 cells by DPH-PC anisotropy. Results are averages with standard deviations from at least three experiments. Plots: (△) POPC; (●) DPPC; (□) DPPC + cholesterol (33 mol %); (◆) plasma membrane vesicles; (◇) lipid extract of plasma membrane vesicles; (▲) DRM.

RBL-2H3 cells by sucrose gradient flotation and labeled with DPH-PC show anisotropy values (▲) with a temperature dependence similar to those for DPH-PC in DPPC/33 mol % cholesterol. The values are somewhat lower than those for the DPPC/33 mol % cholesterol model membranes, indicating a moderately high degree of lipid ordering relative to the POPC membranes. By comparison, plasma membrane vesicles prepared by chemically induced vesiculation and

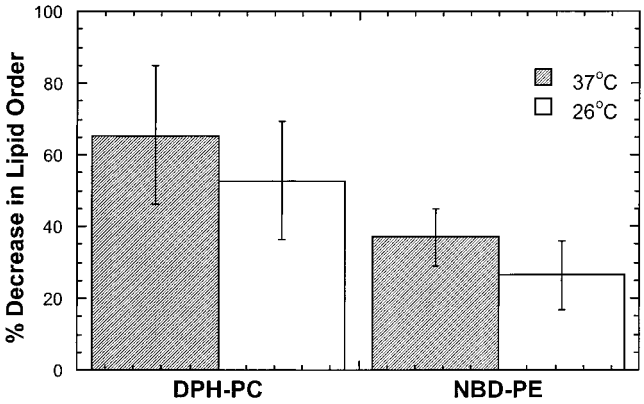

FIGURE 5: Effect of cholesterol depletion from plasma membrane vesicles of RBL-2H3 cells. Plasma membrane vesicles were prepared and treated or not with methyl-β-cyclodextrin to deplete cholesterol and then labeled with DPH-PC or NBD-PE for anisotropy measurements. Percent decrease in lipid order due to cholesterol depletion was calculated according to eq 1. Open bar: measurements at 26 °C. Hatched bar: measurements at 37 °C.

labeled with DPH-PC showed similar temperature dependence with even lower anisotropy values (◆) corresponding to less lipid order. Lipids recovered from these membranes by organic extraction and reconstituted into liposomes showed anisotropy values (◇) that are indistinguishable from the intact membranes, indicating that the protein content of these plasma membrane vesicles did not significantly influence the anisotropy results. Furthermore, these results provide additional confidence that quantitation of lipid order by these anisotropy measurements is relatively insensitive to other nonlipid components that could cause dynamic quenching and thereby alter the fluorescence lifetime of DPH-PC. Anisotropy results for these plasma membrane preparations are intermediate between fully L_o (DPPC/33 mol % cholesterol) and L_α (POPC) phases (Figure 4). Comparisons at 37 °C indicate that lipids in these plasma membrane vesicles are ~40% in the ordered state.

Brown and London showed that detergent insolubility correlates with ordered phases (L_o and gel) (*30*). On this basis we employed an organic phosphate assay to evaluate the lipids in plasma membrane vesicles. Direct comparison of the total lipids sedimented in the presence and absence of 0.5% Triton X-100 showed in two separate experiments that 29 ± 1.5% of the plasma membrane lipids are detergent-resistant, which provides a lower limit for the amount of ordered lipids in the plasma membrane (*31*).

To evaluate the role of cholesterol in ordering of the plasma membrane lipids, we measured the fluorescence anisotropy of DPH-PC and NBD-PE before and after cholesterol depletion of vesicles by MβCD treatment. Under these conditions, 75 ± 10% cholesterol was removed from the plasma membrane vesicles as determined by spectrofluorometric measurement of unesterified cholesterol (data not shown). As summarized in Figure 5, we found that this reduction in cholesterol decreased the amount of lipid order by ~65% for DPH-PC at 37 °C and ~53% for the same probe at 26 °C. These decreases indicate that the membranes become more fluid, consistent with a shift from L_o to L_α (rather than L_o to gel) upon cholesterol depletion. The smaller effect of cholesterol depletion at the lower temperature suggests that phospholipids with high melting temperatures

may contribute more substantially to ordering as the temperature is reduced.

For NBD-PE, we found that the anisotropy values in plasma membrane vesicles are 0.122 ± 0.017 at 37 °C and 0.136 ± 0.006 at 26 °C, and these are intermediate between the values of 0.075 ± 0.007 and 0.139 ± 0.007 for this probe in DPPC liposomes at 45 °C in the absence (L_α) and presence (L_o) of 50 mol % cholesterol, respectively (Figure 3B). Cholesterol depletion by MβCD results in somewhat smaller decreases in lipid order as detected by this probe as compared to values observed with DPH-PC (Figure 5). This is consistent with other indications that NBD-PE is less sensitive to cholesterol-dependent lipid order than DPH-PC. However, results with both probes confirm that cholesterol causes ordering of fluid lipids in the plasma membrane vesicles, consistent with predictions from model membrane studies summarized above.

DISCUSSION

Our fluorescence anisotropy measurements with DPH-PC demonstrate that it is a useful probe for quantifying cholesterol-dependent ordering in biological membranes. As shown in Figure 3A, this anisotropy is highly sensitive to cholesterol content for DPPC liposomes above the gel-to-L_α phase transition temperature of 41 °C but virtually insensitive to cholesterol below the phase transition temperature where the phospholipids have a high degree of order. Furthermore, anisotropy values for DPH-PC in DPPC vesicles are essentially constant above the molar ratio of 2:1 DPPC:cholesterol (Figure 3A) and relatively insensitive to temperature in a physiologically relevant range (Figure 4). These results indicate that the absolute anisotropy value of DPH-PC provides an order parameter that is directly measured. Interestingly, anisotropy values measured for liposomes containing PC phospholipids with one or two unsaturated acyl chains also show sensitivity to cholesterol content. Cholesterol has the greatest ordering effect on the disaturated DPPC and the least on the diunsaturated DOPC, with the monounsaturated POPC being intermediate (Figure 3A), as reported earlier with DPH (*32*). Consistent with previous studies on model membranes, our results indicate that the amount of lipid order is a function of both the cholesterol content and the extent of saturation of the phospholipid acyl chains.

The use of DPH-PC anisotropy as an order parameter depends on the insensitivity of its fluorescence lifetime to cholesterol content or acyl chain (un)saturation. The fluorescence lifetime of DPH-PC has been shown to increase by as much as 10% due to the presence of cholesterol in a 1:2 molar ratio with phospholipid (*27*). Thus, the measured increases in anisotropy due to increased cholesterol content may be slightly reduced by the expected small changes in fluorescence lifetime. Similar small differences in DPH-PC lifetimes as a function of acyl chain composition (*33*) are expected to have similar small effects on measured anisotropy values. The utility of DPH-PC anisotropy as an order parameter for cholesterol-containing phospholipid bilayers also depends on similar partitioning between cholesterol-rich and cholesterol-poor regions. Our results for DPH-PC yield a value of 1.1 for the apparent partition coefficient, indicating that DPH-PC partitions nearly equally between cholesterol-rich and cholesterol-poor regions. The utility of DPH-PC anisotropy as a direct measure of membrane order further depends on the insensitivity of this parameter to small changes (within a factor of 2) in probe concentration used in the experiments, and this was confirmed for measurements on model membranes as well as biological membrane preparations (unpublished observations).

NBD-PE shows qualitatively similar anisotropy changes as a function of cholesterol concentration in model membranes (Figure 3B). However, the changes are smaller than those observed for DPH-PC, and greater temperature sensitivity is evident in both model membranes (Figure 3B) and plasma membrane vesicles (Figure 5). It is not yet clear whether changes in fluorescence lifetime of NBD-PE significantly affect the anisotropy changes measured or, indeed, why NBD attached to the PE headgroup should be sensitive to cholesterol-dependent ordering of the hydrocarbon interior of the bilayer. Overall, the anisotropy values for NBD-PE appear to be not as useful as DPH-PC for measuring cholesterol-dependent ordering. A previous study compared anisotropy measurements with DPH-PC to those with DPH in lymphocyte plasma membranes (*34*). In this study, DPH-PC anisotropy was found to be higher and sensitive to membrane lipid composition changes, consistent with our results.

Using fluorescence anisotropy measurements for DPH-PC, we estimate that the isolated lipid rafts (DRMs) are about 60% as ordered as 2:1 DPPC:cholesterol at 37 °C (Figure 4), using the anisotropy for POPC at 37 °C as a reference point representing the L_α phase. This comparison is consistent with previous lipid compositional analysis of this DRM preparation by Fourier transform mass spectrometry, which showed that ~20% of the phospholipids have saturated acyl chains, ~45% have a single unsaturated bond in their acyl chains (like POPC), and the remainder have two or more double bonds (*17*). Ordering of unsaturated lipids in these DRMs is caused by cholesterol, and we determined a phospholipid:cholesterol ratio of 1.67:1 by quantitation of total phospholipids with organic phosphate assay and total cholesterol with a spectrofluorometric assay. These results are consistent with estimates of lipid order from the anisotropy values for DPH-PC, and together they indicate that lipid rafts isolated from cells need not be fully ordered to resist solubilization by 0.04% Triton X-100. In a recent study (*35*), DPH anisotropy was used to measure the lipid order of DRMs prepared from melanoma cells using higher concentrations of Triton X-100. In these preparations, DPH anisotropy was very similar to that in 2:1 DPPC:cholesterol liposomes, thus indicating more ordered DRMs than those produced with lower detergent concentrations.

Plasma membrane vesicles isolated from RBL cells by chemically induced vesiculation exhibit about 40% as much order as 2:1 DPPC:cholesterol liposomes, indicating that they also have a relatively high degree of order, albeit somewhat less than that for the isolated DRMs. These results are summarized in Table 1, which compares lipid order for different preparations to lipid composition and detergent insolubility. As with the DRMs, lipid compositional analyses of the plasma membrane vesicles are consistent with fluorescence anisotropy results: ~13% of the phospholipids containing fully saturated acyl chains and ~37% with a single unsaturated bond in their acyl chains (*17*). In addition,

Table 1: Comparison of Lipid Order, Phospholipid Saturation, and Detergent Insolubility

	relative lipid order[a]	% lipids (S + M)[b]	% detergent insoluble[c]
gel	100		
L_o	100	100	100[d]
DRM	60	65	100
PM[e]	40	50	30
PM lipids[f]	40		
PM (chol−)[g]	15		
L_α	0		<5[d]

[a] Lipid order determined by steady-state anisotropy of DPH-PC at 37 °C, normalized with respect to L_α (POPC) as 0 and L_o (DPPC: cholesterol, 2:1) as 100 (see Figures 3 and 4). The estimated uncertainty in these values, based on standard deviations for anisotropy measurements and the uncertainty in the anisotropy value for L_α, is ±10. [b] For cholesterol-containing membranes, sum of phospholipids with saturated (S) and monounsaturated (M) fatty acyl chains as defined by model membrane composition (L_o) or determined by mass spectrometry (DRM and PM) (*17*). [c] Detergent insolubility of phospholipids in 0.5% Triton X-100, except for DRM sample (0.04% Triton X-100). [d] Schroeder et al. (*31*). [e] Plasma membrane vesicles isolated from blebbing cells (see Materials and Methods). [f] Lipids extracted from plasma membrane vesicles and reconstituted into vesicles. [g] Cholesterol-depleted plasma membrane vesicles.

quantification of the total phospholipids and cholesterol yielded a phospholipid:cholesterol ratio of 2:1 for these plasma membrane vesicles. These results suggest that a large percentage of the plasma membrane lipids are as ordered as those in the isolated lipid rafts. Consistent with this conclusion, ~30% of the phospholipids in the plasma membrane vesicles were found to be insoluble in 0.5% Triton X-100, similar to results obtained with other plasma membrane preparations (*36*) or red cell ghosts (*37*).

Lipid order detected by DPH-PC anisotropy in plasma membrane vesicles is highly sensitive to the amount of cholesterol, further strengthening its utility as a quantitative indicator of lipid raft content within fluid membranes. It is not sensitive to the protein content of the plasma membrane vesicles (Figure 4), indicating that potential interactions of DPH-PC with proteins or annular lipids do not significantly perturb the lipid order measurements. Decreased anisotropy following cholesterol depletion indicates that the phospholipids remaining in the plasma membrane vesicles are mostly in the liquid disordered phase at the measured temperatures of 26 and 37 °C (Figure 5). This is qualitatively consistent with the lipid composition previously reported (*17*), but it does not preclude the possibility that localized domains of lipids could be enriched in gel-like lipids in cholesterol-depleted membranes. A limitation of this steady-state anisotropy method is that it only provides average values for the degree of lipid order, and it does not indicate whether ordered regions are segregated from more fluid regions on a scale that would constitute separate phases. Furthermore, it does not indicate the dynamics under which such separate phases might form and dissipate. Time-dependent fluorescence anisotropy decay measurements of DPH-PC might provide some of the answers to these more detailed questions.

In conclusion, our results show that DPH-PC is a useful probe for measurements of cholesterol-dependent acyl chain order in biological membranes. Our results further indicate that a substantial percentage of the plasma membrane (~40%) is in an ordered state. Thus, lipid raft-like structures appear to make up a large fraction of the plasma membrane. This latter conclusion is consistent both with biochemical data of phospholipid detergent insolubility and with estimates from fluorescence microscopy co-redistribution studies (*38*). Whether ordered regions of the plasma membrane form domains truly separate from more disordered regions under certain physiological conditions remains to be determined. On the basis of past (*34*) and present results, investigation of this and related questions by incorporation of DPH-PC into the plasma membrane of live cells and real time measurements of fluorescence anisotropy changes as a function of stimulation or other conditions is a promising approach. Fluorescence imaging should permit quantitative assessment of the distribution of such probes in cellular membranes, and this will be essential for proper interpretation of alterations in fluorescence anisotropy that may occur during cellular response processes.

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