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Anisodamine causes acyl chain interdigitation in phosphatidylglycerol

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The effect of anisodamine on the structure of the gel phase and the properties of the acyl chain disordering transition of dipalmitoylphosphatidylglycerol (DPPG) has been studied through high-sensitivity differential scanning calorimetry (DSC) and fluorescence polarization measurements of 16-(9-anthroyloxyl)-palmitic acid (16AP) and 3-(9-anthroyloxyl)-stearic acid (3AS), labeling, respectively, the ends and the third carbon of the acyl chains. The non-interdigitated DPPG multilamellar vesicles formed in HEPES buffer show clear fluidity gradient in their acyl chains, whereas the fluidity gradients are completely abolished in the presence of anisodamine. The DSC results showed that the phase transition temperature (T_m) of DPPG is decreased and the enthalpy (ΔH) is increased by anisodamine, while the pre-transition vanishes. At 3 mM anisodamine, the ΔH of DPPG reaches 9.6 kcal/mol. It can be concluded that DPPG forms an interdigitated gel phase in the presence of anisodamine. A molecular scheme for the interaction of anisodamine with DPPG is proposed.

Anisodamine; Interdigitated gel phase; Dipalmitoylphosphatidylglycerol; Fluorescence polarization; Differential scanning calorimetry

1. INTRODUCTION

The interdigitated structure of the lipid bilayer, in other words the insertion of acyl chains of one layer into the other, which exists only in the gel phase, has attracted much attention. It is believed that the existence of an interdigitated phase in a biological membrane may be much more common than previously considered. It is well known that the interdigitated phase can be detected by X-ray diffraction [1], electron paramagnetic resonance [2], Fourier-transform infrared spectroscopy [3] and differential scanning calorimetry, etc. Many factors have been found to induce lipid interdigitation. Some amphiphilic molecules, such as glycerol [1], chlorpromazine [4], methanol, ethanol [5] and thiocyanate [6] can induce an interdigitated gel phase in saturated symmetrical phosphatidylcholines. Polymyxin B[7], myelin basic protein [8,9] and $Tris^+$ [10] can cause interdigitation in phosphatidylglycerols. All these factors have certain molecular volumes and can bind the phospholipids through electrostatic and hydrophobic interactions.

Hyoscyamus niger L., a medicinal herb recorded in the famous ancient Chinese medical book, *Compendium of Materia Medica*, is widely dispersed throughout China. Anisodamine is isolated from these medicinal herbs and was synthesized first by Chinese scientists. Drugs also belonging to this group are scopolamine and atropine. These drugs, showing an inhibitory effect on the cholinergic nerve function, as well as an improvement of the microcirculation, are extensively used in clinics, especially in the case of toxic shock and organophosphorus intoxication. The interaction of anisodamine with membranes has been extensively studied by our laboratory. The results showed that anisodamine increases the fluidity of membranes [11], causes phase separation of DPPA [12], and induces the hexagonal phase of cardiolipin and dioleoylphosphatidylcholine liposomes [13]. The drug shows strong interactions with biomembranes, especially with the acid phospholipids.

In this paper, our research indicates that anisodamine completely abolishes the fluidity gradient of DPPG bilayers at temperatures below T_m, which can be found in the gel phase as well as the liquid–crystalline phase of a non-interdigitated lipid lamella. These results enable us to elucidate further the molecular mechanism of the interaction of the drug with biomembranes and to explain the phenomenon that anisodamine affects the conformation and function of transmembrane proteins in acid phospholipid bilayers [14,15].

2. MATERIALS AND METHODS

2.1. *Materials*

Dipalmitoylphosphatidylglycerol, 16-(9-anthroyloxyl)-palmitic acid (16AP) and 3-(9-anthroyloxyl)-stearic acid (3AS) were purchased from Sigma Chemical Co. Anisodamine was obtained from Chengdu First Pharmaceutical Factory and purified before use. The chemical structure of the drug is shown in Fig. 4. HEPES was obtained from Fluka, Switzerland, and re-distilled water was used.

2.2. *Preparation of DPPG vesicles*

10 mg DPPG were made into a thin film by dissolving in 1 ml chloroform/methanol (1:1 v/v), evaporating under a stream of nitro-

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Abbreviations: DPPG, dipalmitoylphosphatidylglycerol; DSC, differential scanning calorimetry; 16AP, 16-(9-anthroyloxyl)-palmitic acid; 3AS, 3-(9-anthroyloxyl)-stearic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

gen and evacuating in a lyophilizer overnight. The samples containing various amounts of anisodamine were hydrated with 1 ml of 10 mM HEPES buffer solution, pH 7.4. The lipid vesicles were prepared by vigorously vortexing the samples for 5 min at 45°C.

2.3. *Fluorescence polarization measurements*

Fluorescence polarization measurements were determined on an HITACHI F-4010 fluorescence spectrophotometer fitted with a polarization attachment. The fluorescence probes of 16AP or 3AS dissolved in ethanol (about 0.3 mg/ml) were dispersed in buffer as above to a final concentration of probe of 0.01 mg/ml. The probes in buffer were thoroughly mixed with the DPPG suspension at a probe-to-lipid molar ratio of 1:1,000, then incubated at 45°C for 2 h and at room temperature for 1 h. After diluting to a lipid concentration of 0.1 mg/ml, the samples were excited at 388 nm, and the emissions at 460 nm were recorded. The degree of fluorescence polarization (P), which reflects the motion and viscosity of lipid molecules, was calculated according to the following formula:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

Where I_{VV} and I_{VH} are the fluorescence intensities measured with parallel and perpendicular oriented polarizers, respectively, and G is the calibration factor. Here, $G=I_{HV}/I_{HH}$.

2.4. *Differential scanning calorimetry*

All DSC measurements were made with a high-resolution MC-2 differential scanning microcalorimeter interfaced to an IBM microcomputer(Microcal Inc., Northampton, MA). The samples were carefully degassed before the calorimeter was loaded. The calorimetric data were collected and stored automatically by the microcomputer using the DA-2 digital acquisition system provided by Microcal. Each sample was scanned at a rate of 60°C/h. The lipid content was accurately determined by phosphorous assay [16] and the lipid concentration of all samples undergoing calorimetry was adjusted to 1 mg/ml. The transition temperature, T_m, of the assigned main phase transition was obtained from the endothermic peak in the DSC curve at maximal excess heat capacity. The transition enthalpy (ΔH) was calculated from the area under the endothermic peak using the software sub-routines provided by Microcal.

3. RESULTS

In an interdigitated structure, the phospholipid molecules insert into the opposite lipid layer with the ends of the acyl chains near to the polar head groups of the opposing lipid molecules. The carbon chains of lipid molecules in non-interdigitated lamellae display a fluidity gradient (i.e. the fluidity becomes greater from polar head to methyl end). However, the gradient tends to be abolished in the interdigitated bilayer, especially in the fully interdigitated phase because the insertion of acyl chains of one lipid layer into the other makes the methyl ends of acyl chains display similar physical properties to the carbons near the polar head groups. As an application of this argument, Wang, H.Y. et al. used spin labels, 16-doxyl-stearic acid, to determine the interdigitated phase of DPPG induced by polymyxin B [2]. Here the fluorescence probes of 3AS and 16AP were employed, respectively, to detect the mobility of sites either near the exterior surface of the lamellae or at the ends of the carbon chains of various DPPG vesicles.

It is obvious that the increasing of polarization results from the enhancing of viscosity and diminishing of mobility and fluidity. Fig. 1A shows that non-interdigitated DPPG vesicles formed in HEPES buffer in the absence of anisodamine always have a fluidity gradient in both gel and liquid–crystalline phases, as the fluidity of the ends of acyl chains is much greater than that near to the polar heads, while in the presence of 0.2 mM anisodamine (Fig. 1B), however, the polarization of 16AP and 3AS below about 37°C is similar, i.e. the fluidity of the acyl chain ends is decreased. When the temperature is raised above 40°C (about the T_m) and the phase transition occurs, the polarization of 16AP decreases dramatically, exhibiting the fluidity gradient, the characteristic of non-interdigitation. These results indicate that 0.2 mM anisodamine makes DPPG vesicles an interdigitated structure in the gel phase. In fact, interdigitation usually does not occur at temperatures above T_m, and C(18):C(10)PC is the first example in which the acyl chain interdigitations have been found for the phospholipid bilayer in both the gel and liquid–crystalline states [17]. In the presence of high concentrations of anisodamine, the interdigitations of DPPG molecules are also observed at temperatures below T_m, although some differences exist. We note from Fig.1C and D that, along with the increasing concentration of anisodamine, the phase transition temperatures of DPPG are de-

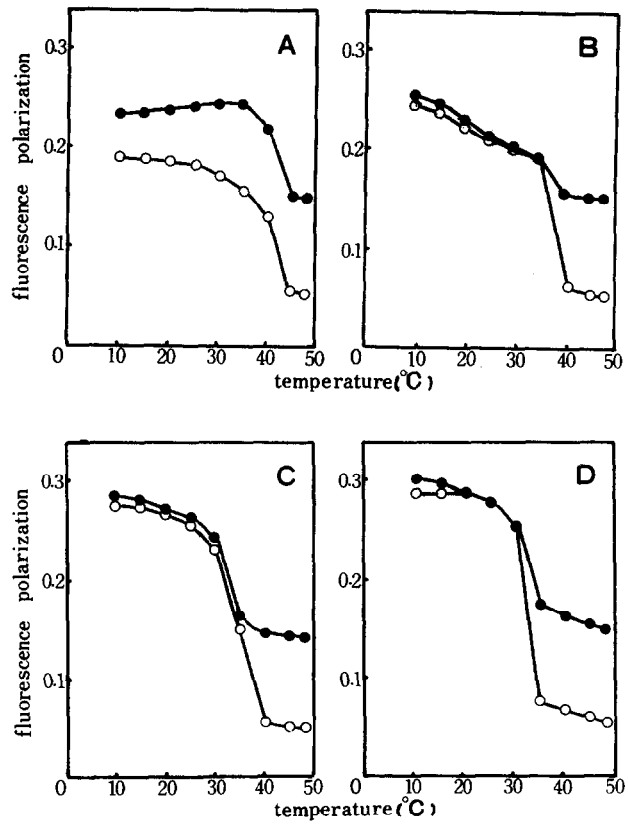

Fig. 1. Fluorescence polarizations of 3AS and 16AP probes in DPPG vesicles by the presence of various concentrations of anisodamine. (●) 3AS; (○) 16AP. (A) Control; (B) 0.2 mM anisodamine; (C) 3 mM anisodamine; (D) 25 mM anisodamine.

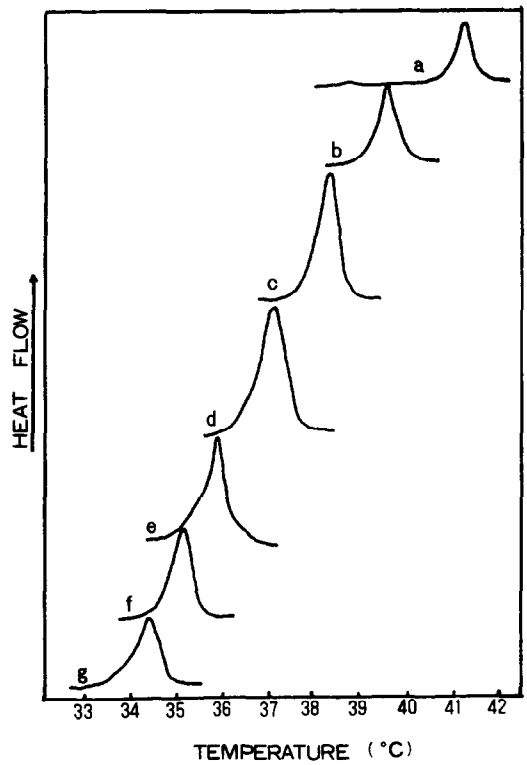

Fig. 2. DSC thermograms of DPPG vesicles in the presence of various concentrations of anisodamine: a, control; b, 0.2 mM; c, 1 mM; d, 3mM; e, 6mM; f, 10 mM; g, 25 mM. Each sample contained 1 mg/ml of phospholipid.

creased and the fluorescence polarizations of the gel phase are increased, which implies that the DPPG lamellae become more ordered.

Fig. 2 shows the DSC thermograms of DPPG vesicles in the presence of various concentrations of anisodamine. A transition centered at 41.2°C was obtained with pure DPPG in HEPES buffer(Fig. 2a). Anisodamine, at a concentration of 0.2 mM, causes a shift in the gel-to-liquid–crystalline phase transition temperature to a lower temperature, 39°C, and abolishes the pre-transitional peak. The pre-transition occurs only in lipids with tilted chains [18], whereas in the interdigitated phase, the acyl chains are perpendicular to the bilayer surface. T_m decreases gradually to 34.5°C as the concentration of anisodamine increases from 0.2 mM to 25 mM. The peak remained sharp, showing high cooperativity in phase transition. Significant increases in peak areas are observed when the concentrations of anisodamine are in the range of 1–6 mM. At 25 mM anisodamine, the transition peak of DPPG is slightly broadened.

The detailed information on the effect of anisodamine on DPPG phase transition behavior is given in Fig. 3. The phase transition enthalpy (ΔH) of DPPG is increased from 7.1 kcal/mol to 9.6 kcal/mol by 3 mM anisodamine, very similar to the enthalpies, 10.6 kcal/mol [2] and 9.1 kcal/mol [10], of the interdigitated DPPG vesicles caused by polymyxin B and $Tris^+$, respectively. Fig. 3 also shows that the binding sites of DPPG vesicles are almost saturated by 10 mM anisodamine. It is obvious that the DSC results also support the idea that DPPG is interdigitated in the presence of anisodamine.

4. DISCUSSION

Anisodamine clearly causes complete interdigitation and restricts the motion of 16AP in DPPG bilayers. It is believed that a molecule may induce lipid interdigitation if it meets the three following requirements. First, the molecule must be amphiphilic with a constraint size; second, the molecule must displace water from a particular location in the interfacial region; and third, its non-polar moiety cannot extend too deeply into the bilayer interior. When small amphiphilic molecules are located in the interfacial region of gel state phospholipid vesicles, they anchor to the interface by virtue of their polar moiety, with the non-polar part of the molecule intercalating between the rigid acyl chains. In the case of short amphiphilic molecules, the non-polar moieties of which are not as long as the lipid hydrocarbon chains, this would potentially cause voids between chains in the bilayer interior. Since the energy of formation of holes in hydrocarbons is extremely large, interdigitation must occur to decrease the potential energy and stabilize the membrane.

Fig. 4A give the structure of the anisodamine molecule. It shows that anisodamine has a monovalent trialkylamine cation, two hydrophilic hydroxyl groups and a hydrophobic benzene ring. Thus, the drug can be regarded as a big amphiphilic molecule with one posi-

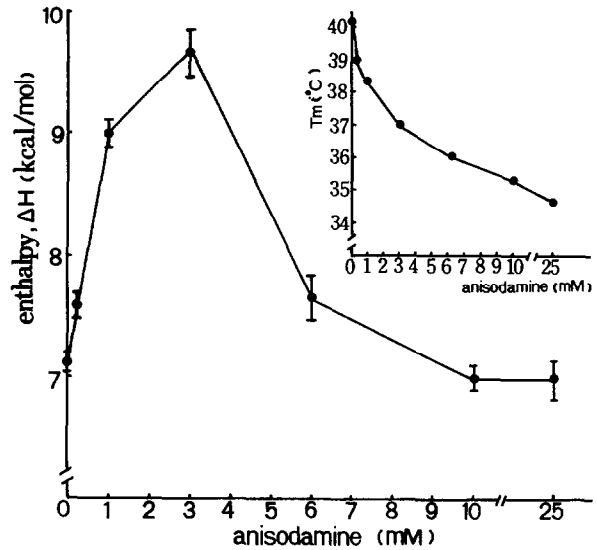

Fig. 3. Effect of anisodamine on the phase transition behavior, ΔH and T_m, of DPPG vesicles. The transition temperature, T_m, of the assigned main phase transition was obtained from the endothermic peak in the DSC curve at maximal excess heat capacity, and the transition enthalpy (ΔH) was calculated from the area under the endothermic peak.

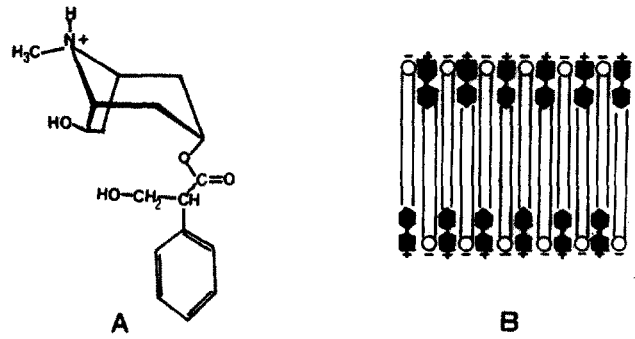

Fig. 4. Diagrammatic representation of the structure of anisodamine (A) and the interaction between the drug and DPPG molecules (B), showing the electrostatic force between the trialkylamino cation of the drug and the negatively-charged head group of the acid lipid, and the intercalation of the aromatic ring into the bilayer surface.

tive charge and a short non-polar moiety. It was proved by Raman spectroscopy that an electrostatic interaction exists between the trialkylamino group of the drug and the headgroup of acid lipids, while the benzene ring of the drug inserts into the phospholipid bilayer [19]. A diagram in Fig. 4B demonstrates the interactions between anisodamine and DPPG molecules.

Our previous studies showed that anisodamine inhibits the activity of Ca^{2+}-ATPase from sarcoplasmic reticulum, and $(Na^+ + K^+)$-ATPase from the kidneys of rabbits by particularly acidic phospholipids [14,15]. We also proved that the interdigitated phase of DPPG inactivated Ca^{2+}-ATPase from rabbit sarcoplasmic reticulum [20]. It is indicated by our work that the two interdigitated and non-interdigitated phases can be converted reversibly by changing either the temperature or buffer composition; consequently the conformation of the integral membrane protein confined to the bilayer can be modulated readily by the lipid matrix [21]. So, it is probably the interdigitated phase induced by anisodamine that inhibits these transmembrane enzymes.

Anisodamine, which belongs to a class of drugs that act on the central nervous system, has the ability to preferentially interact with acidic phospholipids of membranes [22]. It was reported that anisodamine can increase the permeability of liposomes containing acid phospholipids to Ca^{2+}[23]. It is generally believed that acidic phospholipids play an important role in the membranes of neural cells. We propose that anisodamine may causes these phospholipids to form interdigitated structure. In vivo, this structure probably exists in some small regions of gel phase formed by di-saturated membrane lipids because interdigitation of saturated lipids only occurs in the gel phase and cell membranes are usually in liquid–crystalline phase at physiological temperature. It is suggested that anisodamine displays extensive effects, such as improvement of the microcirculation and therary of nephritis and heart-lung failure, through this mechanism, besides its inhibitory effect on cholinergic nerve function. Atropine, a well-known anaesthetic, has a structure very similar to anisodamine with an alkyl group instead of the hydroxyl radical at the cycloalkane. This molecule has been shown to disorder the lipid bilayer [24]. Does the anaesthetic induce lipid interdigitation and affect the function of membranes through this mechanism? The question remains interesting.

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