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Biochim Biophys Acta. Author manuscript; available in PMC 2007 October 18.

Published in final edited form as:

Biochim Biophys Acta. 2007 September ; 1768(9): 2213–2221.

The Phenyltetraene Lysophospholipid Analog PTE-ET-18-OMe as a Fluorescent Anisotropy Probe of Liquid Ordered Membrane Domains (Lipid Rafts) and Ceramide-Rich Membrane Domains

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Abstract

The conjugated phenyltetraene PTE-ET-18-OMe (all-(*E*)-1-*O*-(15'-Phenylpentadeca-8',10',12',14'-tetraenyl)-2-*O*-methyl-*rac*-glycero-3-phosphocholine), is a recently developed fluorescent lysophospholipid analog of edelfosine, (Quesada et al. (2004) *J. Med. Chem.* 47, 5333–5335). We investigated the use of this analog as a probe of membrane structure. PTE-ET-18-OMe was found to have several properties that are favorable for fluorescence anisotropy (polarization) experiments in membranes, including low fluorescence in water and moderately strong association with lipid bilayers. PTE-ET-18-OMe has absorbance and fluorescence properties similar to those of diphenylhexatriene (DPH) probes, with about as large a difference between its fluorescence anisotropy in liquid disordered (Ld) and ordered states (gel and Lo) as observed for DPH. Also like DPH, PTE-ET-18-OMe has a moderate affinity for both gel state ordered domains and Lo state ordered domains (rafts). However, unlike fluorescent sterols or DPH (Megha and London (2004) *J. Biol. Chem.* 279, 9997–10004), PTE-ET-18-OMe is not displaced from ordered domains by ceramide. Also unlike DPH, PTE-ET-18-OMe shows only slow exchange between the inner and outer leaflets of membrane bilayers, and can thus be used to examine anisotropy of an individual leaflet of a lipid bilayer. Since PTE-ET-18-OMe is a zwitterionic molecule, it should not be as influenced by electrostatic interactions as are other probes that do not cross the lipid bilayer but have a net charge. We conclude that PTE-ET-18-OMe has some unique properties that should make it a useful fluorescence probe of membrane structure.

Introduction

Lipid rafts are generally defined as sphingolipid and sterol-rich domains that exist in the liquid ordered phase (Lo). In cell membranes, rafts are thought to co-exist with liquid disordered (Ld) domains rich in lipids with unsaturated acyl chains [1–4]. The Lo phase is an intermediate state having tight lipid packing, similar to the solid-like gel phase, as well as high lipid lateral diffusion rates that are just slightly smaller than in the Ld phase [5,6]. Rafts have been proposed to be important in many cellular processes [7–12]. Although the formation of co-existing liquid ordered and disordered domains in model membranes containing high cholesterol and sphingolipid concentrations is now well-established, the details of raft behavior in cells remain controversial [13–16].

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Novel membrane binding fluorescent lipid probes should be useful in this regard. Previous studies have already shown that fluorescent probes are useful for the detection of lipid rafts in cell membranes. For example, fluorescent probes that can reveal their lipid environment via environment-sensitive emission spectra or lifetimes have been developed for this purpose [17–20]. Probes that detect their lipid environment via fluorescence anisotropy (polarization) can also be of use for raft studies [3,21,22]. Such probes are sensitive to membrane physical state because anisotropy depends directly upon the degree to which the probe is able to reorient after excitation, and probe reorientation is a function of local lipid packing, a parameter which is dependent upon membrane physical state. Reorientation in both the solid-like gel state and liquid ordered state is much more limited than in liquid disordered domain [23–25]. A number of probes containing the diphenylhexatriene (DPH) group have proven valuable for such experiments [25–27].

1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (edelfosine, ET-18-OMe) is a synthetic ether lipid with high metabolic stability and a well-known anti-neoplastic activity, which takes place by eliciting selective apoptosis of tumor cells, sparing normal cells [28]. This unique property of ET-18-OMe is of great interest because the lipid should bind initially in a rather unspecific way to the outer layer of the plasma membrane of both cell types. A fluorescent analog of edelfosine, PTE-ET-18-OMe, was recently introduced as a fluorescent molecule with an anti-neoplastic activity similar to that of the parent ether-lipid, and thus useful for the detection of the drug distribution in cell membranes [29]. In addition, it was shown that the emission of this analog co-localized with Fas protein and the raft marker, cholera toxin B subunit, in the plasma membrane of cancer cells [30]. PTE-ET-18-OMe has a conjugated phenyltetraene (PTE) fluorescent group that is structurally related to DPH. Although PTE chemical and photochemical stability are not as high as those of DPH, edelfosine analogs containing diphenylhexatriene as the emitting tag were inactive against tumor cells. Apparently, the more compact, chain-like structure of PTE is crucial for preserving the antitumor activity of the parent drug. In this report, we investigate the properties of PTE-ET-18-OMe as a fluorescence anisotropy probe. We find that PTE-ET-18-OMe has suitable properties for anisotropy measurements and, unlike DPH, has a relatively high affinity for ceramide-rich ordered domains.

Materials & Methods

Materials

Sphingomyelin (porcine brain, SM), cholesterol, *N*-palmitoyl-*D*-erythro-sphingosine (C16:0 ceramide), *N*-stearoyl-*D*-erythro-sphingosine (C18:0 ceramide), and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL), and used without further purification. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Sigma-Aldrich (St. Louis, MO). PTE-ET-18-OMe (all-*E*)-1-*O*-(15'-Phenylpentadeca-8',10',12',14'-tetraenyl)-2-*O*-methyl-*rac*-glycero-3-phosphocholine) was prepared as described previously [30]. Methyl- β -cyclodextrin (M β CD) was purchased from (Sigma-Aldrich, St. Louis, MO). Lipids and probes were stored dissolved in ethanol at -20° C. M β CD was stored in an aqueous solution at 4° C. Concentrations were determined by dry weight or, in the cases of DPH and PTE-ET-18-OMe, absorbance using an ϵ of $88,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 358 nm in ethanol, and $62,200 \text{ M}^{-1} \text{ cm}^{-1}$ at 341 nm in ethanol, respectively. LW peptide [31], sequence: acetyl-K₂W₂L₈AL₈W₂K₂-amide, was purchased from Anaspec (San Jose, CA) and used without further purification. Its concentration was determined using an ϵ of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm in ethanol.

Vesicle preparation

Small unilamellar vesicles (SUVs) were formed either by ethanol dilution or by sonication of multilamellar vesicles (MLVs). Ethanol dilution SUVs were prepared similarly to previous studies. Mixtures of the appropriate lipids dissolved in ethanol were dried with nitrogen gas and then re-dissolved in 20 μ l ethanol. Unless otherwise noted 980 μ L of PBS (1 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 137 mM NaCl and 2.7 mM KCl at pH 7.4) warmed to 70 °C was then added [32]. Final samples contained 200 μ M lipid and the desired amount of DPH or PTE-ET-18OMe (0.5 mol%, unless otherwise noted). Background samples lacking fluorescent probes were also prepared. MLVs were prepared as follows: the desired amounts of lipids (and fluorescent probe) dissolved in ethanol were mixed. These mixtures were then dried with nitrogen gas and re-dissolved in chloroform. The samples were again dried under nitrogen gas and then under high vacuum for 45 min. PBS warmed to 70 °C was added to the dried lipid-containing mixtures to give a final concentration of 10 mM lipid, and then the samples were vortexed 20 min using a multi-tube vortexer (VWR, West Chester, PA) placed in a 70 °C incubator. Sonication was then carried out for 30 min in a bath sonicator containing water kept from heating with ice. Then the SUV were diluted with PBS as desired.

For samples where PTE-ET-18-OMe was desired in only the outer leaflet, PTE-ET-18-OMe dissolved in ethanol was mixed with preformed SUV.

Fluorescence and Absorbance Measurements

Fluorescence at room temperature (about 23 °C) was measured at room temperature on a Fluorolog 3 spectrofluorimeter (Jobin-Yvon, Edison, NJ) using quartz semi-micro cuvettes (excitation path-length 10 mm and emission 4 mm). Unless otherwise noted, slit widths for fluorescence intensity measurements were set to 4.2 nm bandwidth for excitation and 8.4 nm for emission. DPH fluorescence was measured at an excitation wavelength of 358 nm and emission wavelength 430 nm, and PTE-ET-18-OMe fluorescence was measured at an excitation wavelength of 349 nm and emission wavelength 450 nm. The reported values have been corrected for background fluorescence.

Anisotropy measurements were made at room temperature using a SPEX automated Glan-Thompson polarizer accessory with slit widths set to 8.4 nm bandwidth (excitation) and 10.5 nm (emission). Anisotropy values (A) were calculated from the fluorescence intensities with polarizing filters set at all combinations of horizontal and vertical orientations, after subtraction of fluorescence intensity in background samples lacking fluorophore when necessary. Anisotropy (A) was calculated from the equation $A = \frac{[(I_{vv} \times I_{hh}) / (I_{vh} \times I_{hv})] - 1}{[(I_{vv} \times I_{hh}) / (I_{vh} \times I_{hv})] + 2}$, where I_{vv} , I_{hh} , I_{vh} , and I_{hv} are the various fluorescence intensities with the excitation and emission polarization filters, respectively, in vertical (v) and horizontal (h) orientations.

For Förster resonance energy transfer (FRET) experiments, ethanol dilution vesicles containing 200 μ M lipid and 2mol% LW peptide (FRET donor), were prepared in PBS with, and without, 1 mol% DPH or PTE-ET-18-OMe (FRET acceptors). The ratio of Trp fluorescence (excitation wavelength 280 nm, emission wavelength 340 nm) in the presence of the acceptors (F) to that in the absence of the acceptors (F_0) was calculated after background values were subtracted. Slit widths were set to 8.4 nm bandwidth (excitation) and 10.5 nm (emission). Absorbance was measured on a Beckman 640 spectrophotometer.

Results

Interaction of PTE-ET-18-OMe with Lipid Vesicles

PTE-ET-18-OMe is a lysolipid analog with a fluorophore group similar in structure to that of DPH (Figure 1). The absorption spectra and absorption coefficients, as well as the shape of the emission spectrum of the phenyltetraene chromophore are very similar to those of diphenylhexatriene [29]. To characterize its potential as a fluorescence probe, we first determined whether PTE-ET-18-OMe had a suitable affinity for model membranes. Partition into membranes was assessed by the change in the fluorescence intensity of PTE-ET-18-OMe dissolved in buffer as a function of lipid vesicle concentration (Figure 2A, top). This experiment showed that PTE-ET-18-OMe had much higher fluorescence intensity in the presence of lipid vesicles than in aqueous solution. The increase in fluorescence intensity was greater for vesicles in the L_o state (circles) (SM/cholesterol) than for vesicles composed of DOPC (without cholesterol) (squares). The association with DOPC vesicles appeared to be somewhat tighter than for vesicles composed of SM/cholesterol, since association with SM/cholesterol vesicles still appeared to be incomplete at the highest lipid concentrations tested.

Figure 2B (bottom) shows the effect of lipid concentration upon steady-state fluorescence anisotropy. Association of the probe with lipid changed the observed anisotropy relative to that in buffer (0 mM lipid point). There is an increase in anisotropy in the presence of SM/cholesterol vesicles, while in the presence of DOPC vesicles anisotropy decreases. These changes are consistent with the fact that the former are in the more tightly packed L_o state while the latter vesicles are in the loosely packed L_d state.

The observation that PTE-ET-18-OMe shows considerable anisotropy in aqueous solution can be explained in two ways. One possibility is that monomeric species reorient slowly in aqueous solution. This is unlikely, and a more plausible explanation is that a large fraction of PTE-ET-18-OMe molecules may be aggregated forming micelles. It is known that the critical micelle concentration of the parent ether-lipid ET-18-OMe is quite low ($0.5\text{--}1.1 \times 10^{-6}$ M) [33,34]. In addition, the short fluorescence lifetime observed in these conditions (≈ 270 ps) (Acuña, A. U., unpublished results), would result in a weak but highly polarized emission of the PTE group in aqueous solution.

It is noteworthy that anisotropy reaches a limiting value corresponding to that of the lipid-bound probe at a lower lipid concentration than is necessary to reach a limiting fluorescence intensity value. The reason for this is that the fluorescence from PTE-ET-18-OMe in aqueous solution is much weaker than that of membrane-bound PTE-ET-18-OMe, so that the former does not contribute significantly to fluorescence when there is more than $50 \mu\text{M}$ lipid. This means that anisotropy experiments can be carried out under conditions in which PTE-ET-18-OMe is partially membrane-bound without interference from the PTE-ET-18-OMe in aqueous solution.

Response of PTE-ET-18-OMe Anisotropy to Lipid Physical State

To assess the sensitivity of PTE-ET-18-OMe anisotropy to lipid physical state in more detail, we measured its fluorescence anisotropy in several different lipid mixtures, and compared its anisotropy behavior to that of DPH (Figure 3). Anisotropy was measured in model membrane vesicles containing lipid mixtures forming the liquid disordered (L_α , L_d) state (100 mol% DOPC or 75 mol% DOPC/25 mol% cholesterol), mainly liquid ordered (L_o) state (75 mol% SM/25 mol% cholesterol), or solid-like gel state (SM) [35,36]. Anisotropy was also measured in vesicles with ordered state mixtures containing ceramide, and in vesicles forming a mixture of ordered and disordered states (1:1 SM:DOPC with or without 25 mol% cholesterol).

Overall, Figure 3 shows that the response of steady-state fluorescence anisotropy to lipid physical state was very similar for samples containing DPH (shaded bars) or PTE-ET-18-OMe (open bars). In the liquid disordered state anisotropy was very low, while in samples in an ordered state (Lo or gel) anisotropy was high. The range of anisotropy values for PTE-ET-18-OMe was similar to that of DPH, showing that PTE-ET-18-OMe is as sensitive a probe of membrane physical state as is DPH. PTE-ET-18-OMe and DPH anisotropy was also similar in mixtures containing co-existing ordered and disordered domains (SM/DOPC and SM/DOPC/cholesterol), giving values between those in bilayers containing only ordered or disordered domains. Comparison of anisotropy values in these samples to those in samples containing only SM or DOPC (with or without cholesterol) suggests that both these probes partitioned nearly equally between these domains, as has been noted previously for DPH [24, 37]. However, an exact partition coefficient could not be calculated for two reasons. First, we do not know the exact fraction of the bilayer that is in the ordered and disordered states in these samples. Secondly, the fluorescence intensity arising from molecules in ordered or disordered domains is weighted by their intensity (quantum yield) in that environment, as well as being a function of the fraction of fluorescence molecules in that environment.

PTE-ET-18-OMe Undergoes Slow Transverse Diffusion (Flip-Flop)

It was also important to determine whether the PTE-ET-18-OMe molecule undergoes rapid or slow transverse diffusion (flip-flop) between the leaflets (monolayers) of the lipid bilayer. Slow transverse diffusion would allow use of PTE-ET-18-OMe to assess “fluidity” in a specific membrane monolayer. Transverse diffusion of PTE-ET-18-OMe should be slow because of the charged groups within its polar headgroup. Its slow transverse diffusion is consistent with the observation that the parent drug ET-18-OMe microinjected into normal cells was able to stimulate apoptosis, while it had no effect if added externally [30].

To directly demonstrate slow transverse diffusion of PTE-ET-18-OMe, DOPC vesicles were prepared with PTE-ET-18-OMe initially residing in either both the inner and outer leaflets, or in just the outer leaflet. The “both leaflet” samples were made by adding the PTE-ET-18-OMe with the lipid stock solutions from which the vesicles were prepared. PTE-ET-18-OMe “outer leaflet” samples were made by adding PTE-ET-18-OMe dissolved in ethanol to preformed vesicles. The amount PTE-ET-18-OMe removed from the membrane by methyl- β -cyclodextrin (M β CD) was then measured by monitoring the effect of M β CD on both fluorescence intensity and anisotropy. M β CD is used to extract sterols from membranes [38–40], and Figure 4A shows that low concentrations of M β CD also can bind PTE-ET-18-OMe. The PTE-ET-18-OMe bound to M β CD had a lower fluorescence intensity and anisotropy than the membrane-associated PTE-ET-18-OMe (Figure 4B). This drop in fluorescence intensity is consistent with the transfer of fluorescent PTE group from the extremely hydrophobic environment in the core of the lipid bilayer, to a somewhat less hydrophobic environment in PTE-ET-18-OMe/M β CD aqueous complexes, and the decrease in anisotropy is consistent with the expected faster reorientation of PTE group in aqueous complexes than that of membrane-bound PTE-ET-18-OMe. It should be noted that M β CD should not extract the unlabeled lipids, which have two acyl chains, at the concentrations being used [41].

The fluorescence intensity and anisotropy decrease was even greater when the M β CD was added to PTE-ET-18-OMe that was restricted to the outer leaflet, indicating that the PTE-ET-18-OMe in the inner leaflet was inaccessible to the M β CD, at least over the 1h time course of the experiment (Figure 4B). Consistent with this interpretation, the anisotropy value when M β CD was added to outer leaflet PTE-ET-18-OMe was equal to that the anisotropy value measured when excess M β CD was added to PTE-ET-18-OMe in aqueous solution (Figure 4A). This confirms that the amount M β CD added to the lipid vesicles was sufficient to bind all of the PTE in the outer leaflets.

The anisotropy and intensity decrease upon M β CD extraction in the samples containing PTE-ET-18-OMe in both leaflets was about 80% of that observed when PTE-ET-18-OMe was in the outer leaflet (Figure 4B). Even if the vesicles, which were prepared by a method that forms SUV, had the maximum imbalance in terms of total lipid content in the inner and outer leaflet (about 66% of lipid in the outer leaflet), this value (80%) exceeds what would be expected (66%) for a random distribution of PTE-ET-18-OMe in the inner and outer leaflets. Given the water solubility of PTE-ET-18-OMe, a likely explanation is that some of the PTE-ET-18-OMe binds to vesicles after vesicle formation, and is thus restricted to the outer leaflet. Another possibility is that the shape of the PTE-ET-18-OMe molecule favors its incorporation into the outer leaflet, because SUV are curved in a fashion that thermodynamically tends to favor the incorporation of lipids that have polar headgroups with a large cross-section relative to that of their non-polar groups [42], as does PTE-ET-18-OMe.

Anisotropy of PTE-ET-18-OMe and DPH in Ceramide-Containing Membranes

The possibility that ceramide-rich rafts might have important functions and properties different than those of other rafts has received much recent attention [21,43,44]. Ceramide has a high affinity for ordered domains and stabilizes them [45,46]. We have shown that ceramide displaces cholesterol from ordered domains [21], and this has been confirmed our later studies and studies by other groups [47–49]. We proposed that displacement of cholesterol is partly due to the fact that it and ceramide have small polar headgroups, which results in their competition for locations within ordered domains in which they can be shielded from water by the large headgroups of other ordered domain lipids, as predicted by the umbrella model [50]. Cholesterol displacement may also be partly due to extremely tight packing between ceramide and the acyl chains of other raft lipids. Consistent with both of these explanations, DPH is also displaced from ordered domains by ceramide [21].

The former hypothesis predicts that molecules with especially large headgroups, like PTE-ET-18-OMe, should partition favorably into ceramide-rich domains because they would effectively help shield ceramide molecules from water. To test this, we prepared samples containing co-existing disordered domains and ordered domains, and then compared the anisotropy of DPH and PTE-ET-18-OMe fluorescence as a function of ceramide concentration in the bilayer. As shown in Figure 5, the anisotropy of PTE-ET-18-OMe increases as ceramide content increases. In contrast, DPH anisotropy decreases as ceramide content increases, consistent with our previous results [21]. Notice that although the error bars are large for PTE-ET-18-OMe at high ceramide concentrations, the values are clearly much higher than without ceramide. These results support the hypothesis that lipids that have some ability to pack tightly and have large polar headgroups can partition into ceramide-rich rafts more favorably than molecules without large polar headgroups [21].

FRET Assay of the Effect of Ceramide on PTE-ET-18-OMe Association with Ordered Domains

The increase in PTE-ET-18-OMe anisotropy in the presence of ceramide could be due to an increase of the fraction of PTE-ET-18-OMe molecules in ordered domains as ceramide content increases. An alternate interpretation is that the degree of order in the ordered domains increases with increasing ceramide content. Indeed, Figure 3 shows that ceramide does induce a modest increase in PTE-ET-18-OMe anisotropy in model membrane vesicles that only contain ordered domains. Another interpretation is that the quantum yield of the PTE group in ceramide-rich ordered domains is higher than it is in ordered domains lacking ceramide. This would bias the measured anisotropy to disproportionately reflect the more fluorescent form.

To determine the extent to which PTE-ET-18-OMe enters ceramide-rich ordered domains, we designed a FRET assay. In this assay, the tryptophan-containing transmembrane LW peptide [49] was incorporated into lipid vesicles with coexisting ordered and disordered domains. LW

peptide resides within disordered membrane domains [31]. Both PTE-ET-18-OMe and DPH have absorbance spectra that overlap Trp emission (Figure 6), and so are good FRET acceptors for Trp. In samples containing co-existing ordered and disordered domains, the level of FRET, as measured by the degree of FRET-induced quenching of Trp fluorescence, is a measure of the concentration of PTE-ET-18-OMe or DPH in the disordered domains.

Figure 7 shows a comparison of FRET-induced quenching by PTE-ET-18-OMe and DPH in vesicles containing disordered and ordered domains with and without ceramide. Upon the addition of 20 mol% ceramide (replacing an equal mol% of SM with ceramide), the LW peptide to DPH energy transfer increases (Trp F/F_0 decreases), indicating an increase in DPH concentration in the disordered domains. This is consistent with our previous results showing that DPH is displaced from ordered domains by ceramide [21]. In contrast, FRET from the LW peptide and PTE-ET-18-OMe is almost unaffected by ceramide, or increases very slightly. This indicates that PTE-ET-18-OMe is not displaced from ordered domains by ceramide. We conclude that the increase in PTE-ET-18-OMe anisotropy predominantly reflects a difference in motion or fluorescence intensity in ceramide-rich ordered domains rather than an increase in PTE-ET-18-OMe in the ordered domains.

Discussion

PTE-ET-18-OMe as a Membrane Probe

This study shows that PTE-ET-18-OMe is a useful fluorescence anisotropy probe of membrane structure. Its fluorescence properties are similar to those of DPH in many respects, apart from a lower emission yield. In particular, it shows differences in anisotropy in fluid vs. ordered states that is about as large as that observed with DPH. This similarity is not surprising, since the fluorescent group of PTE-ET-18-OMe is similar to that of DPH. However, there are also distinct differences between PTE-ET-18-OMe and DPH that PTE-ET-18-OMe a better probe of membrane structure. First, PTE-ET-18-OMe does not quickly flip across membranes, and thus can be used to assess order in an individual lipid leaflet in a bilayer. While it is true that the DPH derivative such as trimethylammonium DPH (TMA-DPH) also does not easily flip across the lipid bilayer, TMA-DPH carries a net charge, while PTE-ET-18-OMe is zwitterionic. Thus, PTE-ET-18-OMe behavior should not be complicated by electrostatic interactions with charged lipids or proteins. Furthermore, the difference in PTE-ET-18-OMe anisotropy in ordered and disordered state membranes is somewhat larger than that observed with TMA-DPH [27], making it a more sensitive anisotropy probe. An advantage of PTE-ET-18-OMe, relative to fluorescent lipids with two acyl chains, is that as a lysolipid analog it should be relatively more water soluble, and so can be incorporated into membranes or cells much more easily than ordinary lipids. In addition, as we noted above, the insertion of the PTE emitting group into the edelfosine structure does not interfere with key functional properties of the original drug, as is frequently the case for lipid fluorescent probes [51]. The final advantage of PTE-ET-18-OMe over DPH concerns its affinity for different types of ordered domains. This is addressed below.

Affinity of PTE-ET-18-OMe for Ordered Domains

In cellular studies, PTE-ET-18-OMe appears to have a substantial affinity for lipid rafts, as was observed before for edelfosine [29,52]. In model membranes containing co-existing ordered and disordered domains, fluorescence anisotropy suggests PTE-ET-18-OMe has a tendency to partition nearly equally between ordered and disordered domains. However, its anisotropy behavior may actually reflect a slightly stronger affinity for disordered domains, because fluorescence properties are a weighted average, dependent on both the amount of probe in different domains and the relative fluorescence of a probe in different types of domains, and PTE-ET-18-OMe appears to fluoresce more strongly when bound to ordered domains than

disordered domains (see Results). Thus, the fraction of PTE-ET-18-OMe molecules in ordered domains should be less than the fraction of PTE-ET-18-OMe fluorescence arising from disordered domains. This does not mean that PTE-ET-18-OMe fluorescence is not a suitable marker for ordered domains in model membranes or rafts in cells. In terms of being a fluorescence raft marker the important parameter is whether the fluorescence comes primarily from rafts, not the location in which the probe molecule is most abundant. It should also be noted that the relative association of PTE-ET-18-OMe with ordered domains, i.e. its partition coefficient between ordered and disordered domains, might be dependent upon domain lipid composition, which could be significantly different in model membranes and cells.

In this regard, the observation that PTE-ET-18-OMe was able to associate with ceramide-rich ordered domains to a much greater degree than DPH is of particular interest. Ceramide-rich rafts have been reported to participate in a number of biological processes [43,53–57]. We previously found that molecules with small polar headgroups (sterols), or totally lacking a polar headgroup (DPH), tend to be displaced from ceramide-rich ordered domains [21]. As noted above, this has been confirmed by a number of studies [47–49], and suggests that the physical and functional properties of ceramide-rich rafts may differ significantly from ordinary rafts [44]. Thus, PTE-ET-18-OMe should be particularly valuable for studying ceramide-rich raft properties in both model membranes and cells.

Acknowledgements

This work was supported by NIH grant GM 48596 to EL and a Spanish MEC grant BQU2003/04413 to AUA

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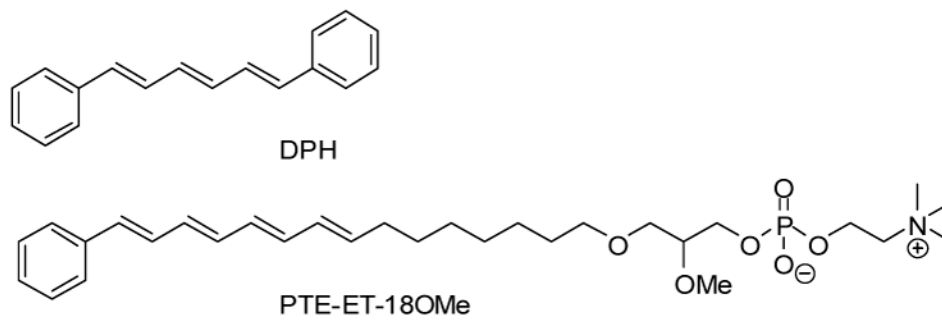


Figure 1.
Chemical structures of DPH and PTE-ET-18-OMe.

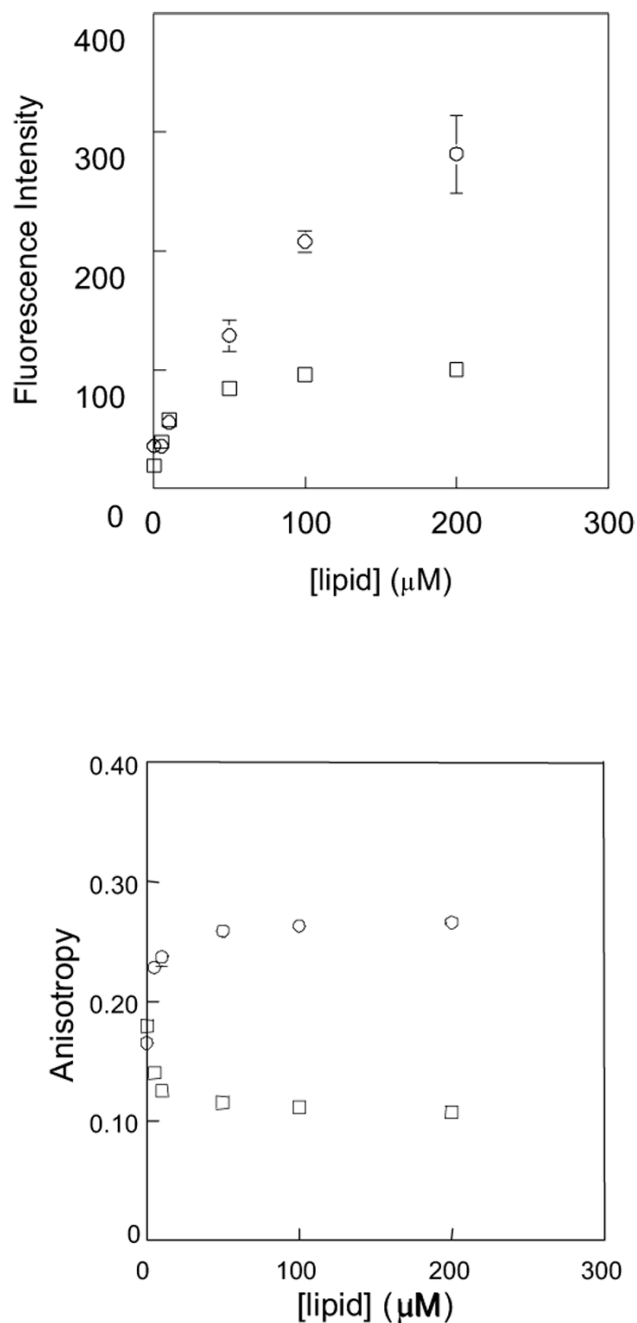


Figure 2. Dependence of PTE-ET-18-OMe Fluorescence Upon Lipid Concentration. A. (Top) Fluorescence Intensity. B. (Bottom) Steady state fluorescence anisotropy. Samples contained 1 μM PTE-ET-18-OMe dissolved in PBS in the presence of various concentrations of SUV composed of either DOPC (squares) or 3:1 (mol:mol) SM/cholesterol (circles). To prepare these samples a small aliquot of concentrated SUVs (10 mM or 1 mM diluted from 10 mM) was added and fluorescence was measured within one minute of each addition. Fluorescence values are not corrected for dilution by SUV aliquots, but dilution was minimal. Experiments here and in the following figures were carried out at room temperature. Results shown here and in following figures are the average and range of duplicates, unless otherwise noted.

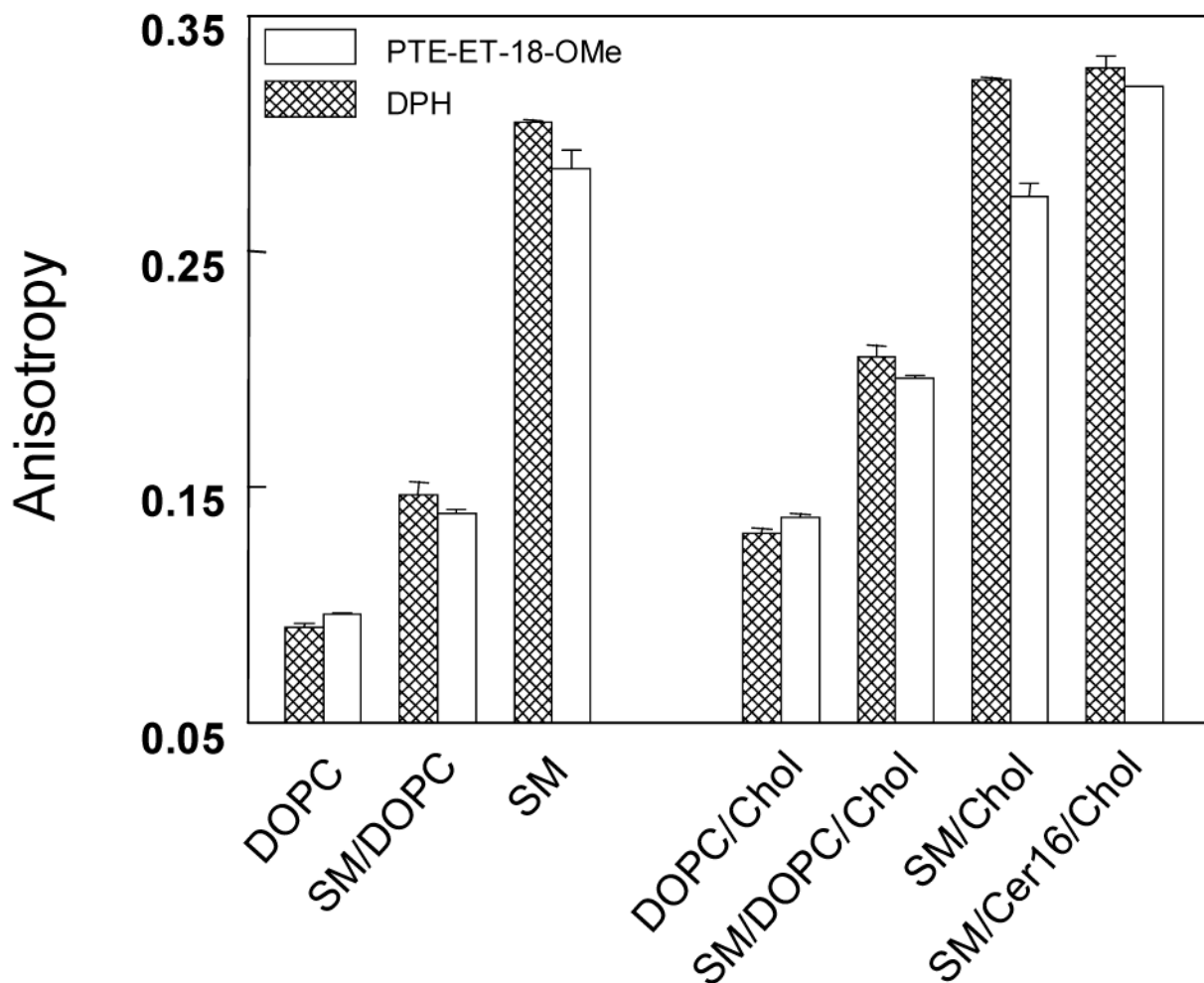


Figure 3. Anisotropy of DPH and PTE-ET-18-OMe Fluorescence in Lipid Bilayers With Different Compositions and Physical States. Samples contained ethanol dilution SUVs with 50 μM lipid dispersed in PBS and contained of either 0.25 μM DPH or PTE-ET-18-OMe at room temperature. From left to right, lipid composition (and physical state) was DOPC (Ld), SM/DOPC 1:1 mol:mol (Ld+gel), SM (gel); DOPC/cholesterol (Ld) 3:1, SM/DOPC/cholesterol 3:3:2 (Ld+Lo); SM/cholesterol 3:1 (Lo); SM/C16:0 ceramide/cholesterol 2.42:0.48:1 (ordered). DPH anisotropy (cross-hatched bars); PTE-ET-18-OMe (open bars).

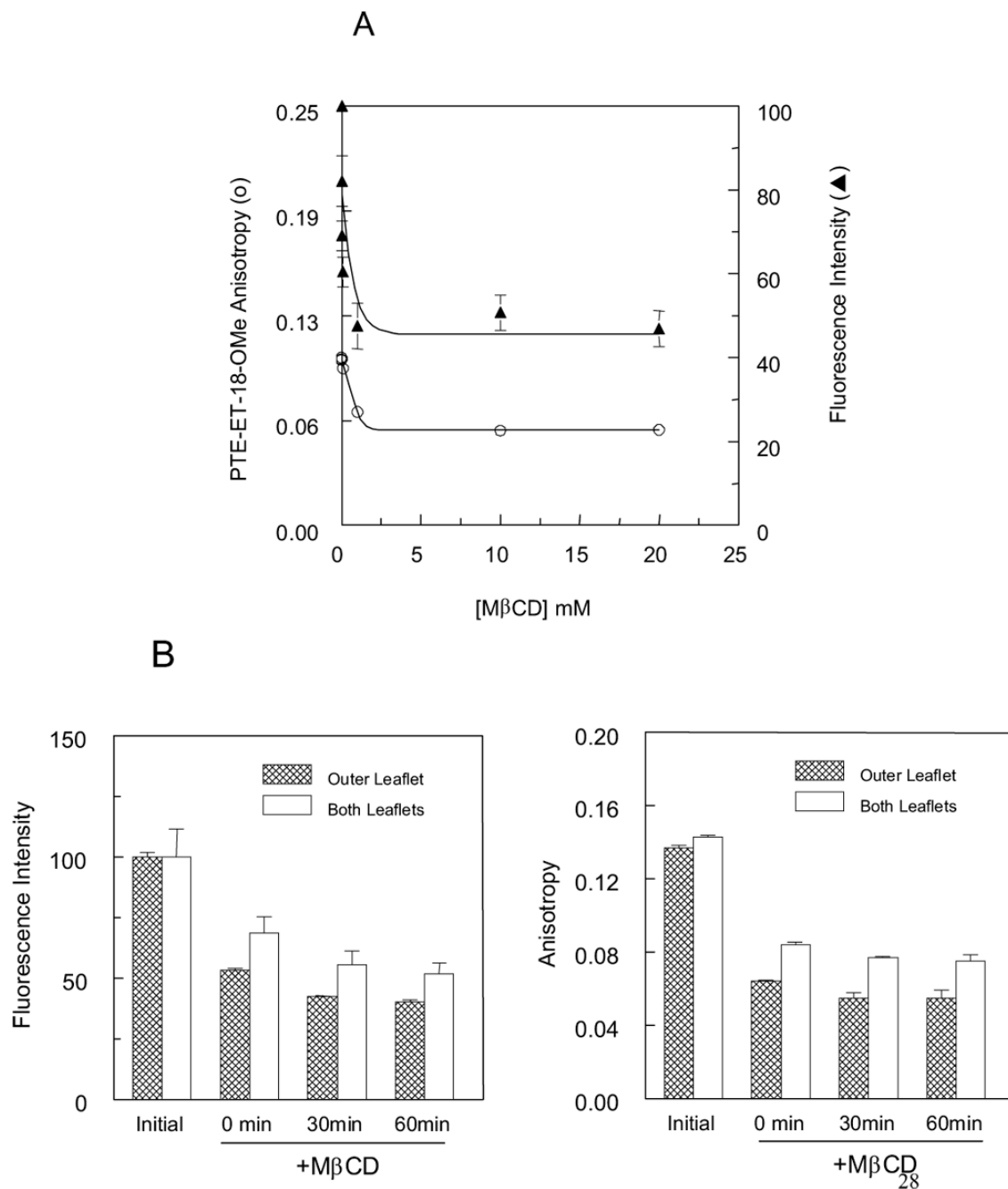


Figure 4. Dependence of PTE-ET-18-OMe Fluorescence Upon M β CD Treatment Indicates Slow PTE-ET-18-OMe Transverse Diffusion (Flip-Flop). A. Effect of M β CD concentration upon fluorescence intensity and anisotropy for samples with PTE-ET-18-OMe in the both leaflets. Samples contained SUV prepared by ethanol dilution and composed of 1 μ M PTE-ET-18-OMe and 200 μ M DOPC dispersed in PBS. Separate samples were prepared at each M β CD concentration. Fluorescence intensity (triangles); fluorescence anisotropy (circles). B. Comparison of fluorescence vs. time after M β CD addition for samples with PTE-ET-18-OMe in either both leaflets or just the outer leaflet. Samples contained SUV prepared by sonication and composed of 1 μ M PTE-ET-18-OMe and 200 μ M DOPC dispersed in PBS. In the (cross-

hatched bars) “outer leaflet” samples PTE-ET-18-OMe was added to preformed vesicles. PTE-ET-18-OMe bound to the vesicles within seconds (data not shown). For the (open bars) “both leaflet” samples PTE-ET-18-OMe was mixed with lipid stock solutions prior to vesicle formation. In both cases, a 100 μ l aliquot of 100 mM M β CD was added to the samples (final M β CD concentration of 10 mM). Fluorescence was measured at 0, 30, and 60 minutes after the addition of M β CD. Left panel. Fluorescence intensity. Right panel. Steady state fluorescence anisotropy.

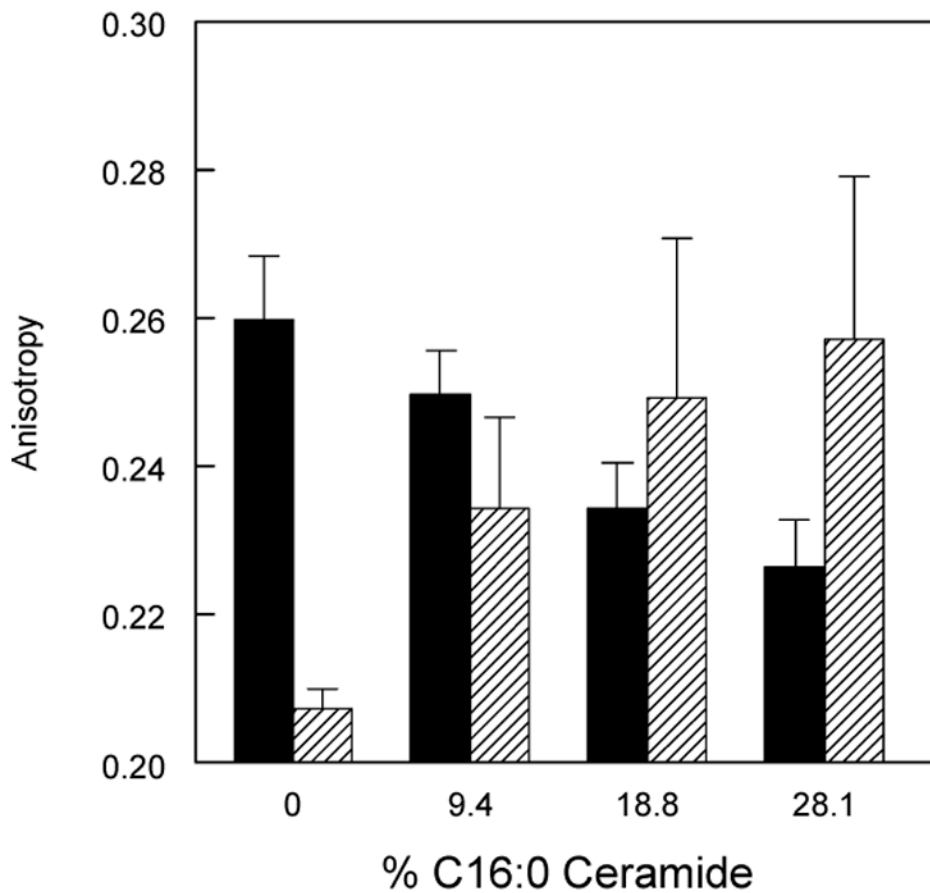


Figure 5. DPH and PTE-ET-18-OMe Steady State Fluorescence Anisotropy in Bilayers with Increasing Ceramide Levels. Samples contained SUV prepared by ethanol dilution with 50 μ M total lipid plus (solid bars) 0.2 mol% DPH; or (striped bars) 0.2 mol% PTE-ET-18-OMe dispersed in PBS. Lipid composition was 37.5 mol% DOPC, 25 mol% cholesterol and 37.5 mol% (SM + C16:0 ceramide). Data is the average and standard deviation from six samples.

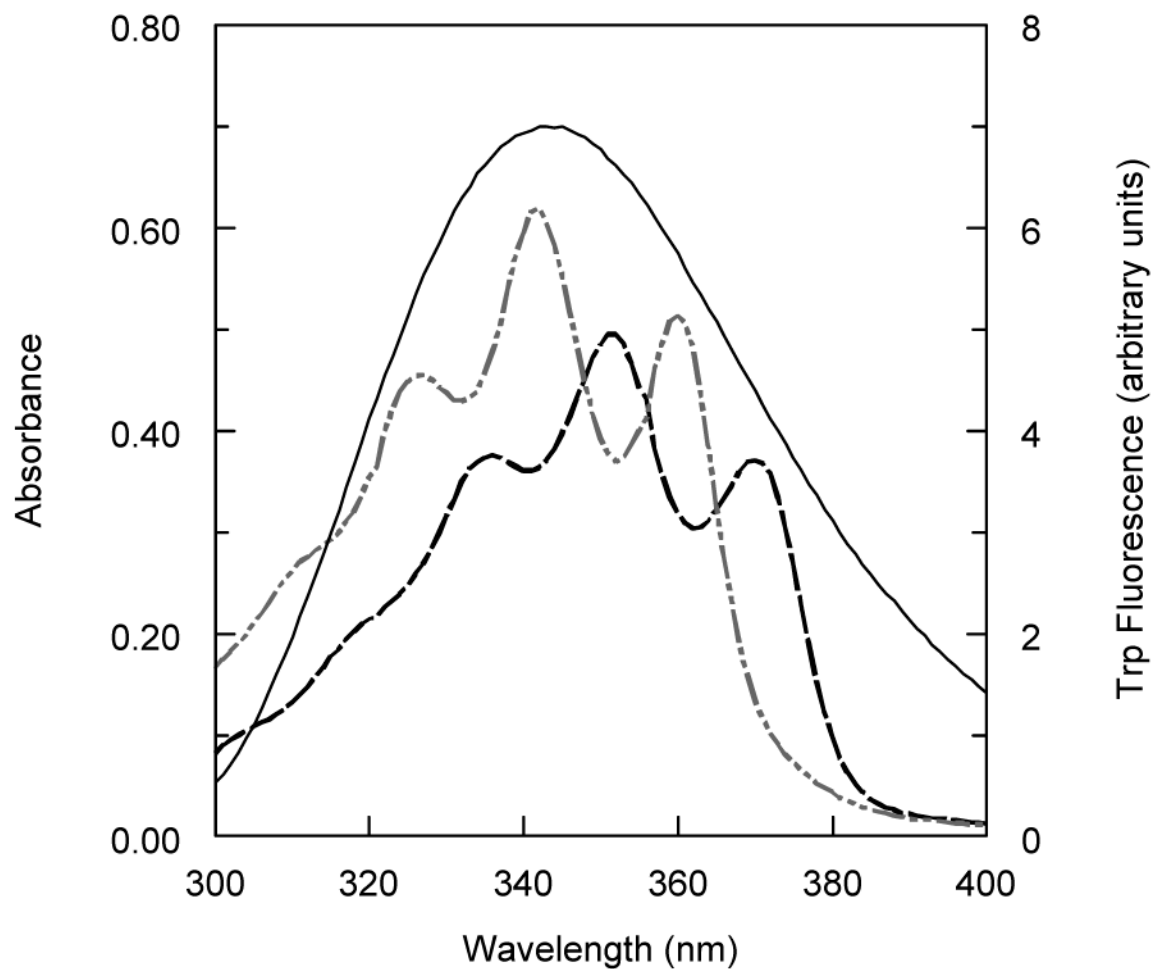


Figure 6. Absorbance of DPH and PTE-ET-18-OMe and Fluorescence Emission Spectrum of LW peptide Trp. DPH and PTE-ET-18-OMe samples contained 5–10 μM of probe dissolved in ethanol. LW peptide sample contained 1 μM peptide incorporated into DOPC ethanol dilution SUV (50 μM lipid) dispersed in PBS. (solid line) LW fluorescence, (dashed line) DPH absorbance, (dashes and dots line) PTE-ET-18-OMe absorbance.

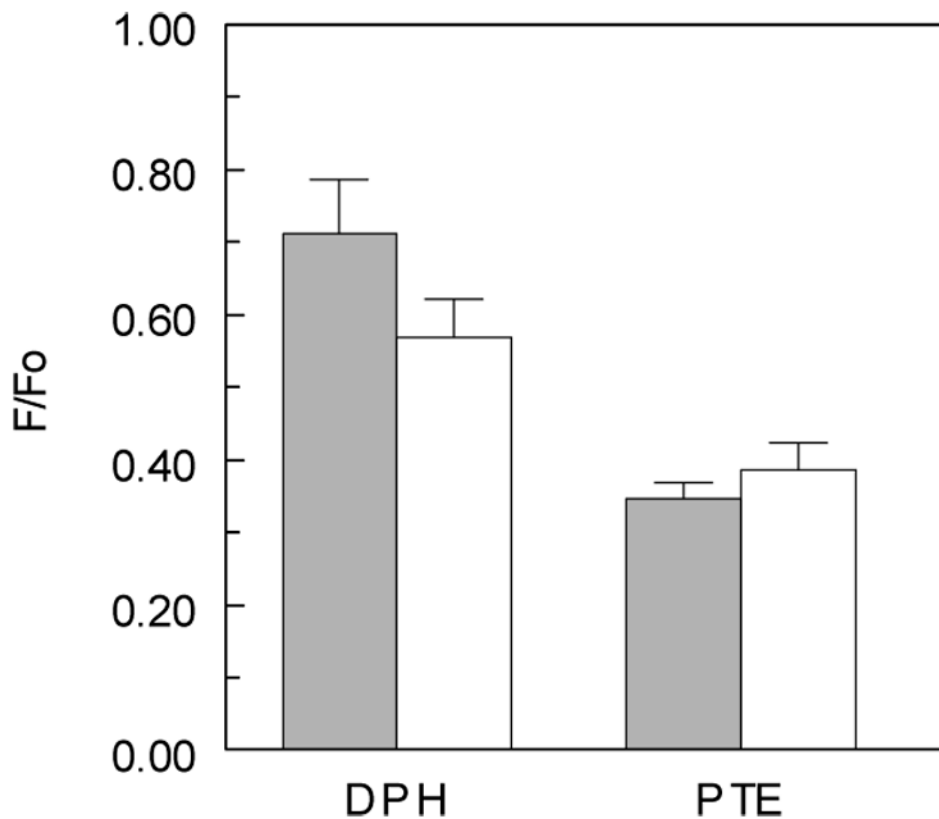


Figure 7.

Tryptophan to Fluorophore (DPH and PTE-ET-18-OMe) Energy Transfer in Bilayers Containing or Lacking Ceramide. Samples contained 4 μ M LW peptide incorporated into ethanol dilution SUV (200 μ M total lipid dispersed in PBS). Sample lipid compositions were either 37.5mol% SM, 37.5 mol% DOPC and 25 mol% cholesterol (shaded bars), or 17.5 mol % SM, 20 mol% C18:0 ceramide, 37.5mol% DOPC, 25mol% cholesterol (open bars). FRET acceptor-containing samples had 1 mol% DPH or PTE-ET-18-OMe. Y-axis gives the ratio of fluorescence intensity in samples containing acceptor to that in samples lacking acceptor (F/F_0). Energy transfer efficiency is given by $1-F/F_0$. The average and standard deviation for quadruplicates is shown. PTE=PTE-ET-18OMe.