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Trifluoperazine induces domain formation in zwitterionic phosphatidylcholine but not in charged phosphatidylglycerol bilayers

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Abstract

The interaction of trifluoperazine with the zwitterionic lipids dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine and with anionic dimyristoylphosphatidylglycerol was studied by means of microcalorimetry and fluorescence spectroscopy. Intercalation of drug molecules into the lipid bilayers was confirmed by the observed differential scanning calorimetry peak broadening and the decrease in chain-melting temperatures. For trifluoperazine:lipid mole ratios higher than 0.4 and 0.6 (for dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine, respectively) the deconvolution of transition profiles into two Gaussian components was possible, which suggests phase separation in the studied mixtures. Deconvolution of the thermograms was not possible for any of the drug:dimyristoylphosphatidylglycerol mole ratios studied. To confirm the existence of phase separation in trifluoperazine-phosphatidylcholine mixtures fluorescence spectroscopy experiments were performed using Laurdan as a probe. From the generalised polarisation versus excitation wavelength dependences, recorded at different temperatures, we conclude that a phase separation occurs in the gel state of the studied trifluoperazine-phosphatidylcholine mixtures. We attribute the existence of domains in the bilayer to the dissimilar interactions of two protonation forms of trifluoperazine with phosphatidylcholine molecules. Structural defects present at domain boundaries could be related to the trifluoperazine induced increase of membrane permeability and fluidity. This may partially explain the mechanism of multidrug resistance modulation by trifluoperazine.

Keywords: Trifluoperazine; Phospholipid bilayer; Differential scanning calorimetry; Laurdan fluorescence; Phase separation

1. Introduction

Trifluoperazine (TFP) is a widely known calmodulin antagonist [1] and a very effective antipsychotic drug. The drug is also known to affect the gating mechanism of voltage dependent sodium [2] or potassium channels [3], to inhibit protein kinase C [4] and adenylate cyclase [5], and to interact with ATP synthase [6]. All of these processes require TFP binding to specific binding sites on target molecules. However, within the wide spectrum of known biological effects of TFP there are some that do not

Abbreviations: CPZ, chlorpromazine; DMSO, dimethyl sulphoxide; ΔH, molar enthalpy change of phase transition; GP, generalised polarisation; I_R, fluorescence intensity at the red edge of Laurdan emission spectrum; I_B, fluorescence intensity at the blue edge of Laurdan emission spectrum; λ_{ex}, excitation wavelength; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; T_M, temperature of phospholipid main phase transition; TFP, trifluoperazine

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need specific binding. Such phenomena as antioxidant TFP activity in hydrophobic environment [7,8], erythrocyte haemolysis [9] or reversal of multidrug resistance (MDR) by this drug [10] are good examples of such a non-specificity. They may be explained by the alterations induced by TFP in phospholipid bilayers.

Interactions between phenothiazines and phospholipid bilayers or membrane models were studied by a variety of experimental techniques. Frenzel et al. [11] have shown by means of microcalorimetry, ^{13}C NMR and ^{31}P NMR that chlorpromazine (CPZ) molecules are incorporated into dipalmitoylphosphatidylcholine bilayers and are located close to the polar/hydrophobic interfacial region of the bilayer. Experiments on the influence of CPZ on erythrocyte shape changes [12,13] have demonstrated that CPZ preferentially interacts with anionic phosphatidylserine (PS) and induces redistribution of this lipid between the inner and the outer layer of the membrane. The interaction of CPZ and TFP with erythrocyte membranes was confirmed by ESR experiments [14]. The authors of this work suggested, however, that also erythrocyte skeletal proteins are involved in the modulation of membrane fluidity by phenothiazines. More recent calorimetric and ^{13}C NMR studies [15] have also proved that interaction of CPZ with phosphatidylserine is much stronger than with phosphatidylcholine (PC). It seems, however, that TFP, being more hydrophobic than CPZ, does not distinguish between charged and dipolar phospholipids to such an extent. In a calorimetric and NMR study on the influence of MDR reversing compounds on PS and PC bilayers, Pajeva et al. [16] have found that TFP and CPZ perturb both types of phospholipids almost equally. This work was mainly devoted to screening the interactions of tested drugs with phospholipids and therefore it contains only a limited discussion of the effects of TFP on lipid bilayers.

The aim of the present study was to characterise the influence of TFP on lipid bilayers composed of zwitterionic (1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC)) or anionic (1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol (DMPG)) phospholipids. Calorimetric measurements provided us with information about the thermotropic phase behaviour of TFP-lipid systems. Fluorescence spectroscopic measurements were performed using Laurdan as a fluorescent probe. This probe offers the possibility to detect phase separation occurring in phospholipid bilayers due to its different spectral properties in gel and liquid crystalline phases. We found that trifluoperazine effects on phospholipid bilayers are similar to those described by other authors for various phenothiazine derivatives. Moreover this report is probably the first to show that trifluoperazine induces domain formation in zwitterionic phosphatidylcholine but not in charged phosphatidylglycerol bilayers.

2. Materials and methods

DMPC and DPPC were purchased from Sigma (St. Louis, MO, USA). DMPG was from Avanti Polar Lipids (Alabaster, AL, USA). Lipids were used without further purification. Trifluoperazine was purchased from ICN Biomedicals (Costa Mesa, CA, USA). Laurdan was from Molecular Probes (Eugene, OR, USA). All other chemicals used in the experiments were of analytical grade.

2.1. Microcalorimetry

TFP was dissolved in 20 mM Tris-HCl buffer (150 mM NaCl, 0.5 mM EDTA, pH 7.4). Concentrations of trifluoperazine stock solutions were chosen to obtain the required TFP:lipid mole ratio in the samples. For each sample 2 mg of lipid were suspended and hydrated in 20 μl of appropriate TFP solution. So the lipid molar concentrations were 0.136 M for DPPC, 0.147 M for DMPC, and 0.150 M for DMPG. Mixtures were heated to a temperature about 10°C above the gel-liquid crystalline phase transition temperature of the respective lipid and shaken in the thermostatted mechanical shaker operating at high frequency (>20 Hz) for several minutes. When optical homogeneity was obtained, the mixtures were transferred to aluminium pans and sealed.

Calorimetric measurements were performed using a DSC 600 microcalorimeter (Unipan, Warsaw, Poland) at a scan rate of 1°C/min. For each TFP:lipid mole ratio at least two separate sample preparations were made, and each sample was scanned at least

three times. Samples were scanned immediately after preparation. Calorimetric data were computer analysed off-line using software developed in our laboratory. The temperature at which maximal deviation of the transition peak from the baseline was recorded was taken as the phase transition temperature in cases when the transition profiles were not deconvoluted into two separate Gaussian peaks. The temperatures of Gaussian maxima were taken as the transition temperatures for profiles which were deconvoluted into two components. The area under the transition profiles was used to calculate the molar enthalpy change accompanying phase transition (called further the transition enthalpy).

2.2. Fluorescence spectroscopy

Unilamellar DPPC or DMPG liposomes were obtained by sonication of 2 mM phospholipid suspensions in 20 mM Tris-HCl buffer, 50 mM NaCl (pH 7.4) using a UP 200s sonicator (Dr. Hilscher, Berlin, Germany). Laurdan stock solution (1 mM) was prepared in DMSO. A stock solution of TFP (1 mM) was prepared in double distilled water. Liposomes were incubated with fluorescent probe in darkness for 30 min at room temperature. For the TFP containing samples incubation with Laurdan was preceded by 15 min incubation with phenothiazine. In all experiments the final phospholipid concentration was 200 μM. The concentration of Laurdan was either 1.5 μM or 5 μM in experiments without and with TFP, respectively. The TFP concentration in the samples was 100 μM.

Steady-state fluorescence emission spectra were measured with an LS 50B spectrofluorimeter (Perkin-Elmer, Beaconsfield, UK) equipped with a xenon lamp using emission and excitation slits of 5 nm. Temperature was controlled by a water-circulating bath and the actual temperature was measured directly in the sample cuvette using a platinum thermometer. Excitation wavelength was 390 nm for the fluorescence temperature, and 320–400 nm for the fluorescence excitation wavelength dependence studies. The fluorescence emission spectra of 100 μM TFP-phospholipid mixtures were also recorded at all excitation wavelengths and temperatures examined but their intensity was found to be negligible in comparison with Laurdan emission spectrum intensity. Data were processed with FLDM Perkin-Elmer software.

The Laurdan generalised polarisation was calculated according to the equation given by Parasassi at al. [17]:

$$\mathrm{GP} = \frac{I_B - I_R}{I_B + I_R}$$

where I_R and I_B are the fluorescence emission intensities at the blue and red edges of the emission spectrum, respectively. The GP values were calculated using emission wavelengths $I_B = 440$ nm and $I_R = 490$ nm.

3. Results

3.1. The effect of trifluoperazine on phosphatidylcholine phase behaviour

The thermograms of DPPC in the absence and at increasing contents of TFP are presented in Fig. 1. For pure DPPC (the upper trace) pretransition and gel-liquid crystalline transition were recorded at 34.3°C and 41.5°C, respectively. In the presence of TFP the pretransition almost vanished at the lowest of the TFP:DPPC mole ratios (0.01) – only a broad

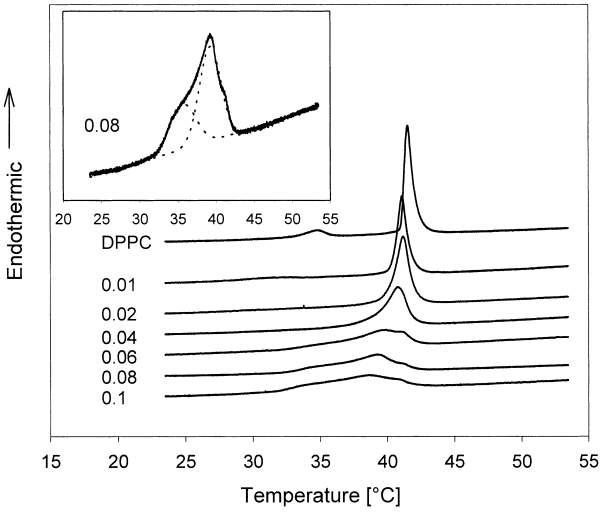

Fig. 1. Thermograms of DPPC (upper profile) and TFP-DPPC mixtures. Numbers in the figure represent TFP:DPPC mole ratios. DPPC concentration was 0.136 M. The thermograms were normalised to an equal amount of lipid for each profile. (Insert) An example of thermogram (0.08 TFP:DPPC) deconvolution.

and flat deviation from the baseline was observed near the temperature 31°C (effect not seen in the scale used in Fig. 1). For none of the higher TFP:DPPC mole ratios the pretransition was observed.

Increasing the TFP:DPPC mole ratio resulted in broadening of the gel-liquid crystalline transition peaks and shifting of the transition temperature to lower values. In addition, transition peaks became asymmetric – with low temperature shoulders broader than the high temperature ones. Since for TFP:DPPC mole ratios higher than 0.04 transition profiles clearly consist of two overlapping components, thermograms for all studied TFP:DPPC mole ratios were fitted with one or two (if possible with reasonable relevance) Gaussian function(s). The deconvolution of the transition peaks into two separate Gaussian components was possible for TFP:DPPC mole ratios equal to 0.04 and higher.

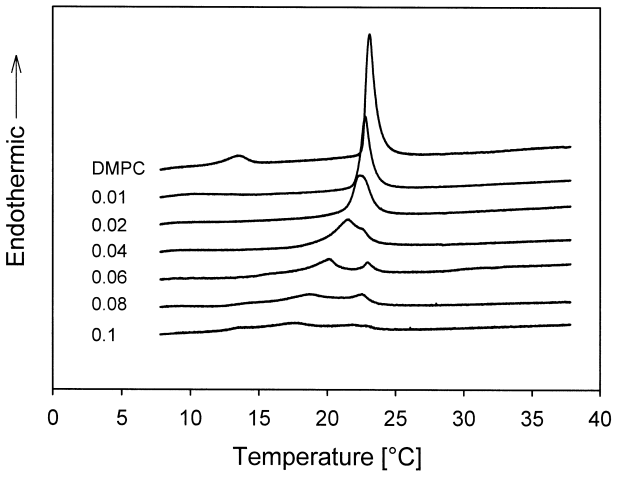

Fig. 3. Thermograms of DMPC (upper profile) and TFP-DMPC mixtures. Numbers in the figure represent TFP:DMPC mole ratios. DMPC concentration was 0.147 M. The thermograms were normalised to an equal amount of lipid for each profile.

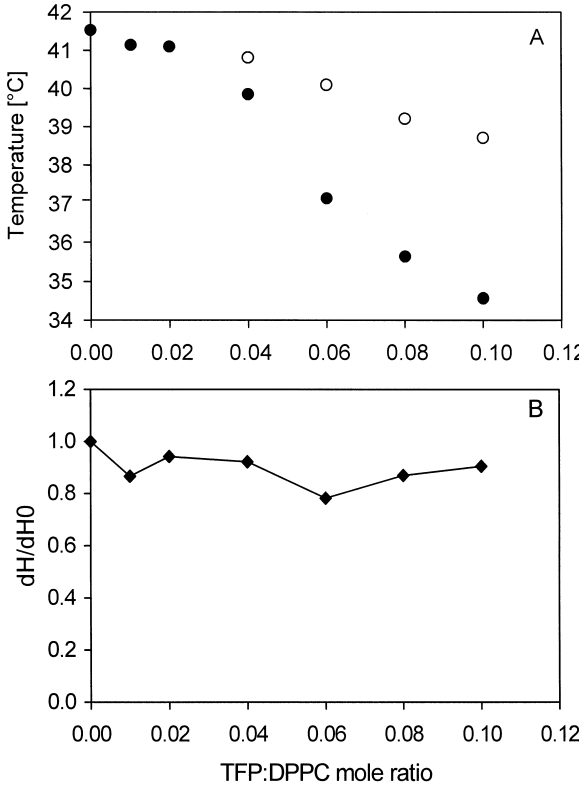

Fig. 2. Influence of TFP on DPPC phase transition parameters: dependence of (A) transition temperatures (Gaussian fit maxima) and (B) relative molar transition enthalpy, $\Delta H/\Delta H_0$, on TFP:DPPC mole ratio. ΔH_0 is transition enthalpy for pure lipid.

An example of such fitting is presented in the insert of Fig. 1. The temperatures at which Gaussian components reach their maximum were calculated and plotted versus the TFP:DPPC mole ratio (Fig. 2A). Apart from the already mentioned lowering of transition temperatures an increase of the distance between the maxima of double Gaussian fits was found (see Fig. 2A). As the TFP concentration is elevated the gap between the two Gaussian components becomes more pronounced.

DPPC molar transition enthalpy (ΔH) was not affected by increasing TFP concentration – ΔH values recorded for all studied TFP:DPPC mole ratios were similar to that for pure lipid. This is illustrated in Fig. 2B in which the relative enthalpy ($\Delta H/\Delta H_0$, where ΔH_0 is a transition enthalpy obtained for pure lipid) is plotted versus the TFP:DPPC mole ratio.

Results similar to those for DPPC were obtained for TFP-DMPC mixtures. For pure DMPC two transitions, pretransition and main gel-liquid crystalline, were recorded at 13.4°C and 23.1°C, respectively. Pretransition was abolished by the lowest of the examined TFP concentrations. Increasing TFP content caused a shift of gel-liquid crystalline transition peaks to lower temperatures. Elevation of TFP concentration also caused asymmetric broadening of the transition peaks (for a TFP:DPPC mole ratio

less than 0.06), followed by splitting of the transition profiles into two almost separate peaks (starting at a TFP:DMPC mole ratio of 0.06), as shown in Fig. 3.

As for DPPC, also for DMPC a deconvolution of thermograms into two Gaussian components was possible, in this case for mole ratios higher than 0.06. The temperatures of (single or double) Gaussian maxima are plotted in Fig. 4A as a function of TFP:DMPC mole ratio. Similarly as for DPPC the distance between the transition temperatures of the higher- and lower-melting components increased. The only difference between these two examined phosphatidylcholine species was that the transition enthalpy of DMPC was lowered by the increasing content of TFP (Fig. 4B). At the highest of the TFP:DMPC mole ratios studied, molar transition enthalpy was reduced to about 65% of the value recorded for pure DMPC.

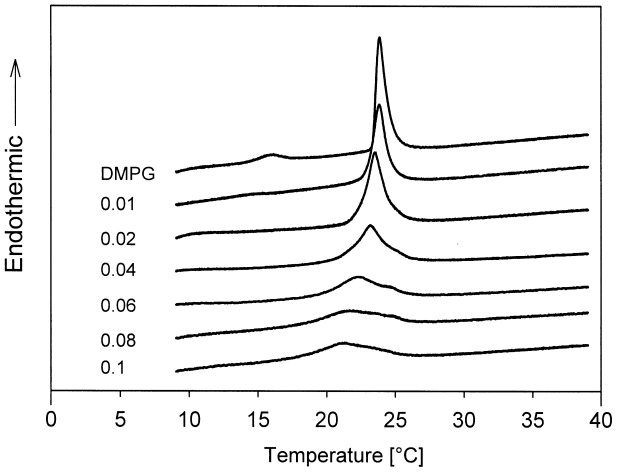

Fig. 5. Thermograms of DMPG (upper profile) and TFP-DMPG mixtures. Numbers in the figure represent TFP:DMPG mole ratios. DMPG concentration was 0.150 M. The thermograms were normalised to an equal amount of lipid for each profile.

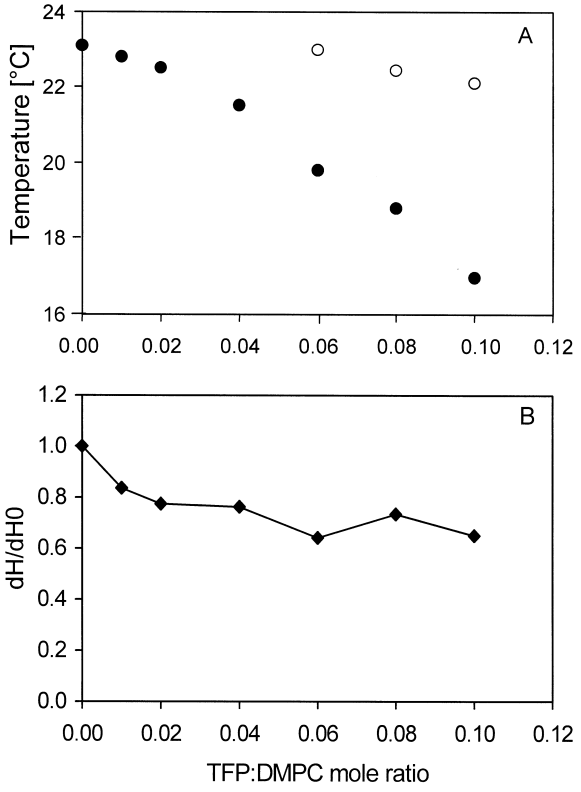

Fig. 4. Influence of TFP on DMPC phase transition parameters: dependence of (A) transition temperatures (Gaussian fit maxima) and (B) relative molar transition enthalpy, $\Delta H/\Delta H_0$, on TFP:DMPC mole ratio. ΔH_0 is transition enthalpy for pure lipid.

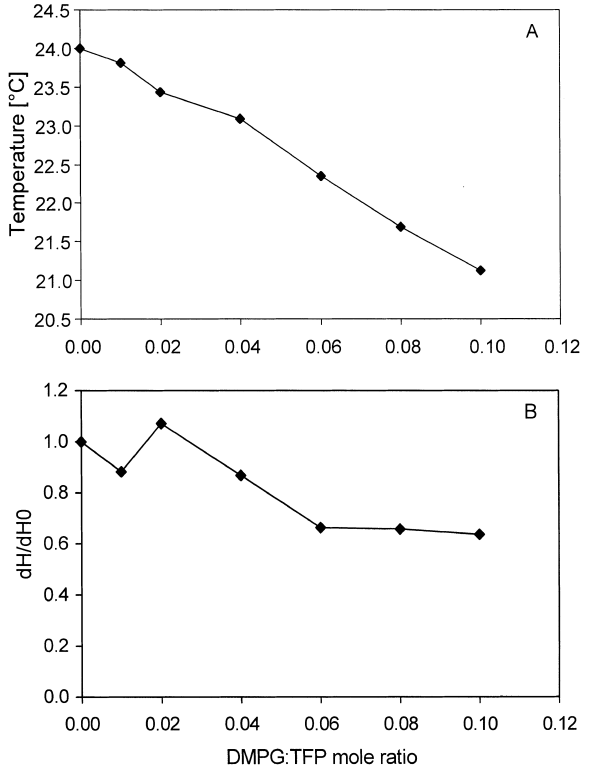

Fig. 6. Influence of TFP on DMPG phase transition parameters: dependence of (A) transition temperature and (B) relative molar transition enthalpy, $\Delta H/\Delta H_0$, on TFP:DMPG mole ratio. ΔH_0 is transition enthalpy for pure lipid.

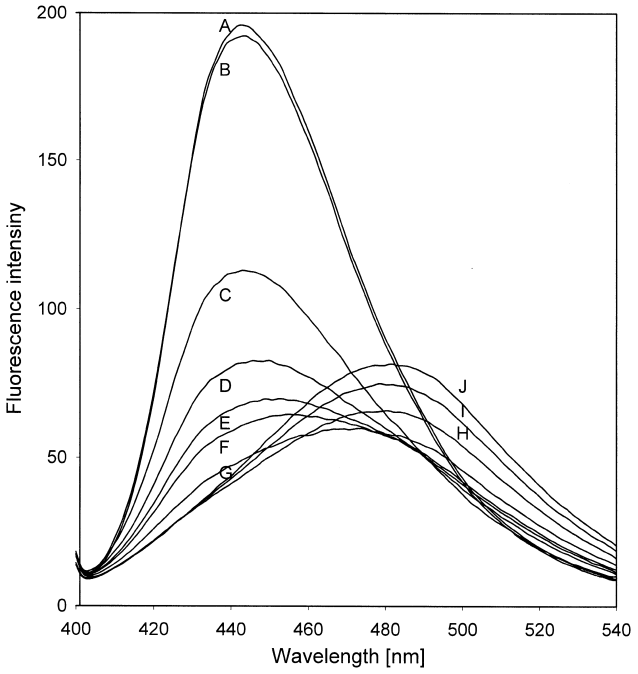

Fig. 7. Fluorescence emission spectra of Laurdan (not normalised) in DPPC liposomes with addition of 100 μM TFP measured at different temperatures: (A) 16°C, (B) 22, 25 and 30°C, (C) 34°C, (D) 36°C, (E) 37°C, (F) 38°C, (G) 40, 41 and 42°C, (H) 50°C, (I) 56°C, (J) 61°C. Excitation wavelength was 390 nm. Buffer was 20 mM Tris-HCl (pH 7.4) with 50 mM NaCl. DPPC concentration was 200 μM, Laurdan concentration was 2.5 μM.

3.2. The effect of trifluoperazine on phosphatidylglycerol phase behaviour

Trifluoperazine was able to affect also the phase behaviour of DMPG. Pretransition, present at 16.5°C for pure DMPG, was abolished at the lowest TFP:DMPG mole ratio used (0.01). For pure DMPG the main gel-liquid crystalline transition was recorded at 24.0°C. Increasing TFP concentrations we observed that DMPG main transition profiles became broader and shifted to lower temperatures, as shown in Fig. 5.

The dependence of main transition temperature on the TFP:DMPG mole ratio is presented in Fig. 6A. The broadening of transition profiles was much more symmetrical than for DMPC and DPPC and therefore deconvolution of thermograms into two separate Gaussian components was not possible for any of examined TFP-DMPG mixtures. Trifluoperazine also caused a decrease of molar transition enthalpy (Fig. 6B). At the highest of TFP concentrations studied the transition enthalpy was reduced to about 60% of the value obtained for pure DMPG.

3.3. Laurdan fluorescence studies

To further study the effect of trifluoperazine on phase state of the phospholipid bilayers and on possible phase separation we employed Laurdan, a fluorescent probe whose spectral properties depend on the phase state of the bilayer [18]. Fluorescence emission spectra of Laurdan in TFP-DPPC liposomes obtained at different temperatures are presented in Fig. 7. As the temperature was raised, we observed changes in position, shape, and intensity of the emission spectra. Apart from the intensity decrease, a red shift of the spectrum maximum of about 40 nm was seen above 40°C. The temperature induced changes of Laurdan emission spectra in pure DPPC liposomes were similar to those observed for TFP-DPPC samples (data not shown).

On the basis of the emission spectra recorded at different temperatures we have calculated the generalised polarisation values using the method given in [17] (see Section 2). Fig. 8 presents GP values plotted as a function of temperature for pure DPPC and TFP-DPPC liposomes. The GP shows an abrupt change at the main phase transition temperature of phospholipid (T_M). The value of T_M is higher for pure DPPC liposomes than for TFP-DPPC samples,

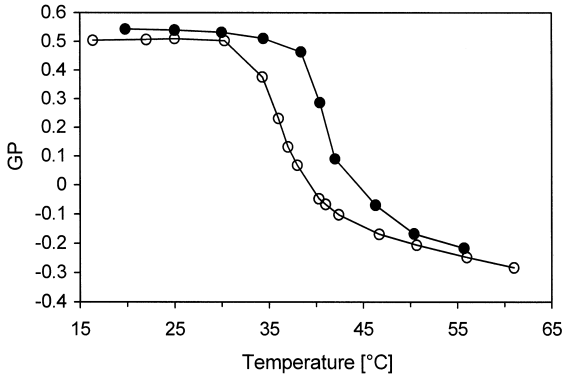

Fig. 8. Generalised polarisation values for Laurdan in DPPC liposomes as a function of temperature: ●, DPPC; ○, DPPC with addition of 100 μM TFP. Excitation wavelength was 390 nm. Buffer was 20 mM Tris-HCl (pH 7.4) with 50 mM NaCl. DPPC concentration was 200 μM, Laurdan concentration was 1.5 μM and 2.5 μM for pure DPPC and DPPC with TFP, respectively.

in agreement with the calorimetric data presented above. We can also observe that the GP vs. temperature course becomes steeper (in the transition range) for pure DPPC than for DPPC with TFP. This could be the result of the same processes that cause the broadening of DSC peaks in TFP-DPPC thermograms.

A recognised method for demonstrating phase coexistence in lipid systems is examining the generalised polarisation as a function of excitation wavelength [18,19]. We performed such experiments for pure DPPC liposomes (results shown in Fig. 9) and also for TFP-DPPC samples (Fig. 10). Laurdan emission spectra recorded in pure DPPC liposomes below the main phase transition temperature show high (over 0.5) GP values and generalised polarisation is not dependent on excitation wavelength. At 41°C (T_M) the GP values become lower (about 0.2) and the slope of the GP versus λ_{ex} relationship is positive. This dependence changes again at temperatures above the main phase transition: GP becomes a descending function of λ_{ex} (● in Fig. 9). Simultaneously the generalised polarisation values are negative (less than −0.2). The situation for TFP-DPPC samples is different (Fig. 10). At the three lowest temperatures studied (up to 37.1°C) the GP(λ_{ex}) dependence is ascending. Differently from pure DPPC, the generalised polarisation values obtained for TFP-DPPC mixtures at temperatures below T_M were significantly lower (in the range 0–0.48). At temperatures in the range 39–43°C the GP(λ_{ex}) dependences become almost flat. The generalised polarisation values lie mostly below zero in this temperature range (Fig. 10: □, ● and ○ for 39.3, 41.3 and 43.4°C, respectively). Only at a temperature well above the main phase transition (55°C) the GP versus λ_{ex} behaviour observed for TPP-DPPC strongly resembles that for pure DPPC in similar conditions. In both cases the slope of the GP(λ_{ex}) line is negative and the values of generalised polarisation are similar (less than −0.2). Taking into account the fluorescence results presented above, we can conclude that the effect of TFP on DPPC bilayers is more pronounced at lower temperatures.

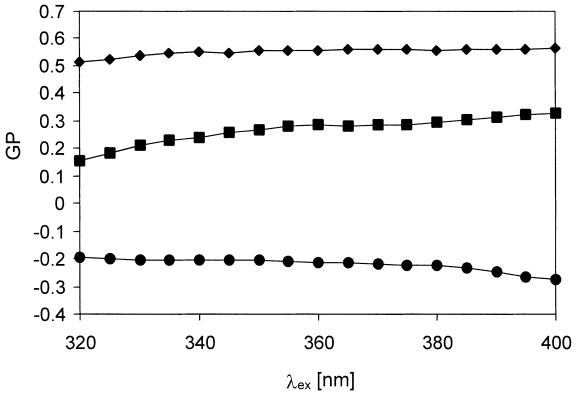

Fig. 9. Generalised polarisation values for Laurdan in DPPC liposomes as a function of excitation wavelength at 19.5°C (♦), 41°C (■) and 55°C (●). Buffer was 20 mM Tris-HCl (pH 7.4) with 50 mM NaCl. DPPC concentration was 200 μM, Laurdan concentration was 1.5 μM.

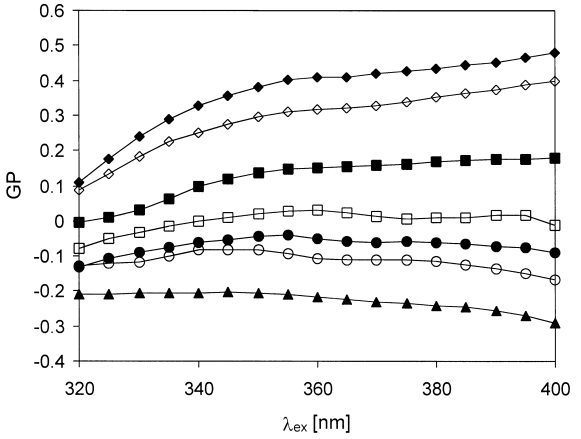

Fig. 10. Generalised polarisation values for Laurdan in DPPC liposomes with addition of 100 μM TFP as a function of excitation wavelength at 21°C (♦), 35.4°C (◇), 37.1°C (■), 39.3°C (□), 41.3°C (●), 43.4°C (○) and 55°C (▲). Buffer was 20 mM Tris-HCl (pH 7.4) with 50 mM NaCl. DPPC concentration was 200 μM, Laurdan concentration was 5 μM.

To check the effect of trifluoperazine on charged phospholipid bilayers we have examined a TFP-DMPG mixture. Similarly to the case of pure DPPC the generalised polarisation was not dependent on excitation wavelength at temperatures below T_M. The GP values, however, resembled more the ones gained for a TFP-DPPC mixture (data not shown). These features of the GP(λ_{ex}) behaviour indicate that trifluoperazine affects DPPC and DMPG bilayers in a different way.

4. Discussion

The microcalorimetric studies show that even the lowest TFP concentration used strongly perturbs

(DPPC) or abolishes (DMPC, DMPG) phospholipid pretransition. Since pretransition is a result of changes in lipid acyl chain packing [20–22], its perturbation and/or vanishing indicates that TFP alters the structure of the lipid bilayer in the gel state. Trifluoperazine also causes a shift of transition temperatures to lower values of both phosphatidylcholines and phosphatidylglycerol. Furthermore the increase in the TFP:lipid mole ratio is accompanied by the broadening of DSC peaks that suggests lower cooperativity of phospholipid main phase transition in the presence of TFP. In its influence on the features of phospholipid DSC profiles described above trifluoperazine is found to be similar to other phenothiazine derivatives such as chlorpromazine and diethazine [11,15,16]. Chain-melting temperature decrease and transition peak broadening observed in phenothiazine-phospholipid mixtures are commonly recognised to be the signs of increased mobility of phospholipid acyl chains, i.e. membrane fluidisation [11,23].

Our results also show that in phospholipid model membranes TFP affects mainly the transition temperature, while its influence on the transition enthalpy is weaker. Such a behaviour is acknowledged to indicate that the modifier molecule interacts with the polar head group region of phospholipids and partially penetrates into the hydrocarbon region [24,25]. Our results suggest that TFP positioning in the phospholipid bilayer is analogous to that proposed for CPZ interaction with phospholipid bilayers in molecular models of Frenzel et al. [11] and Nerdal et al. [15]. Both models assume the phenothiazine moiety to penetrate into the acyl chain region and to be positioned along the chain direction whereas the amino side chain with its positive charge is located in the vicinity of a phospholipid polar head group.

In both DPPC and DMPC samples the elevated content (mole ratio above 0.06) of trifluoperazine causes the DSC peak to become asymmetric with the low temperature shoulder broader than the high temperature one. Such an asymmetry suggests that the gel-like phase is more perturbed by the presence of TFP than the liquid crystalline one. This effect is more pronounced in DMPC where splitting of transition profiles into two almost separate peaks can be observed. Also, the same concentrations of TFP decrease the main phase transition temperature of DMPC more than that of DPPC ($\Delta T_{M[\text{pure PC}-0.1\ \text{TFP}-\text{PC mixture}]}$ is 4.4°C for DMPC and 3.1°C for DPPC). It is clearly visible that trifluoperazine affects the short chain phosphatidylcholine stronger than the long chain one. This may be explained by the fact that the partition coefficient of phenothiazines is reduced in long-chain lipids [26] as a result of increased interactions between their acyl chains. Chain length dependence was also found for other drug-phospholipid systems. Theodoropoulou and Marsh [27] demonstrated by microcalorimetry and ESR spectroscopy that the non-peptide angiotensin antagonist losartan perturbs DMPC bilayers more than DPPC ones. Also Thomas and Verkleij's [28] DSC studies on the calcium antagonist flunarizine indicated that the phosphatidylcholine species with diminished chain-chain interactions (short chain and/or unsaturated PC) are more sensitive to the drug presence. These authors also proposed that the acyl chain dependence of drug effect reflects the degree to which the chains themselves influence the phase transitions of the studied phospholipids. In our experiments we have observed that increased TFP concentration alters the DMPG transition enthalpy to a similar extent as the ΔH of DMPC. Since these lipids differ exclusively in their polar head groups, the detected similarity additionally supports the idea of chain length role in drug-lipid interactions.

The distinct asymmetry of TFP-PC transition peaks prompted us to try fitting these thermograms with two Gaussian functions. The deconvolution into two separate Gaussian components was possible for TFP:lipid mole ratios equal or higher than 0.04 and 0.06 for DPPC and DMPC, respectively. The presence of two Gaussian components in transition peaks of samples with high TFP content could be explained in two ways. It means either that the whole system undergoes two separate transitions one after another, or that there are two distinct populations of species undergoing the same transition. The latter hypothesis seems to be more probable since the possibility that phenothiazine derivatives induce phase separations in phospholipid model bilayers has already been suggested by Frenzel et al. [11] for CPZ-PC systems.

The DSC peaks of TFP-DMPG samples also become broader as the trifluoperazine concentration increases. However, they remain symmetric and even in the highest TFP:DMPG mole ratio studied

(0.1) a peak deconvolution into two Gaussian components is impossible. Thus, in spite of decreased transition co-operativity caused by TFP in DMPG bilayers, there is no phase separation visible in phosphatidylglycerol membranes. Also the downward shift of T_M is smaller for DMPG than for both PC species ($\Delta T_{M[\text{pure DMPG}-0.1\text{ TFP}-\text{DMPG mixture}]}$ is 2.9°C).

It is well known that many organic compounds can induce an interdigitated gel phase in phosphatidylcholine or phosphatidylglycerol bilayers [29]. Interdigitation was also shown in chlorpromazine-DPPC mixtures by means of X-ray diffraction [30] at a CPZ:DPPC mole ratio higher than 0.2. The same experimental model was studied by [11] using DSC. Rising the chlorpromazine:lipid mole ratio in the range 0.02 up to 0.41 they observed first a broadening of the transition peaks, which became narrow again when the CPZ:DPPC mole ratio was higher than 0.25. Similar calorimetric picture of the new phase induction was also recorded in the case of phthalic acid-DPPC mixtures [31]. It is characteristic for all the studies presented above that interdigitation occurred at relatively high concentrations of new phase inducer molecules. Since TFP and CPZ are chemically related one cannot exclude the possibility that an interdigitated phase should also be induced by trifluoperazine at high enough concentrations. In our work this was not observed because the TFP concentrations chosen for this study were well below the interdigitation threshold. In this situation the appearance of a pure interdigitated phase does not seem to be the proper explanation of the observed thermotropic behaviour.

To further investigate the putative phase separation induced by trifluoperazine in phosphatidylcholine model membranes we performed fluorescent studies using Laurdan. The spectral properties of this fluorescent probe are highly sensitive to the amount of dipolar relaxation process that undergoes in its environment [18,19,32]. As the gel and liquid crystalline phases differ in hydration of the phospholipid bilayer, there is more dipolar relaxation in the latter phase. That causes the decrease in intensity of the Laurdan emission spectrum and the red shift (of about 40 nm) of its maximum in the liquid crystalline phase, as compared to the gel phase. Studying the dependence of Laurdan generalised polarisation values on temperature one can follow the sample thermotropic behaviour. On the other hand the relation between GP and excitation wavelength studied at different temperatures allows to confirm/exclude the coexistence of phases. Shortly, when Laurdan molecules are incorporated into phospholipid bilayers in different phase states there is no GP on λ_{ex} dependence in the gel phase, the GP(λ_{ex}) function is descending in the liquid crystalline phase, and it is ascending when separate domains of both phases coexist under the same conditions (for details see e.g. [18]). The GP values themselves also give information about the environment surrounding Laurdan molecules. These values have been found to depend only on the phospholipid phase state [32], and if calculated for the same excitation and emission conditions as in [18], they are about 0.6 in the gel phase and about −0.2 in the liquid crystalline phase.

As for fluorescence studies, there was more buffer phase present in the samples, i.e. the whole system was more diluted than in the DSC experiments. Therefore in spectroscopic measurements we had to use higher TFP:DPPC mole ratios to observe effects comparable to those recorded by microcalorimetry. Our spectroscopic experiments confirm the results obtained by means of DSC and show that the TFP-DPPC mixture, as compared to pure DPPC, has a lower T_M and a broader main phase transition temperature range. The positive GP(λ_{ex}) slope at temperatures below T_M and GP values in the range 0–0.48 (too low for gel phase) indicate that the TFP-DPPC mixture cannot be described as a pure gel phase. It rather contains a mixture of different quasi-gel phases present in separate domains. At T_M both phases become homogenised (GP does not depend on λ_{ex}). A pure liquid crystalline phase is present at temperatures above T_M – GP becomes a descending function of λ_{ex}, and GP values are typical for this phase (less than −0.2). On the basis of these observations we conclude that trifluoperazine induces phase separation in DPPC bilayers at temperatures below the main phase transition temperature.

Analogous studies performed for TFP-DMPG (0.5 mole ratio) mixtures indicate that TFP at such a concentration does not induce phase separation in DMPG bilayers. As GP does not depend on λ_{ex} at temperatures below T_M, we conclude that the TFP-DMPG samples contain only one, homogeneous phase. This phase, however, cannot be characterised

as a typical gel because the generalised polarisation values are too low (about 0.25).

Phase separation in phospholipid systems has been reported to be caused by a variety of factors, e.g. cholesterol [33], antimicrobial peptides [34], poly(ethylene glycol) [35] and cyclosporin A [36]. To our best knowledge this report is the first that shows phase separation induced by phenothiazine derivatives in model phospholipid bilayers. In the presence of trifluoperazine the two phases exist at temperatures below the chain-melting transition in DMPC and DPPC but not in DMPG bilayers. The occurrence of separate domains inevitably provokes the question of their origin. The simplest event that could result in domain formation would be the presence of TFP-rich and TFP-poor regions inside the membrane, analogously as in cholesterol-phospholipid systems [37]. Such a possibility has already been suggested by Frenzel et al. [11] on the basis of their DSC studies of CPZ-PC mixtures and putative domain formation has been attributed to the incomplete drug-lipid miscibility.

A possible reason for the observed effect of phenothiazine on membranes could be the dissimilar interaction of TFP species being in a different protonation state with phospholipids. Various pK values of TFP amino groups can be found in the literature (ranging from 6.8 [38] to 8.1 [9]), but all authors admit that in the physiologic pH range TFP may occur in both charged and uncharged (or higher/lower protonated) forms. It was shown by Malheiros et al. [9] that the neutral form, being more hydrophobic, interacts stronger with the erythrocyte membrane than the charged one. We can therefore assume the TFP species of lower protonation to enter phosphatidylcholine bilayers deeper and to perturb them stronger than the more protonated form. It was proved that trifluoperazine insertion into a non-polar surrounding, such as erythrocyte membranes [9] or detergent micelles [38], results in a decrease in the TFP pK value as compared to the value obtained for the aqueous phase. It means that at physiological pH there is a higher content of the less protonated TFP form inside the phospholipid bilayer than predicted by the pK value measured outside the membrane.

As stated above, in all types of model membranes (DMPC, DPPC and DMPG) at pH 7.4 trifluoperazine molecules of both protonation forms are present. However, phosphatidylglycerol being negatively charged would accommodate both drug forms similarly whereas in zwitterionic phosphatidylcholine the more protonated TFP species would probably be worse miscible. The more protonated TFP form might locate near the membrane surface in contrast to the less protonated, more hydrophobic TFP molecule population that could bury deeper into the non-polar core of the lipid bilayer. For such differences in localisation inside the membrane it seems likely that the observed domains consist of lipid enriched in one or another drug form. Domains rich in the less protonated form that mixes better with lipid and is deeper buried, would probably manifest their presence as a lower-melting component of the two Gaussian curves into which the TFP-PC thermograms can be deconvoluted. The higher-melting Gaussian component probably corresponds to the domains where more protonated TFP species are abundant. Additionally, as Caetano and Tabak have shown [38], the pK decrease accompanying TFP-detergent micelle binding is bigger for zwitterionic than for negatively charged detergents. We suppose that a similar situation could take place for neutral and anionic phospholipids. If this was the case, a higher content of the more protonated TFP form would be present in PG as compared to PC bilayers at the same pH value. It would further facilitate the drug-lipid mixing. However, more detailed studies on the effect of TFP on phospholipid bilayers, especially in various pH conditions, are required to fully elucidate the role of the pK change in the interactions of higher/lower protonated drug forms with phospholipids.

The phase separation and membrane fluidisation induced by TFP in phosphatidylcholines are likely to be the reason for the alteration in membrane permeability caused by the drug. This change is considered to be due to the structural defects present at the domains' borders [39]. Indeed, trifluoperazine was shown to increase the platelet plasma membrane permeability for small molecules such as ADP and ATP [40]. TFP is also a well known multidrug resistance reversing agent but its mechanisms of action are poorly understood [41]. Since membrane phospholipids are regarded as one of the possible targets for MDR reversing compounds [10,42], and a correla-

tion has been found between potentiation of anticancer-drug cytotoxicity and membrane fluidising (permeabilising) effects for a group of chemosensitisers [43], we conclude that the putative TFP mechanism of action can be related to its ability to induce changes in membrane phospholipid properties.

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