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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.

Keywords: Fluorescence polarization; Fluorescence anisotropy; Edelfosine; Lipid domain; Lipid microdomain; Energy transfer; FRET; Shingomyelin; Shingolipid

1. 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].

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

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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.

2. Materials and methods

2.1. 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 (MβCD) was purchased from (Sigma-Aldrich, St. Louis, MO). Lipids and probes were stored dissolved in ethanol at −20 °C. MβCD 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 M^{-1} cm^{-1} at 358 nm in ethanol, and 62,200 M^{-1} cm^{-1} at 341 nm in ethanol, respectively. LW peptide [31], sequence: acetyl-$K_2W_2L_8AL_8W_2K_2$-amide, was purchased from Anaspec (San Jose, CA) and used without further purification. Its concentration was determined using an ε of 22,000 M^{-1} cm^{-1} at 280 nm in ethanol.

2.2. 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 20μl ethanol. Unless otherwise noted 980 μL 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 μM lipid and the desired amount of DPH or PTE-ET-18O Me (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 SUVs 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.

2.3. Fluorescence and absorbance measurements

Fluorescence at room temperature (about 23 °C) was measured 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 of 430 nm, and PTE-ET-18-OMe fluorescence was measured at an excitation wavelength of 349 nm and emission wavelength of 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 $A=[((Ivv\times Ihh)(Ivh\times Ihv))-1][((Ivv\times Ihh)(Ivh\times Ihv))+2]$, where Ivv, Ihh, Ihv, and Ivh 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 μM lipid and 2 mol% 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 (Fo) 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.

3. Results

3.1. 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 (Fig. 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

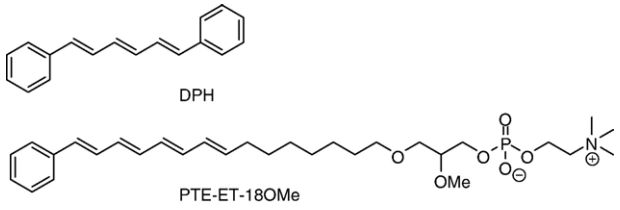

Fig. 1. Chemical structures of DPH and PTE-ET-18-OMe.