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A fluorescence anisotropy study of stabilizing effect of tri- and tetra- nitrovasodilatory drugs on DPPC liposomal membrane

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Glyceryl trinitrate (GT) and pentaerythritol tetranitrate (PT) are two vasodilatory drugs. The physical properties of the membrane lipid matrix, which determine the structure and function of the membrane-bound proteins, generally control the perturbation mechanism of these drugs. Thus, physical interaction of these drugs with membrane lipids is very crucial for their clinical use, different cellular processes, as well as for targetted drug delivery systems. In the present paper, we have reported for the first time the interaction between these drugs and the lipid molecules in the liposomal system of dipalmitoylphosphatidylcholine (DPPC), as measured by steady-state fluorescence anisotropy using 1,6-diphenyl-1,3,5-hexatriene (DPH) as fluorescent probe. Our results show that by dissolving in the lipid matrix these two drugs effectively stabilise the liposomal membrane: the effect being more in case of GT than in PT, indicating that the rigidifying effect is independent of the number of nitrate groups of the two drugs. This effect increases with the increase in drug concentration, implying solubilisation of all drug molecules. Though our in vitro study has more physical significance than a physiological one, the results obtained here may be used to interpret the effects that are observed in vivo.

Introduction

Glyceryl trinitrate (GT) and penta-erythritol tetranitrate (PT), the two organic nitrate compounds, collectively known as nitro-vasodilators, are used for the symptomatic treatment of angina pectoris. These drugs dilate the constricted blood vessels for which the total output of the heart increases and thereby relieves the symptoms [1]. The biochemical mode of vasodilation of these drugs is already established [2–5]. The observed effect of these drugs depends upon the relevant metabolic pathway and the membrane constituent, i.e., proteins, lipids and others. The physical properties of the lipid matrix are solely responsible for perturbation mechanism of the drug [6] and the associated changes in the lipid matrix ordering does affect the functioning of the membrane proteins due to the changes in their configuration. Thus, the drug-induced changes in structure and function of membrane proteins occur secondarily to changes in membrane lipid ordering [7]. However, the effect of these drugs on the physical properties of the lipid matrix has not yet been reported.

Using a fluorescence polarization probe 1,6-diphenyl-1,3,5-hexatriene (DPH), we are reporting for the first time, the effect of these drugs in their intact (unmetabolised) form on the fluidity profile of liposomal membranes of dipalmitoyl phosphatidyl choline (DPPC). The study shows that these two drugs stabilise the lipid matrix and the effect is more in case of GT than in PT. With the incease in drug concentration, the stabilising effect increases indicating the solubilisation of all drug molecules in the lipid matrix.

Materials and Methods

Reagents. Spectral grade solvents (chloroform, methanol, acetone, and *N,N'*-dimethylformamide) were supplied by E. Merck, India. Dipalmitoylphosphatidylcholine (DPPC) and 1,6-diphenyl-1,3,5-hexatriene (DPH) from Sigma were used without further purification.

Drug extraction. GT was extracted with acetone from medically available tablets of Burroughs Wellcome (Bombay, India) and the reduced volume of the extracted material was assayed for GT content. Finally, a 1 mM solution of GT in acetone was made for experimental purposes [8].

PT was extracted with acetone from medically available tablets of Warner Hindustan (Bombay, India) and

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recrytallised with methanol. A 1 mM solution of PT in acetone was made for experimental purposes [9].

Preparation of drug-incorporated liposomes. DPPC in chloroform and drug in acetone, in required proportion, were put in a round bottom flask and dried to a thin film by rotary evaporation (the vacuum was maintained for several hours to remove the residual solvent). Finally, the film was suspended in double distilled water. The final concentration of lipid was 0.1 mM and the ratios of lipid to drug were 1:0, 1:0.25, 1:0.5 and 1:1 (v/v). The recommended clinical dose for these drugs is 2.5–9 mg, 2–4-times daily as chewable tablets or 0.4 mg per spray [1], much less compared to what we have used. But such high concentrations of drugs were chosen as our earlier experiments have shown that effects of drugs on liposomal membrane are more prominent in relatively higher ratio of drug to lipid than the physiological one [10–12]. This has also been supported by others [13].

The aqueous suspension of lipid and drug was sonicated using an Imeco bath sonicator at a temperature of 50°C, which is approx. 5°C above the phase-transition temperature of DPPC in water, for better formation of liposome [10–12].

Fluorescence anisotropy measurement. 25 μl of DPH solution ($4 \cdot 10^{-4}$ M in *N,N'*-dimethylformamide) was added to the sonicated aqueous dispersion of liposome (with or without the drug). DPH/lipid molar ratio was maintained at a very low level (1:200) to avoid competition between drug and DPH molecule for placement in the hydrophobic core of the liposome.

A Perkin-Elmer fluorescence spectrometer (MPF 44B) was used to measure r, the steady-state fluorescence anisotropy [14]:

$$r = (I_1 - I_2)/(I_1 + 2I_2)$$

where I_1 and I_2 are the vertical and horizontal components of 428 nm emission band of DPH in liposome, while the sample was excited by the vertical component of light from an excitation monochromator at 360 nm. In all experimental setups fluorescence intensities were calculated after properly eliminating the light scattering effect. All measurements were made in a thermostatted cell maintained at constant temperatures.

Results and Discussion

In Fig. 1 we have plotted the temperature profile of the fluorescence anisotropy curve of DPH-probed DPPC liposomes and monitored its change due to the incorporation of two different organo-soluble nitrovasodilatory drugs, GT (Fig. 1A) and PT (Fig. 1B) in the liposomal membrane. From the figure and the data presented in Table I it is evident that both the drugs induce an increase in phase-transition temperature T_c

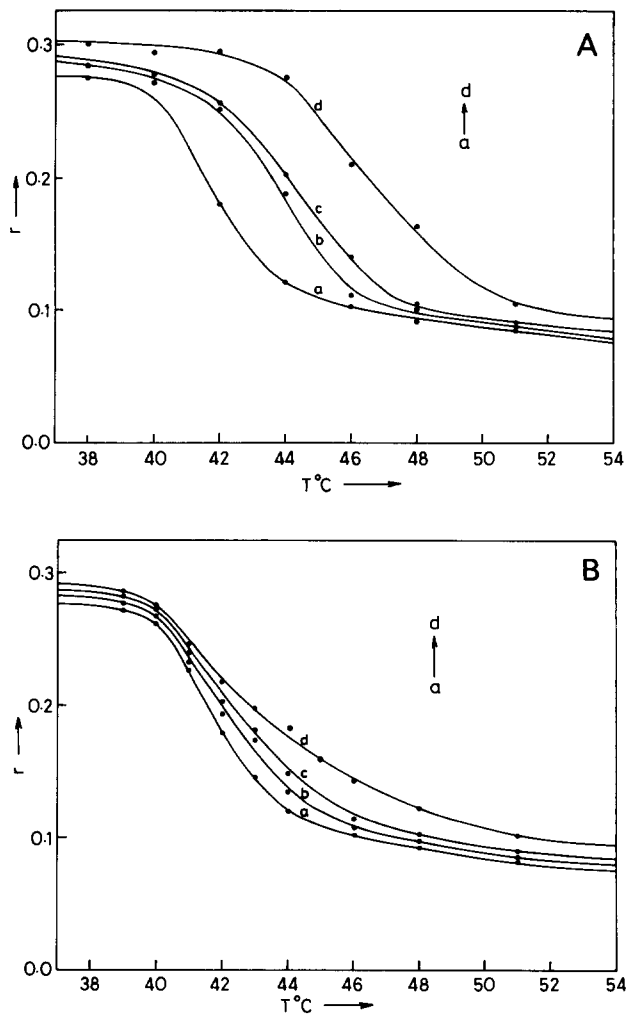

Fig. 1. Fluorescence anisotropy as a function of temperature in DPH-probed DPPC liposome (concentration 10^{-4}M). Molar ratio of lipid to drug (a), 1:0; (b), 1:0.25; (c), 1:0.5 and (d), 1:1. (A) GT-incorporated liposomes; (B) PT-incorporated liposomes

(the temperature corresponding to the point of inflexion), thus implying that throughout the experimental temperature range the drugs effectively stabilize the

TABLE I

Change in phase transition temperature, T_c, with different lipid / drug ratios for GT and PT

Data presented here are the mean of five or six separate measurements, with an average deviation of 5%.

Drug	Lipid/drug molar ratio	T_c (°C)
GT	1:0	42.1
	1:0.25	44.1
	1:0.5	44.7
	1:1	47.1
PT	1:0	42.1
	1:0.25	42.7
	1:0.5	43.1
	1:1	44.3

membrane, resulting in the rigidification of the liquid-crystalline phase of the bilayer. The value of r is also consistently (with respect to drug concentration) more in case of drug incorporated liposome, compared to that for drug-free liposome. Once again this indicates the rigidifying effect of the drug molecules dissolved in the lipid matrix. The change in anisotropy due to change in temperature for drug-free liposome is greater compared to that for drug-incorporated ones. This can be explained from the fact that, due to solubilisation of the drug molecules, the motion of the lipids are restricted throughout the experimental temperature range and so they can not contribute significantly to the change in anisotropy.

To compare the effect of GT and PT on DPPC liposomes, it is evident from Fig. 1 that GT always exerts a greater rigidifying effect than PT and so there is no correlation between the rigidifying effect and the number of nitrate group of the two drugs. This difference probably arises due to the structural differences between the two drugs for which the miscibility of GT in lipid matrix is greater than that of PT and it may be mentioned that GT has a wider spectrum of solubility in organic solvents than PT [15].

Thus, our study shows that due to their solubilisation in the lipid matrix, the two drugs effectively stabilise the membrane even below the lipid phase transition. An interesting point of observation is that this effect is in contrast with the stabilising effect of other small molecules, like cholesterol, where the anisotropy is decreased below the phase transition [10,11]. Another important aspect of this study is to realise the side effects of these drugs on different cellular processes, e.g., mitosis, miosis, etc., which depend on the fluidity of cellular membranes. Besides these, the drug induced change in membrane fluidity also becomes a significant factor for efficient drug release from liposome in the process of targetted drug delivery system [13]. Though our in vitro study has more physical significance than a physiological one, the results obtained here may be used to interpret the effects that are observed in vivo.

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