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THE DISORDERING EFFECT OF HYOSCYAMINE DRUGS ON PHOSPHOLIPID MEMBRANES

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The effect of hyoscyamine drugs on the fluidity of dipalmitoylphosphatidylcholine liposomes has been studied by differential scanning calorimetry (DSC), electron spin resonance spectroscopy (ESR), fluorescence polarization and freeze-fracture electron microscopic techniques. DSC results indicate that anisodamine, anisodine, atropine and scopolamine all increase the fluidity of dipalmitoylphosphatidylcholine liposomes but with different degrees of efficiency. The increasing of fluidity of dipalmitoylphosphatidylcholine liposomes by hyoscyamine drugs is in a dose-dependent way. Increase of the fluidity of phosphatidylcholine liposomes by anisodamine was also shown by the other three methods. The possible mechanism of hyoscyamine-membrane interaction is discussed.

Introduction

Phospholipids are important constituents of biological membranes. Their physicochemical properties have been the subject of extensive studies. As the membrane composition is too complex to allow a simple analysis, liposomes are widely used as models for biological membranes. Many studies dealing with the mode of action of anesthetics focus on the change of fluidity of phospholipids by interacting with the anesthetic molecules [1–5]. Recent studies using DSC, ESR, fluorescence spectroscopy and NMR techniques have shown that local anesthetics can induce significant molecular disordering and increase the fluidity of phospholipid in natural and model membranes. But the mechanism of action of anesthetics is ill-defined.

Hyoscyamus niger L., medicinal herbs recorded in the famous ancient Chinese medical book Compendium of Materia Medica, are widely dispersed all over China. Anisodamine and anisodine are newly isolated from these medicinal herbs and were 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 the improvement of microcirculation, are extensively used in clinic, especially in case of toxic shock and organophosphorus intoxication. Anisodine and scopolamine, if they are in combination with chlorpromazine, can be used as combined intravenous anesthetics.

In this paper we study the effects of hyoscyamine drugs on the fluidity of model membrane in an attempt to elucidate the molecular mechanism of the interaction of the drugs with biomembranes. Four different physical techniques have been used to provide complementary information. All the results show that hyoscyamine drugs increase the membrane fluidity and the effects of the four drugs were compared.

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC) and 1,6-diphenylhexatriene (DPH) were obtained from Sigma Chemical Co. DPPC showed a single spot by thin-layer chromatography, without further purification. Anisodamine, anisodine and scopolamine as their hydrobromides and atropine sulfate were obtained from Chengdu First Pharmaceutical Factory and purified before use. The chemical structures of these drugs are shown in Fig. 1. Hepes was obtained from Fluka, Switzerland and redistilled water was used.

Preparation of drug-lipid mixtures

DPPC and the drug were dissolved in chloroform and redistilled water separately and mixed according to a desired molar ratio. Solvents were removed under vacuum and their last traces were then expelled by placing the samples in vacuum desiccator with phosphorus pentoxide over night. The thin drug/lipid films were suspended in the Hepes solution (with DPPC concentration about 30 mg/ml), transferred to a stoppered small Teflon tube and dispersed by sonication (with CFS-250-5 Ultrasonic generator, 10–15 kHz, 250 W) at about 45°C for 10 min.

Multimembrane liposomes could be observed by freeze-fracture electron microscopy. The drug/lipid mixture was concentrated under reduced pressure to a final concentration of DPPC 120 mg/ml in Hepes (30 mM) solution. The pH was about 5.8. Pure lipid dispersion was used as control.

Liposomes for freeze-fracture electron microscopy were prepared by dispersing with YKH-I Vortex mixer for 8 min above the lipid phase transition temperature. The final concentration of DPPC was 40 mg/ml.

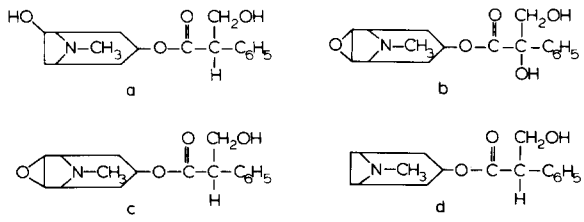

Fig. 1. The chemical structures of the hyoscyamine drugs. (a) anisodamine; (b) anisodine; (c) scopolamine; (d) atropine.

Differential scanning calorimetric measurement

All calorimetric measurements were made in sealed calorimeter pans by using a Perkin-Elmer DSC 2B differential scanning calorimeter supplemented with Intracooler II. Temperature was calibrated with indium (P-E standard). All the scans were obtained with: range setting, 1 mcal/s; heating rate, 5 K/min; nitrogen atmosphere; sample volume, 12 μl. The transition temperature, peak maximum T_m was recorded by a microprocessor.

Electron resonance spectroscopic measurement

TEMPO ($5 \cdot 10^{-3}$ M in water) was added to the DPPC liposome to give a final TEMPO concentration of $5 \cdot 10^{-4}$ M. Samples were transferred to a quartz tube with an inner diameter 1 mm. The temperature was controlled with ± 1 K over a range of 20–60°C. ESR spectra were recorded on a JES-3BX spectrometer.

Fluorescence polarization measurement

$2 \cdot 10^{-3}$ M diphenylhexatriene in tetrahydrofuran was diluted 1000-fold with 0.01 M phosphate-buffered saline (pH 7.4) just before use and then mixed in a 1:1 ratio with liposome suspension to make up a lipid concentration of 0.5 mg/3 ml. The mixture was incubated 30 min at 30°C in the absence or presence of anisodamine for diphenylhexatriene labelling, and then with a further incubation for 1.5 h. Fluorescence polarization measurements of diphenylhexatriene incorporated into liposomes was performed as described by Shinitzky et al. [6] and carried out with a Hitachi MPF-Model 4 spectrofluorimeter, excitation was set at 360 nm and emission at 430 nm. The temperature of the sample was checked by using a thermister with an accuracy of ± 0.5 K. The degree of fluorescence polarization $p = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + GI_{\perp})$ was measured with 5-nm slits, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities measured parallel and perpendicular to the plane polarized of the excitation, respectively; G is the correction factor. Microviscosity was calculated according to the Perrin equation [7].

Freeze-fracture electron microscopy

Prior to freeze-fracturing, glycerol was added into the liposomes with a final concentration of

12%. A small volume of sample was pipetted into specimen block and equilibrated at 35°C for 15 min, quenched rapidly in the liquid nitrogen. The samples were fractured by using a HUS-5GB module and coated with platinum-carbon at a vacuum of $1 \cdot 10^{-5}$ Torr. The replicas were cleaned in chloroform/methanol, picked up on copper grid and examined in a JEM-100CX electron microscope.

Results

Differential scanning calorimetry

Anisodamine, at a concentration of 10 mol%, causes a shift in the gel to liquid-crystal phase transition temperature of DPPC to a lower temperature and vanishing of the 'pre-transitional' peak. T_m decreases gradually to 312.3 K as the concentration of anisodamine increases from 10 mol% to 66.7 mol% (Fig. 2), with a decrease of temperature from 0.5 K to 3.6 K. The peak remains sharp, showing a good cooperativity in phase transition, and no significant change in peak area is observed.

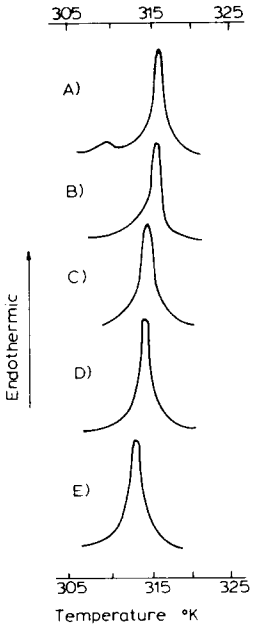

Fig. 2. Differential scanning calorimetry curves showing the effect of different concentrations of anisodamine in mol% on the gel to liquid-crystalline transition of dipalmitoylphosphatidylcholine liposomes. (A) zero; (B) 10; (C) 30; (D) 50; (E) 66.7 mol%.

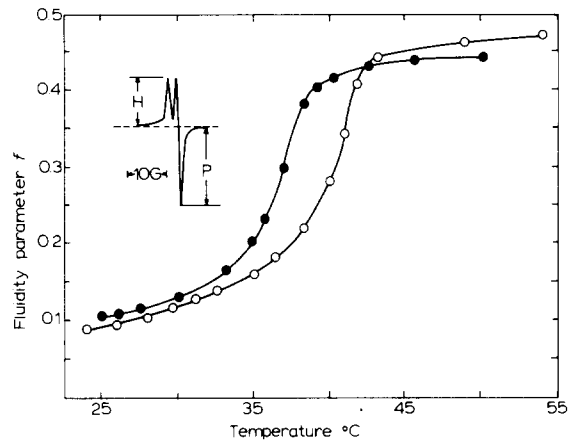

Fig. 3. Phase transition curves of dipalmitoylphosphatidylcholine liposomes with different concentrations of anisodamine. ○, zero; ●, 50 mol%.

Anisodine, scopolamine and atropine affect the transition temperature of DPPC in the same way as does anisodamine. Atropine, at a concentration of 10 mol%, decreases the T_m, while scopolamine and anisodine at the same concentration do not show any effect on the transition temperature of DPPC. When the concentration of scopolamine and anisodine increases to 30 mol%, T_m begins to decrease. T_m decreases with the increase of the drug concentration. The effect of these four drugs on membrane fluidity are in the following order: anisodamine ≈ atropine > scopolamine > anisodine. Drugs at a concentration of 50 mol% result in a decrease of transition temperature of 2.4, 2.3, 1.4 and 0.8 K, respectively, only a slight increase in membrane fluidity can be seen.

TABLE I

EFFECT OF ANISODAMINE ON THE DEGREE OF FLUORESCENCE POLARIZATION (P) AND MICROVISCOSITY ($\bar{\eta}$) OF DPPC LIPOSOMES MEASURED AT 41°C

P and $\bar{\eta}$ are reported as means ± S.D. of eight experiments. * $p < 0.001$, as estimated by Student's t-test with respect to control.

Anisodamine : DPPC (mol%)	Polarization (P)	Microviscosity ($\bar{\eta}$)
0 : 100	0.225 ± 0.009	1.97 ± 0.04
50 : 50	0.202 ± 0.012 *	1.57 ± 0.05 *

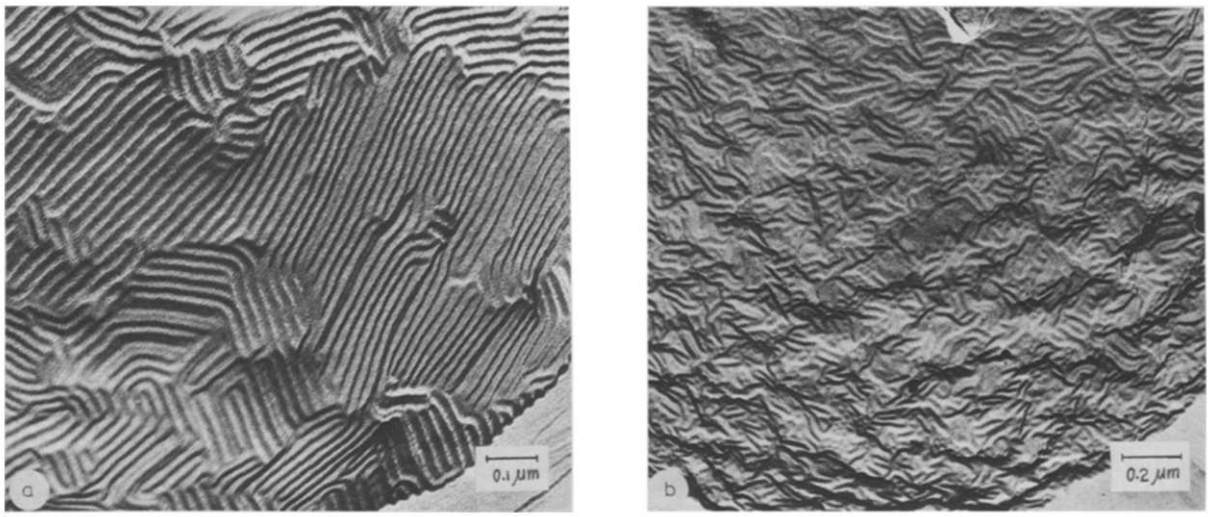

Fig. 4. Freeze-fracture electron micrographs of dipalmitoylphosphatidylcholine liposomes quenched from 35°C. (a) Without anisodamine, magnification about 62400×. (b) With 30 mol% anisodamine, magnification about 38400×.

Electron spin resonance

The phase transition temperature of DPPC liposomes with and without anisodamine was ascertained by determining and plotting the TEMPO spectral parameter f as described by Shimshick and McConnell [8].

Anisodamine enhances the solubility of TEMPO in the hydrophobic region of DPPC liposomes, which reveals the decrease of the phase transition temperature. Phase transition curves of DPPC in the presence and absence of anisodamine are shown in Fig. 3. Anisodamine at the concentrations of 30 mol% and 50 mol% decrease the phase transition temperature of DPPC liposome about 2°C and 4°C, respectively. This indicates that the fluidity of DPPC was increased. The results are in agreement with those obtained by the DSC method.

Fluorescence polarization

The fluorescence excitation and emission maximum of diphenylhexatriene solution are at 375 nm and 440 nm, respectively, and those of anisodamine in phosphate-buffered saline are at 373 nm and 408 nm. When the two additional slits were set at a certain position, the effect of anisodamine could be eliminated. When diphenylhexatriene was incorporated into DPPC liposomes both excitation and emission spectra show a blue shift and the fluorescence intensity is sharply increased. The polarization of diphenylhexatriene fluorescence, P, in DPPC liposomes containing 50 mol% anisodamine has no change at 25°C, but it is decreased at 41°C by comparison with the control. Anisodamine also decreases the microviscosity of DPPC liposomes (Table I). The lower the observed P and $\bar{\eta}$ value, the more fluid the labelled membranes.

Freeze-fracture electron microscopy

The effect of anisodamine on the crystalline phase structure of DPPC liposomes is shown in Fig. 4. Since the phase transition temperature of DPPC liposomes is 41°C, a regular rippling pattern can be observed when DPPC liposomes have been quenched from 35°C. This result is in accord with our previous report [9]. If 30 mol% of anisodamine is added into DPPC liposomes and quenched from 35°C, the regular rippling pattern of liposomes is disturbed.

Discussion

Most drugs must cross biological membranes prior to reaching their target; therefore, a good understanding of the mechanism of the drug-membrane interaction seems essential. More attention has been paid recently to the study of the interac-

tion of drugs with liposomes or biomembranes, which is one of the major problems in molecular pharmacology. It has been found that some drugs can interact directly with specific protein receptor sites, whereas others interact with membrane lipids or with hydrophobic regions of the membranes. Anesthetics have been shown to disorder the lipid bilayer [10] and cationic local anesthetics can affect cytochrome oxidase activity [11]. The mechanism of action of hyoscyamine drugs on membranes is interesting and remains to be elucidated.

Our calorimetric results show that the phase transition temperature of DPPC from the gel to the liquid-crystalline state is shifted to a lower temperature by all these four hyoscyamine drugs. The increase of fluidity of DPPC liposomes by drug is in a dose-dependent way. The higher the drug concentration, the greater the transition temperature shift.

The use of the TEMPO spectral parameter, f, a measure of the relative concentration of the probe in the hydrophobic and aqueous phases of the phospholipid membrane, was reported in the study of the influence of the drugs on the fluidity of membranes [8]. The phase transition curve of DPPC membranes indicates that the incorporation of TEMPO into DPPC liposomes is increased as the concentration of anisodamine increases from 30 mol% to 50 mol%. This result parallels that obtained from DSC.

Fluorescence polarization data further demonstrated a disordering effect of anisodamine in DPPC liposome. The addition of anisodamine in a molar ratio equal to that of the DPPC results in a more decreased polarization of diphenylhexatriene fluorescence at temperature about 41°C by comparison with the control. Since diphenylhexatriene is a small lipophilic molecule which can incorporate spontaneously into the hydrophobic region of the lipid where melting occurs, and can be detected by a dramatic decrease in the polarization [12], the greater the fluidity of the membrane the smaller the polarization and microviscosity. Thus the disordering can be well interpreted.

The DSC results shown in Fig. 2 illustrate that the transition peak remains sharp, showing a good cooperativity in phase transition and no significant change in peak area, which suggests that the interaction of drug with membrane is of 'trigger mechanism'. The drug molecule interacts only with the polar head of the lipid molecule on both sides of lipid bilayer by electrostatic force, which loosens the hydrophobic region of the lipid molecule and thus affects phase transition temperature. Since these drugs are of polyheterocyclic structure, having a larger steric hindrance and polar groups such as hydroxyl group, oxygen bridge, it is probably impossible for the drug molecules to penetrate into the hydrophobic region and affect the degree of crystallization of the lipid bilayer directly. The drugs at a high concentration of 50 mol% give rise to only a slight increase in membrane fluidity. This is probably due to the fact that DPPC, a neutral phospholipid, reacts weakly on drug molecule by electrostatic forces. The small differences between the structures and polarities of these four drugs exhibit only a minute difference in their effects.

Recently, freeze-fracture electron microscopy has become one of the new methods used to study the phase transition and phase separation of liposome. This technique allows a direct visualization of the morphology of synthetic phospholipid liposomes. We found that a regular rippling pattern can be observed on the fractured face with a repeat distance about 150 Å when DPPC liposomes are quenched from 35°C (Fig. 4a). The crystalline phase was perturbed significantly when DPPC liposomes contained 30 mol% anisodamine and quenched from the same temperature (Fig. 4b). This shows that the pretransition had vanished, which is consistent with the results from DSC.

Morphine derivatives and some antidepressants lowered the transition temperature of phospholipid membranes to a different extent. Even at drug/lipid ratio of 1 : 1, no significant change in heat of transition is observed [13–15]. Similar effects were obtained from diethazine, chlorpromazine and dibucaine. Local anesthetics, antiarrhythmic drugs, are able to affect the fluidity of membranes in different ways [16,17]. Results obtained in this study clearly demonstrate that hyoscyamine drugs can increase the fluidity of DPPC liposomes, which is similar to the action of anesthetics, since anisodine and scopolamine can also function as anesthetics. Although the effect of drugs on phase transitions of lipids has been observed only at much higher concentrations of the drug compared to the clinical levels of most of the

drugs reported, the results may be also of pharmacological importance.

As stated above, these hyoscyamine drugs react weakly with DPPC, a neutral phospholipid with trigger mechanism; thus it is quite reasonable to expect a profound effect on acidic phospholipid. Preferential interaction of propranolol, quinidine and dibucaine on acid phospholipid was reported. Our recent work has shown that the effect of hyoscyamine on acid phospholipid liposomes is more marked than on the DPPC liposomes. Further investigations are in progress.

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