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THE USE OF A PHOSPHOLIPID ANALOGUE OF DIPHENYL-1,3,5-HEXATRIENE TO STUDY MELITTIN-INDUCED FUSION OF SMALL UNILAMELLAR PHOSPHOLIPID VESICLES

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A phospholipid analogue incorporating the diphenyl-1,3,5-hexatriene (DPH) chromophore has been synthesized. The compound has been shown to have similar fluorescence properties to DPH itself but, unlike DPH, is unable to exchange freely through solution when incorporated as probe in a subset of phospholipid vesicles of given composition. The non-exchangeability of this probe has been exploited to study the fusion of phospholipid vesicles to form larger structures. The peptide melittin was used to initiate fusion, and it was shown that vesicles which had been induced to fuse by heating in the presence of melittin would not fuse with subsequently added vesicles.

Introduction

DPH has been widely used as a fluorescent probe for both biological membranes and model systems such as phospholipid dispersions [1]. The popularity of DPH has arisen from its sensitivity to environment and from its high quantum yield. Although DPH is highly hydrophobic and partitions readily into lipid structures, it is exchangeable, through solution and will redistribute rapidly when, for example, unlabelled phospholipid is added to a dispersion of DPH-labelled phospholipid.

In this study the use of a phospholipid analogue bearing the diphenyl-1,3,5-hexatriene chromophore is described. This molecule shares with DPH a high quantum yield and great environmental sensitivity, and has additional advantages in that (i) the orientation of the chromophore relative to the bilayer is known and that (ii) the molecule will not spontaneously exchange through solution when it has been incorporated into a phospholipid dispersion. The ability to localize the fluorescent material in a subset of phospholipid vesicles is exploited in this study of the fusion between vesicles of phospholipids of known acyl chain composition. The fusion process is monitored by measurement of polarization of fluorescence of bound phospholipid analogue as a function of temperature. By this means it is possible to study not only fusion between vesicles of differing acyl chain length, but also self-fusion.

Materials and Methods

Fluorescence spectra were obtained using a Schoeffel RRS 1000 spectrofluorimeter. Measurements of the polarization of fluorescence as a function of temperature were performed using an apparatus previously described [2]. This apparatus

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Abbreviations: DPH, diphenyl-1,3,5-hexatriene; DPHPC, diphenylhexatriene-phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; PMR, ^{1}H-NMR.

was interfaced to an Apple II-plus microcomputer through a Synertek 6522 V.I.A. which provided control signals for the flash lamp, and which was wired to an ADC 0817 CCN analogue-to-digital converter (Radiospares Ltd). Temperature measurements were made using a thermistor bead which had been calibrated at known temperatures and which consequently could be corrected for linear output through the microcomputer. The thermistor circuit was connected to the analogue-to-digital converter of the computer through a multiplexer, so that temperature and fluorescence parameters could be simultaneously measured. Plots of fluorescence polarization as a function of temperature were made using the high resolution graphics capability of the microcomputer. The data were stored on a floppy disk and plotted on a TCM 1000 thermal printer when hard copy was required.

Measurements of fluorescence decay times were made using an Edinburgh Instruments single-photon-counting decay time fluorimeter. The instrument is equipped with excitation and emission monochromators and a thyratron-gated flashlamp which was filled with nitrogen for these measurements. Data analysis was performed on the microcomputer system supplied with the instrument. The system programmes use a convolution method with least-squares minimization to fit the acquired data to one or two exponential models [3].

Phospholipid vesicles were prepared by the injection of 40 mM ethanolic lipid solutions into vortexing buffer above the gel-liquid crystalline phase transition temperature for the compound used [4]. The resulting dispersions were maintained at the phase transition temperature for 30 min in the dark before use in order to allow entrapped ethanol to equilibrate with the bulk medium. Final concentrations of ethanol did not exceed 1% by volume. Vesicle dispersions thus prepared were optically clear and appeared to be quite stable over several hours in the absence of fusogen. In some experiments 5% by weight of phosphatidylglycerol was added to the phospholipid before dispersion in order to inhibit spontaneous vesicle fusion, but this addition did not affect the results obtained. Vesicles prepared with added fluorescent probe were routinely kept in darkness during all incubations. Vesicles were always used within 3 h of preparation. Buffers used for all experiments contained 10 mM EDTA in order to inhibit any residual phospholipase A_2 activity which might be present as a contaminant in the melittin samples. The phospholipid and melittin samples were obtained from Sigma, Ltd., and were used without further purification. Phospholipase A_2 (from *Crotalus adamanteus*) was also purchased from Sigma.

The phospholipid analogue incorporating DPH (DPHPC) was synthesized according to the scheme shown (Fig. 1a). 5-Phenylpentadienal was prepared as described [5]. 4-(Hydroxymethyl)phenylpropionic acid (I) was prepared by catalytic hydrogenation of 4-formylcinnamic acid (Aldrich Chemical Co.).

Compound I (10 mM) in dry diethyl ether (30 ml) was treated with phosphorus tribromide (10 mM) at 4°C with stirring. After stirring for 2 h at 25°C, the reaction mixture was treated with methanol (5 ml), and quenched on ice. Crude 4-(bromomethyl)phenylpropionic acid methyl ester was extracted into diethyl ether; the diethyl ether extracts were washed with aqueous sodium bicarbonate, dried with anhydrous sodium sulphate, and evaporated. Treatment of the residue with 5 mM triphenylphosphine in 20 ml toluene at 100°C for 2 h gave the crystalline phosphonium salt (II), which was filtered off, washed with diethyl ether, and recrystallized from methanol/diethyl ether. Compound II had m.p. 186°C (uncorr.); the PMR spectrum (measured in dimethyl sulphoxide-d_6) was fully consistent with the designated structure.

To a solution of 2 mM II and 2 mM 5-phenylpentadien-2,4-al in 5 ml methanol was added 5 mM sodium methoxide with stirring under nitrogen. After 2 h at 25°C, crystalline (2-carboxyethyl)-DPH methyl ester (III) was filtered off, washed with ice-cold methanol, and recrystallized from dimethylformamide/methanol. Compound III, obtained in 60% yield, had m.p. 167°C (uncorr.); λ_{max} (chloroform) 343, 363, 382 nm (ϵ, $58.0 \cdot 10^3$, $75.0 \cdot 10^3$, $54.0 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$); M^+ 318. Conventional alkaline hydrolysis of III gave (2-carboxyethyl)-DPH (IV) m.p. 255°C (uncorr.), recrystallized from acetone.

DPHPC (V): to a stirred suspension of 9.15 mg IV in 2 ml dry methylene chloride 4.5 μl redistilled triethylamine were added, followed by 15 μl

pivaloyl chloride. After incubation for 60 min at 25°C to form the mixed anhydride, the reaction mixture was evaporated to dryness, and held under 1 mmHg vacuum for 60 min at 25°C. The residue was then treated in 2 ml dry methylene chloride with 10 mg egg lysophosphatidylcholine and 4 mg 4-dimethylaminopyridine for 12 h at 25°C with stirring. DPHPC was then isolated by first diluting the reaction mixture with chloroform and then extracting with cold 0.01 M HCl. The chloroform layer was evaporated to dryness, and the residue chromatographed over silica gel (Merck, Kieselgel 60) using chloroform/methanol/acetic acid/water (75:25:7.5:2.5) as eluent. Eluted fractions showing single fluorescent spots on TLC examination, and which also gave positive spray reactions with Dragendorff and Zinzadze reagents were combined and washed with water, and the organic phase was evaporated to dryness under nitrogen, yielding pure DPHPC. Final yields of DPHPC (based on IV) ranged from 5–10%. The material was homogenous on TLC examination in a range of solvents. Incubation of DPHPC with phospholipase A_2 as described [6] yielded IV and lysophosphatidylcholine, both identified by TLC. DPHPC was stored in the dark as an ethanolic solution at −5°C; it was stable under these conditions for several months.

Results

The photophysical properties of DPHPC proved rather similar to those of DPH. Measurements of fluorescence depolarization as a function of temperature indicated, however, that the fluorophore in DPHPC is more 'restricted' in its motion in liquid crystalline phospholipid than is DPH: polarization values at 53°C for DPH and DPHPC were 0.12 and 0.21, respectively. The polarization in gel-phase phospholipid is similar for both compounds, i.e., 0.37 and 0.36 for DPH and DPHPC at 25°C, respectively. Restriction of probe motion is expected in DPHPC due to covalent linkage of the fluorophore. Uncorrected fluorescence excitation and emission spectra of DPHPC dispersed in dipalmitoylphosphatidylcholine (DPPC) are shown in Fig. 1b. The fluorescence decay profile of DPHPC (less than 1 mol%) in DPPC at 23°C was well fitted ($\chi^2 = 1.15$) to a sum of two components

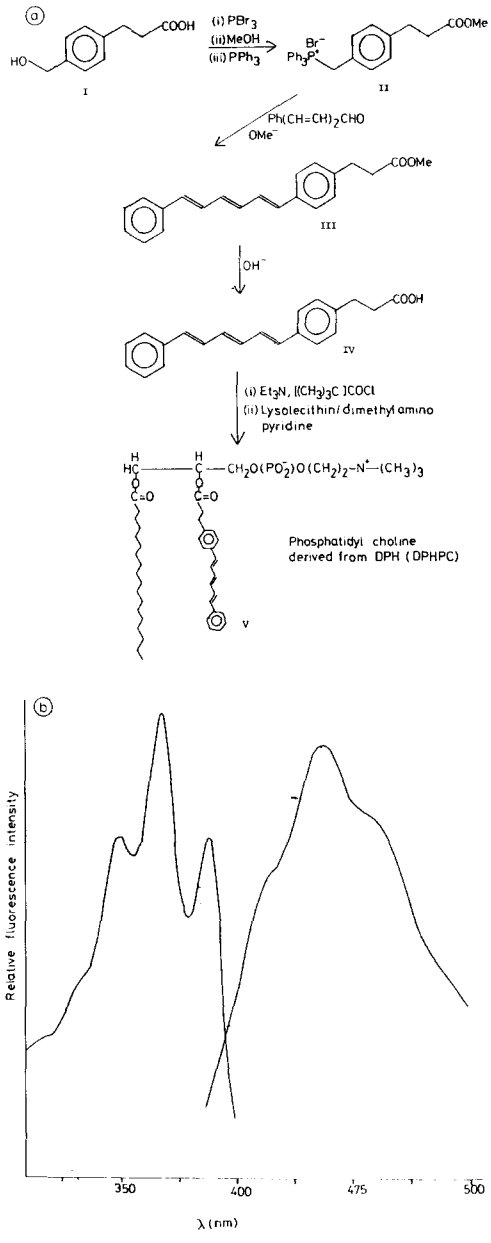

Fig. 1. (a) Synthesis of DPHPC. 4-(Hydroxymethyl)phenylpropionic acid (I), resulting from catalytic hydrogenation of *p*-formylcinnamic acid, was converted to the phosphonium/salt (II). Reaction with phenylpentadienal in the presence of base, followed by hydrolysis and acidification, yielded the carboxylic acid, IV. The phospholipid derivative (V) was prepared by a mixed anhydride synthesis using pivaloyl chloride and lysophosphatidylcholine from egg yolk. The product was purified by column chromatography and stored in ethanol at −5°C. (b) Uncorrected fluorescence excitation and emission spectra of DPHPC (less than 1 mol%) in DPPC vesicles at 20°C. DPPC concentration is approx. 0.2 mg/ml in 50 mM sodium phosphate buffer, pH 7.5.

with decay times of approx. 3.0 ns and 7.6 ns. Similar biphasic behaviour has been obtained for DPH itself [7].

The fluorescence polarization of DPHPC in DPPC vesicles is shown in Fig. 2a as a function of temperature. The phase transition region is rather broad, being typical of the small unilamellar vesicles formed by ethanol injection. If ethanol injection is made into buffer at a temperature below the gel-liquid crystalline phase transition for DPPC, then a sharper transition is seen. Such vesicle preparations are slightly turbid, are presumed to be larger than those used in this study, and are probably multilamellar.

The fluorescence polarization/temperature profile of DPHPC dispersed in DPPC was unaltered when an equal concentration of distearoylphosphatidylcholine (DSPC) vesicles was added. Evidently DPHPC is restricted to the DPPC vesicles and cannot exchange through solution. Similarly, the phase transition of DSPC is seen when an aliquot of labelled DSPC vesicles is mixed with vesicles of DPPC containing no DPHPC (Fig. 2a). If DPH itself is used as a probe under these conditions, both transitions are seen. If an equimolar mixture of lipids labelled with DPHPC is made prior to formation of vesicles then a single broad phase transition is seen as in Fig. 2b. The same result is obtained when DPH is used as probe [8].

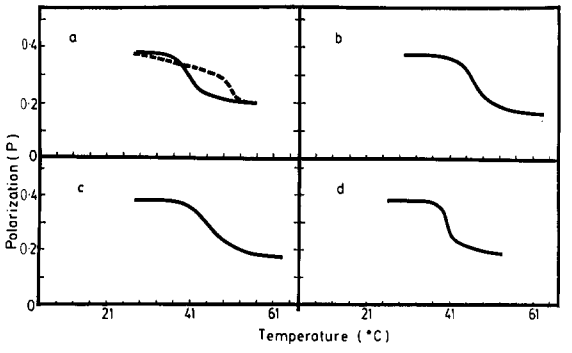

Fig. 2. (a) (Continuous line): Fluorescence polarization of DPHPC in DPPC vesicles as a function of temperature. Conditions as in Fig. 1b. Samples were heated to 60°C and allowed to cool: cooling rate less than 2 C deg/min. (Broken line): Temperature profile for polarization of fluorescence of DPHPC (less than 1 mol%) in vesicles of DSPC. The labelled vesicles were mixed with an equal concentration of unlabelled vesicles of DPPC. Buffer and conditions as in Fig. 1. (b) Fluorescence polarization profile for DPHPC (less than 1 mol%) in vesicles formed from an equimolar mixture of DPPC and DSPC. Lipid concentration was 0.2 mg/ml in 50 mM sodium phosphate buffer, pH 7.4. (c) Effect of melittin on the fluorescence depolarization of DPHPC-labelled DPPC vesicles mixed with unlabelled DSPC vesicles. Total lipid concentration was 0.2 mg/ml. The samples were maintained at 60°C for several minutes and allowed to cool as before. (d) Effect of melittin on the phase transition of DPPC as monitored by DPHPC. The transition is less broad than that shown in (a). Melittin concentration was less than 1 mol% in lipid based on the molecular weight of the monomer.

The fluorescence polarization profile as a function of temperature shown in Fig. 2a does not change when the mixture is kept in the dark for several hours either at room temperature or above 42°C (the 'chain-melting' transition for DPPC). It is therefore clear that neither extensive lipid exchange nor vesicle-vesicle fusion takes place under these circumstances.

Effect of melittin

Melittin is a surface-active peptide from bee venom. It is known to cause cell lysis at low concentrations, and its conformation and interaction with phospholipids has been studied by a variety of techniques [9–11]. It is thought that the lytic effect of melittin is not solely due to detergent action, since surface-active fragments obtained by enzyme digestion are not lytic to cells [12].

The effects of melittin on the interaction between vesicles of phospholipid of the same, and of different acyl chain lengths were studied by fluorescence polarization methods using DPHPC.

Upon addition of melittin (less than 2 mol% relative to phospholipid from a stock solution of 0.1 mg/ml in buffer) to a sample of DPPC vesicles containing DPHPC, no change in fluorescence emission spectrum of the probe was seen, though a small decrease of fluorescence intensity (approx. 5%) was noted. The polarization of fluorescence of the probe remained constant immediately after addition of melittin at room temperature. Addition of a further equal aliquot of melittin produced no further change in fluorescence intensity or polarization. The polarization of fluorescence was unaffected by heating to 55°C for 10 min followed by cooling to room temperature, although the intensity measured then was a few percent lower

than that originally seen before melittin addition. It is possible that the changes in intensity might be due to small changes in light scattering by the solutions, or alternatively might involve alteration of the extent of vesicle binding to cuvette walls. Alternatively, a genuine small change in quantum yield could be responsible. On standing at room temperature in the presence of melittin, phospholipid dispersions became turbid: vortexing reverses this effect to some extent, indicating that aggregation of lipids is partly responsible

Effects of melittin on mixed lipid systems

The effect of addition of melittin (less than 2 mol% relative to phospholipid) at room temperature to a mixture of DPPC (containing DPHPC) and DSPC vesicles (no probe) is shown in Fig. 2c. On heating slowly after incubation for 5–10 min at room temperature, a distorted DPPC transition is seen, while on cooling, the broad transition characteristic of mixed DPPC/DSPC is seen. It is concluded that melittin will cause fusion between the lipid vesicles with mixing of the component lipids on heating.

If this experiment is repeated using DPHPC-labelled DPPC, then the addition of melittin has the effect of sharpening the phase transition as shown in Fig. 2d. The sharp transition is similar to that seen when ethanolic DPPC-containing probe is injected into buffer below 42°C and the temperature profile measured.

The influence of the phase transition region on the fusion is suggested by the following experiments. A mixture of DPPC vesicles and DSPC vesicles, both labelled with DPHPC, is prepared and heated to 65°C. Addition of melittin at this temperature and incubation for up to 10 min prior to cooling does not cause the phase transitions of the individual lipids to be replaced totally by a broad phase transition (Fig. 3a). However, this result is obtained if the vesicles are heated rapidly to 65°C following addition of melittin at room temperature (Fig. 3b). Slow heating of a vesicle mixture immediately after melittin addition gives results intermediate between the limiting cases (Fig. 3c). The DPPC transition is present and sharpened to some extent, suggesting that DPPC vesicles preferentially fuse with each other before the DSPC transition is reached.

Vesicles which had been fused by heating with melittin do not undergo further fusion with small unilamellar vesicles, as demonstrated in the following experiments.

A sample of DPPC containing 2 mol% melittin and no fluorescent probe was heated as before to 65°C and maintained at this temperature for 10 minutes. After cooling to room temperature the sample was mixed with a sample of DSPC vesicles containing probe but no melittin. This mixture was heated to 65°C for 10 min and the fluorescence polarization/temperature profile measured. The result was identical to that given by a sample of DSPC alone showing that under these conditions no further fusion occurred.

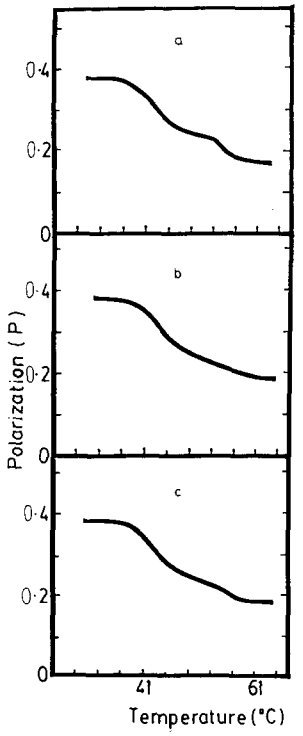

Fig. 3. (a) The effect of melittin (2 mol% relative to monomer and total lipid) on the phase behaviour of a mixture of labelled DSPC vesicles and DPPC vesicles. The melittin was added to a preheated lipid dispersion and the profile recorded on cooling. Conditions are those previously described. (b) The experiment of Fig. 3a is repeated but with melittin addition to the lipid dispersion at room temperature. The sample was heated rapidly to 65°C and the profile recorded on cooling as before. (c) The experiment of Fig. 3b was performed but the sample was heated slowly (2 C deg./min) after addition of melittin and these data were recorded on cooling from 65°C.

This experiment was repeated using a dispersion of DPPC which had been fused as before and which contained no probe, but replacing the DSPC with a dispersion of labelled DPPC which contained no melittin. In this case the DPPC transition was seen, but as a broad profile characteristic of small unilamellar vesicles, showing that the small vesicles had not fused with the larger vesicles. These effects of melittin, as reported by DPHPC, have been confirmed and verified in a parallel study (unpublished data) on DPPC vesicles using electron microscopy.

Discussion

DPHPC is the first of a family of DPH-containing fluorescent probes which we have synthesized for specific types of measurement. The virtue of DPHPC in this set of experiments is its inability to exchange through solution, which allows subsets of a lipid dispersion to be labelled. This has been exploited to study the interaction of small unilamellar vesicles with larger vesicles of the same lipid, as well as to give easily interpreted results in mixed systems. The probe is also suitable for the study of vesicle fusion by fluorescence energy transfer methods.

The fusogenic properties of melittin were first observed recently using a method based on photodimerization kinetics of a fluorescent probe bearing the parinaroyl chromophore [13]. This study demonstrated that the fusion occurred rapidly when lipids were heated through the characteristic phase transition temperature, but that vesicles of 'fluid' lipids such as phosphatidylcholine from egg yolk were not caused to fuse upon heating. The binding of melittin to phospholipid vesicles has been studied by NMR and CD techniques, and it has been suggested that the melittin binds to lipids as a tetramer [14] .and that lipid packing is affected. Phospholipid vesicles formed by ultrasonic irradiation below the phase transition temperature will not fuse spontaneously unless they are first 'annealed' by briefly heating to above the phase transition temperature [15]: defects in acyl chain packing might be involved.

It is reasonable to speculate that the vesicle fusion induced by melittin is the result of perturbation of gel-phase lipid packing and that this effect can be accommodated more readily by lipids in a liquid crystalline phase.

The present study has extended the previous work [13] to mixtures of different phospholipids using an independent method to monitor the fusion of small unilamellar vesicles. It has also demonstrated that the structures produced on fusion do not readily fuse with small unilamellar vesicles added subsequently. It is possible that the structures reach a limiting size and that thereafter the melittin cannot cause further fusion. Alternatively it may be that melittin, once bound, exchanges only slowly and that it must be present in all vesicles for fusion to be favoured. Electron microscopy shows that vesicle aggregation precedes fusion: this can be seen as an increase in the turbidity of melittin-containing samples on standing at room temperature (unpublished data). DPHPC itself cannot distinguish between aggregation and fusion, but lipid derivatives incorporating IV and its nitro analogue are currently being used in this laboratory to distinguish these events by energy transfer measurements.

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