Biochimica et Biophysica Acta, 1030 (1990) 211–222
Elsevier

BBAMEM 75084

The dissimilar interactions of the calcium antagonist flunarizine with different phospholipid classes and molecular species: a differential scanning calorimetry study

Peter G. Thomas [1] and Arie J. Verkleij [2]

[1] *Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Utrecht (The Netherlands)* and [2] *Department of Molecular Cell Biology, University of Utrecht, Utrecht (The Netherlands)*

(Received 19 June 1990)

Key words: Flunarizine; Calcium antagonist; Phospholipid phase behavior; Phosphatidylcholine; Phosphatidylethanolamine; Phosphatidylserine; DSC

The influence of the class IV calcium antagonist flunarizine on the phase behaviour of different species of the major phospholipid classes of mammalian plasma membranes has been examined using differential scanning calorimetry. We show that it has the ability to substantially influence the phase behaviour of phospholipids. Flunarizine significantly influences the gel to liquid-crystalline transition temperature of phosphatidylserines whilst having little effect on those of the phosphatidylethanolamines tested. The liquid-crystalline to inverted hexagonal phase transition of phosphatidylethanolamines is, however, strongly induced by the presence of flunarizine. Examination of the effect of flunarizine on the phase behaviour of different phosphatidylcholine species revealed an acyl-chain dependent influence. Dissimilar results with phosphatidylcholines, phosphatidylethanolamines and phosphatidylserines reveal different locations and ionization states for the drug in the different phospholipid bilayers. These results not only indicate an essential role for the ionization state of the drug in determining drug–phospholipid interactions but also the role of the phospholipid in determining the ionization state of the drug and have important implications for drug–membrane interactions demonstrating that drug interaction with one phospholipid may bear no relation whatsoever to its interaction with another.

Introduction

Calcium antagonists have gained a position of great importance in recent years particularly in the field of cardiovascular pharmacology. However, this large class of drugs with its great diversity of chemical structures clearly does not have one unifying mode of action [1]. These drugs are united by their ability to prevent calcium-induced contracture of vascular tissue preparations by a blockade of calcium entry through voltage-gated calcium channels, the so-called calcium slow channels of muscular tissues: hence their designation as calcium entry blockers or calcium slow channel blockers [2]. This classification is now firmly established and is well-suited to the majority of the calcium antagonists: compounds such as verapamil, nifedipine, diltiazem and their derivatives have been shown to have specific interactions with defined regions of the α_1 polypeptide of the voltage-gated calcium slow channel [3]. However, direct channel interaction cannot be shown for a fourth class of calcium antagonist, the diphenylpiperizines [4].

This group of compounds typified by flunarizine (1-cinnamyl-4-(di-*p*-fluorobenzhydryl)piperazine) apparently does not interfere with normal calcium cellular function but is very effective against the deleterious effects of elevated cellular calcium concentration [5]. We have recently demonstrated that flunarizine has the ability to influence the interaction between calcium and

Abbreviations: DSC, differential scanning calorimetry; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; L_β, phospholipid gel phase; L_α, phospholipid liquid-crystalline phase; H_{II}, phospholipid inverted hexagonal phase; ΔH, enthalpy of the phospholipid gel to liquid-crystalline phase transition; ΔH_H, enthalpy of the liquid-crystalline to inverted hexagonal phase transition; T_m, gel to liquid-crystalline phase transition temperature. The temperature of maximum specific heat capacity; T_{mH}, liquid-crystalline to inverted hexagonal phase transition temperature; ^{31}P-NMR, phosphorus nuclear magnetic resonance spectroscopy; Eld, *trans*-9-octadecenoic acid (elaidic acid); Ole, *cis*-9-octadecenoic acid (oleic acid); Pam, hexadecanoic acid (palmitic acid); Pipes, 1,4-piperazinediethanesulphonic acid.

Correspondence: P.G. Thomas, I.M.B., University of Utrecht, Transitorium III, Padualaan 8, 3584 CH Utrecht, The Netherlands.

membranes and thereby is able to prevent damaging membrane reorganization from occurring in response to high calcium concentrations [6]. It has long been appreciated that cationic amphiphilic drugs have the ability to displace calcium from phospholipid monolayers [7] and that through this have the capability of preventing calcium-induced membrane reorganisation [8]. This then raises the question of whether flunarizine, like these other membrane-active drugs, is also able to influence the phase behaviour of membrane phospholipids.

Flunarizine, like a multitude of pharmacologically active molecules, is a cationic amphiphile. This rather general term represents a wide range of drug classes including local anesthetics, β-blockers, neuroleptics and antidepressants amongst others. In general, cationic amphiphiles are characterized by a common molecular structure: a hydrophobic aromatic ring system which is linked to an amine group by a more hydrophilic moiety; usually a short alkyl side chain. A great deal of attention has been paid to their interactions with membrane phospholipids. This is because the plasma membrane features prominently in their proposed modes of action: the plasma membrane, or more specifically its protein or lipid constituents often being the cellular targets of these drugs.

There is a wealth of information about the influence of such compounds on the physicochemical properties of phospholipids. A great deal of this information has been acquired using the technique of differential scanning calorimetry (DSC) which provides a rapid, simple method of assessing the interactions of molecules with model and natural biomembranes (for reviews, see Refs. 9 and 10). However, much of this work, particularly the earlier studies, concentrated on the effect of cationic amphiphiles on the phase behaviour of a small, rather non-representative group of phospholipids, the saturated phosphatidylcholines (PtdCho) (for examples, see Refs. 11–15). More recent studies have examined the effect of variation in the phospholipid headgroup on the influence of such drugs on phospholipids phase behaviour. Of particular interest is the effect of these positively charged drugs on the phase behaviour of anionic phospholipids and to this end several authors have reported the influence of cationic amphiphiles on saturated phosphatidic acids [16], phosphatidylglycerols [15,16] and phosphatidylserines (PtdSer) [17].

In this study we have examined the interaction of the class IV calcium antagonist flunarizine with three different phospholipid classes (PtdCho, phosphatidylethanolamine (PtdEtn) and PtdSer) and show that flunarizine has a distinct effect with each of the three phospholipid classes. These three are the major phospholipid classes of mammalian plasma membranes including the myocyte plasma membrane [18]; the target membrane for cardiovascular calcium antagonist action. We have also studied the effect of molecular species on flunarizine–phospholipid interaction and show that the ability of the drug to influence the phase behaviour of PtdCho's is greatly affected by acyl chain length and unsaturation. These results are discussed in relation to the localization of the drug in the phospholipid bilayer and their implications for drug–membrane interactions. Some of these results have been presented previously in a preliminary form [19].

Materials and Methods

Phospholipid synthesis and purification

1,2-Diacyl-*sn*-glycero-3-phosphocholines were synthesized essentially according to the method of Baer and Buchnea [20] by coupling the cadmium chloride adduct of glycerophosphocholine, derived from egg PtdCho as previously described [21,22], with fatty acid chlorides in the presence of pyridine. The crude synthesis products were purified by partition chromatography followed by preparative HPLC on a Polygosil Si60 (5–20 μm) column using a chloroform/methanol/ammonia/water (68 : 28:2 : 2, v/v) eluent system [23,24]. Purified lipids were stored under N_2 at $-80°C$.

1,2-Diacyl-*sn*-glycero-3-phosphoethanolamines were synthesized from the corresponding PtdCho via a base exchange reaction using phospholipase D, isolated from cabbage leaves [25], as previously described [26]. The reaction was terminated by adding a two times excess of EDTA and adjustment of the pH to 8.0 (NaOH). The crude products were purified by preparative HPLC and stored as described above.

1,2-Diacyl-*sn*-glycero-3-phosphoserines were also synthesized from the corresponding PtdCho via a base exchange reaction, as described by Comfurius and Zwaal [27]. The crude lipid products were converted to their sodium salt by initial conversion to the calcium salt, followed by a Bligh and Dyer type extraction to remove non-acidic phospholipids, which was then followed by a Bligh and Dyer type extraction in the presence of 0.1 M EDTA and 0.1 M NaCl at pH 8.2. The sodium salt of the PtdSer was then purified by preparative HPLC methods [23] using a 2,2,2-trichloroethanol/propane-2-ol/ethanol/water (45 : 19 : 28 : 8, v/v) eluent system and stored as described above.

Flunarizine was a gift from Janssen Pharmaceutica, Beerse, Belgium. All other reagents were of analytical grade.

DSC

The effects of flunarizine on the phase behaviour of phospholipids were examined by DSC. Flunarizine/phospholipid mixtures were prepared from stock chloroform solutions of known concentration (approx. 20 mM) which were stored under N_2 at $-30°C$. Phospholipids were tested for purity by thin layer chromatography prior to use. 10 μmoles phospholipid and various

amounts of flunarizine were mixed and evaporated to dryness using a rotary evaporator. Samples were then dried further overnight under vacuum in a vacuum desiccator over phosphorous pentoxide.

The flunarizine/phospholipid film was suspended in 1.5 cm^3, 100 mM NaCl, 25 mM Pipes (pH 7.4, NaOH), 40 μM EDTA by vortexing at room temperature or at approximately 10°C higher than the gel-liquid crystalline phase transition of the respective phospholipid (when higher than room temperature). Lipid samples were pelleted by centrifugation (30 min, 40000 × g), the pH of the supernatant was checked and the wet pellets were transferred to aluminum sample pans. The supernatant was found to be pH 7.2 to 7.3 for all samples tested. Under similar experimental conditions flunarizine has been shown to have a partition coefficient of 6000 in liposome systems [28] and 12000 in erythrocyte membranes [29] and so it has been assumed that virtually all of the added drug is incorporated into the membrane.

The thermotropic phase behaviour of the flunarizine/phospholipid dispersions was measured using a DSC4 Perkin-Elmer differential scanning calorimeter. Indium was used to calibrate the apparatus. All scans were carried out at a sensitivity range of 0.5–1 mcal · s^{-1} and a scanning rate of 2 K · min^{-1}. Each flunarizine/phospholipid sample was subjected to at least three cooling and heating temperature cycles. Differences were never observed between the second and third cycles. All experiments were repeated using two separate preparations.

The phase transition temperatures and enthalpies of transition were determined using a TADS program on a Perkin-Elmer 3600 data station and were normalized for phosphorus content following phosphorus analysis of sample pan contents [30]. Cryoprotectant was not used for dioleoylphospholipid samples as it is known that such substances can affect the bilayer to hexagonal II phospholipid phase transition [31], the measurement of this phase transition being essential to these studies. Moreover, it has been shown previously that the phase transition of parameters of Ole_2PtdCho are equivalent in the absence and presence of cryoprotectant [32].

The phase transition temperature of a phospholipid is normally defined as the temperature of maximum excess specific heat; that is the temperature at which deviation from the baseline is greatest, denoted by T_m. This definition of T_m has been used in the studies presented here.

Results

The DSC study of the effects of flunarizine on phospholipid physicochemistry was initially carried out using dielaidyl-(Eld_2) phospholipids because of their convenient phase transition temperatures. Typical DSC thermograms of dispersions of Eld_2PtdCho in the absence and in the presence of increasing amounts of flunarizine are shown in Fig. 1A. The phase transition of Eld_2PtdCho occurred at a temperature of $T_m = 11.0$°C, with a total enthalpy value for the transition of $\Delta H = 7.2$ kcal · mol^{-1}. These values are in good agreement with previ-

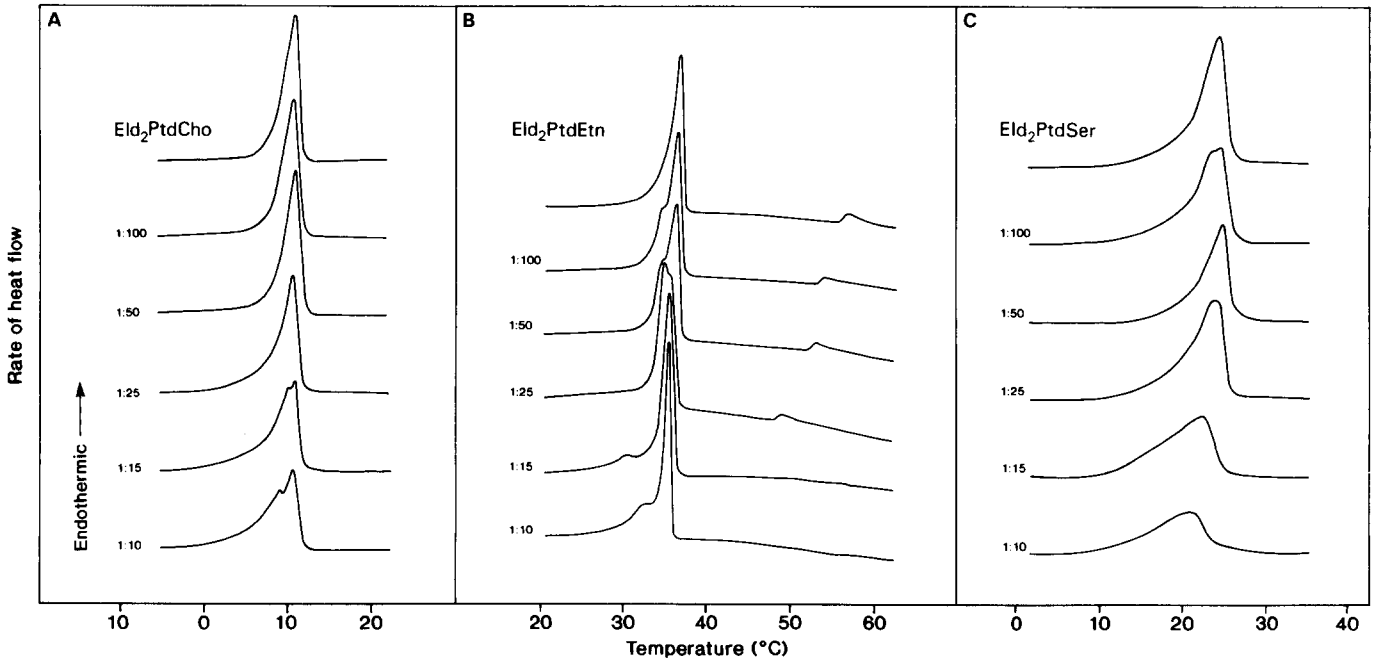

Fig. 1. Typical DSC of flunarizine/dielaidoylphospholipid dispersions; (A) phosphatidylcholine, (B) phosphatidylethanolamine, and (C) phosphatidylserine. Numbers on the figures represent flunarizine to phospholipid mole ratios.

ously reported data [33]. It is clear that although there is a slight downward shift in T_m flunarizine has little effect on the thermotropic phase behaviour of $Eld_2PtdCho$ excepting a slight broadening of the transition peak.

Fig. 1B shows a typical DSC thermogram of dispersions of $Eld_2PtdEtn$ and illustrates the effect of increasing amounts of flunarizine. The L_β-L_α transition for a control sample of $Eld_2PtdEtn$ occurred at a temperature of $T_m = 36.6°C$ with an enthalpy of transition of $\Delta H = 7.8$ kcal · mol^{-1}. These values are in good agreement with values published by Van Dijck [32] but somewhat lower than those published by Epand [34]. Although there are complex alterations in the profile of the thermograms it can be seen that, once again, there is a slight downward shift in T_m with effectively no change in ΔH values. However, Fig. 1B reveals the striking effect that increasing amounts of flunarizine have on the L_α-H_{II} phase transition of $Eld_2PtdEtn$. Control values for this transition are a transition temperature T_{mH} of 57.0°C and an enthalpy for the transition of $\Delta H_H = 0.50$ kcal · mol^{-1}. The enthalpy value is in good agreement with that published by Epand [34] but the transition temperature is significantly lower than his value (cf. 65.6°C). The transition temperature for the L_α-H_{II} transition (T_{mH}) for $Eld_2PtdEtn$ is, however, in agreement with values previously obtained using phosphorus nuclear magnetic resonance (^{31}P-NMR) [35]. The L_α-H_{II} phase transition was no longer detectable by DSC at the highest flunarizine mole ratio ($x = 0.1$).

The effects of flunarizine on DSC thermograms of $Eld_2PtdSer$ are shown in Fig. 1C. The L_β-L_α phase transition of $Eld_2PtdSer$ was observed at a temperature of $T_m = 24.4°C$. This value is in good agreement with previously reported data [27,36]. The enthalpy for the transition was determined to be $\Delta H = 6.6$ kcal · mol^{-1} To our knowledge this is the first report of this parameter. This value is, however, of a similar magnitude to those reported for other PtdSer's [36]. As with the corresponding PtdCho and PtdEtn increasing amounts of flunarizine cause a downward shift in the T_m for the L_β-L_α transition of $Eld_2PtdSer$, although in this case the shift in T_m is significantly greater than with the other two phospholipid classes. Unlike its lack of effect on the enthalpy of the L_β-L_α transition for the corresponding PtdCho and PtdEtn, flunarizine does have a large effect on this value for $Eld_2PtdSer$ reducing the enthalpy by over 50%.

Fig. 2A summarizes the influence of flunarizine on the phase transition temperatures of the dielaidoylphospholipids. The dramatic effect of increasing the mole ratio of flunarizine on the temperature at which the L_α-H_{II} phase transition of $Eld_2PtdEtn$ occurs is immediately apparent. From the DSC thermograms (see Fig. 1B) it was not possible to determine the hexagonal II phase transition (T_{mH}) at the highest mole ratio

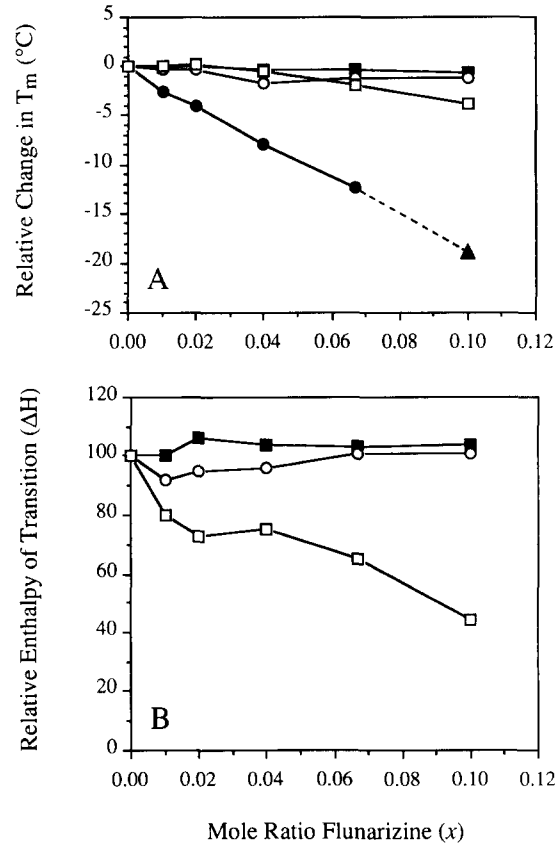

Fig. 2. (A) A DSC study of the effect of increasing flunarizine mole ratios on the gel to liquid-crystalline phase transition temperatures of dielaidoylphospholipid dispersions (phosphatidylcholine (■), phosphatidylethanolamine (○) and phosphatidylserine (□)) and on the liquid-crystalline to hexagonal II phase transition temperature of phosphatidylethanolamine (●,▲). Value at highest mole ratio (▲) was estimated using ^{31}P-NMR. (B) The effect of flunarizine on the gel to liquid-crystalline phase transition enthalpies of dielaidoylphospholipid dispersions: phosphatidylcholine (■), phosphatidylethanolamine (○) and phosphatidylserine (□). Values represent the average of four determinations.

tested ($x = 0.1$). However, ^{31}P-NMR experiments (results not shown) indicate that at higher mole ratios of flunarizine ($x \geqq 0.10$) $Eld_2PtdEtn$ undergoes either a transition directly from the L_β to the H_{II} phase or a transition from the L_β to the H_{II} phase via the L_α phase where the L_α phase is very short lived. In either case this would require a shift in the onset of the L_α-H_{II} transition (T_{mH}) of the order of -18 to $-20°C$. This is represented in Fig. 2A and indicates a linear reduction in T_{mH} with increasing flunarizine mole ratios. The relative lack of effect of flunarizine on the L_β-L_α phase transitions (T_m) of $Eld_2PtdCho$ and $Eld_2PtdEtn$ can clearly be seen as can the moderate effect of flunarizine on the T_m of $Eld_2PtdSer$ at higher mole ratios ($x > 0.04$).

The influence of flunarizine on the relative enthalpies of the L_β-L_α phase transitions of the dielaidoylphospholipids is illustrated in Fig. 2B. The effect of flunarizine on the enthalpy values of the L_α-H_{II} phase transition (ΔH_H) has been omitted from this figure due to limita-

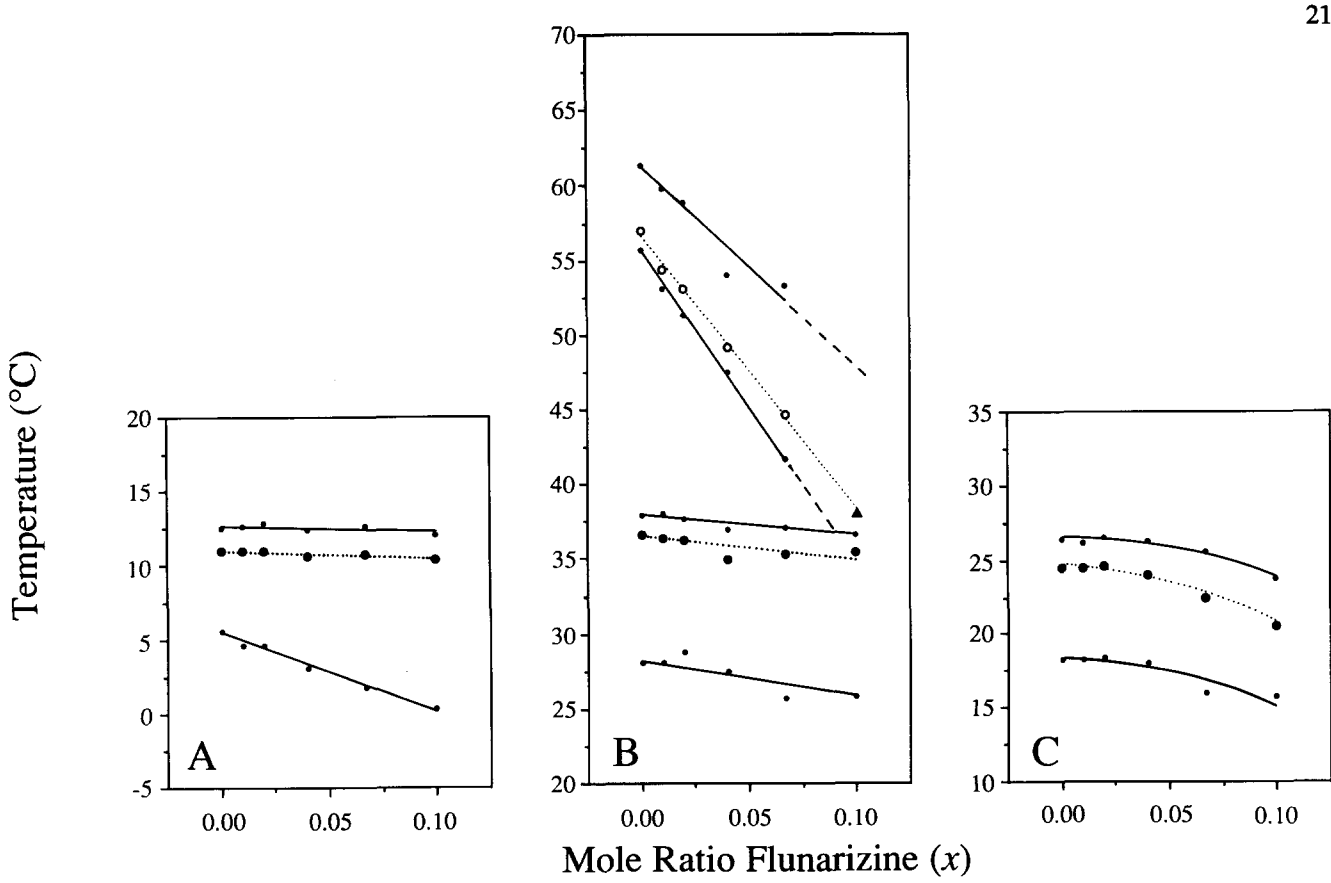

Fig. 3. Temperature-flunarizine mole ratio phase diagrams for (A) dielaidoylphosphatidylcholine, (B) dielaidoylphosphatidylethanolamine, and (C) dielaidoylphosphatidylserine. The lower and upper solid lines represent the beginning and end point of the phase transition, respectively, the stippled line the phase transition temperature. The highest flunarizine/phosphatidylethanolamine value (▲) was determined using ^{31}P-NMR.

tions in accurately determining changes in the relatively small enthalpy of this transition with our calorimeter. This effect was, however, observed to be quite small. Fig. 2B shows that increasing amounts of flunarizine cause a progressive, marked reduction in the ΔH for $Eld_2PtdSer$ resulting in a decrease of over 50% at $x = 0.10$. In contrast, these high flunarizine: phospholipid mole ratios have little effect on the ΔH of the L_β-L_α phase transitions of $Eld_2PtdCho$ and $Eld_2PtdEtn$.

The effect of agents on the phase behaviour of phospholipids can best be appreciated using phase diagrams. Phase diagrams of the effects of flunarizine on the phase behaviour of $Eld_2PtdCho$, $Eld_2PtdEtn$ and $Eld_2PtdSer$ are presented in Fig. 3. Examination of this figure clearly reveals now flunarizine has a distinctly different effect on each of the phospholipid classes studied here. Fig. 3A shows how flunarizine, whilst having little effect on the T_m of $Eld_2PtdCho$ and the point of 100% conversion of the phospholipid to the L_α phase, markedly reduces the temperature at which the onset of the phase transition from the L_β to the L_α phase occurs; that is to say that there is a significant broadening of the L_β-L_α phase transition with increasing flunarizine mole ratio. The phase diagram for $Eld_2PtdEtn$/flunarizine mixtures (Fig. 3B) emphasizes how, despite only a small effect on the L_β-L_α phase transition, increasing the mole ratio of flunarizine dramatically causes a downward shift of the L_α-H_{II} phase transition of the PtdEtn. As mentioned previously the T_{mH} for the highest mole ratio of flunarizine has been estimated from ^{31}P-NMR data. However, it can be seen from Fig. 3B how this estimation is in good agreement with the rest of the data and supports the ^{31}P-NMR observation that the H_{II} phase can be observed at temperatures immediately above the L_β-L_α phase transition temperature at $x = 0.10$. If we examine Fig. 3C we can see a different type of profile in the phase diagram for $Eld_2PtdSer$/flunarizine mixtures. Here we observe that the downward shift in T_m of the L_β-L_α phase transition is accompanied by a shift in the entire phase transition profile. This is in marked contrast to the profile of the phase diagram for the PtdCho/flunarizine mixtures where a downward shift in the onset of the phase transition was not accompanied by a similar shift in the other phase transition parameters.

The effect of flunarizine on dioleoylphospholipids

The study of the effect of flunarizine on phospholipids was extended to cover the dioleoyl-(Ole_2) phospholipids. These were chosen because they have quite different thermal properties from the dielaidoylphospholipids whilst having virtually the same molecular

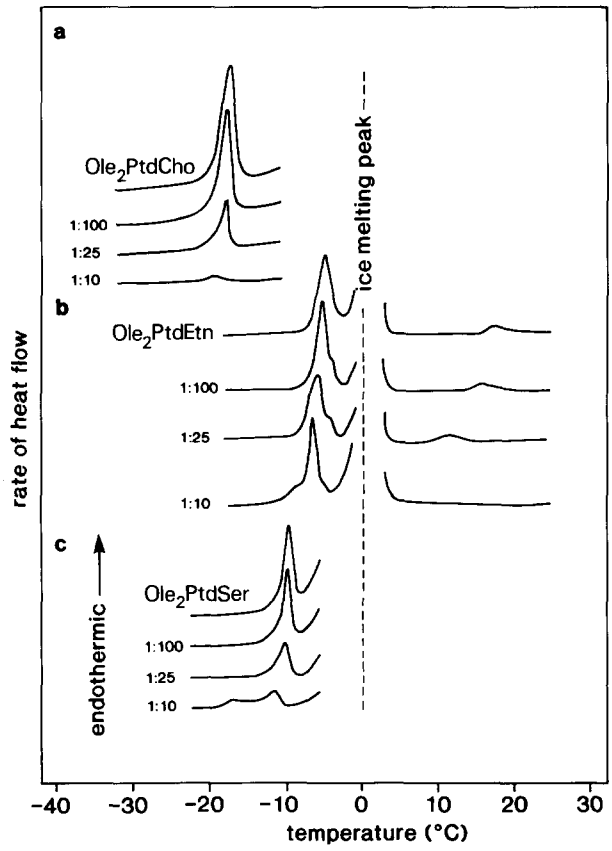

Fig. 4. Typical DSC of flunarizine/dioleoylphospholipid dispersions; (a) phosphatidylcholine, (b) phosphatidylethanolamine, and (c) phosphatidylserine. Numbers on the figures represent flunarizine to phospholipid mole ratios.

structure, differing only in the configuration of the acyl chain double bond. The same three phospholipid classes as were used above were chosen to allow comparison of the results. Typical thermograms of $Ole_2PtdCho$ in the absence and in the presence of increasing mole ratios of flunarizine are given in Fig. 4a. The L_β-L_α phase transition of $Ole_2PtdCho$ occurred at a temperature of $T_m = -17.7°C$ with a total enthalpy for the transition of $\Delta H = 8.0$ kcal · mol^{-1}. These results are in good agreement with previously reported data [32]. As was observed with $Eld_2PtdCho$ there is a slight downward shift in T_m with increasing flunarizine mole ratios. However, in this case flunarizine has had a remarkable effect on the enthalpy of the transition (ΔH) which at a flunarizine mole ratio of $x = 0.25$ has been reduced by over 50% and at a mole ratio of $x = 0.1$ by over 90%.

Fig. 4b shows a typical DSC thermogram of a $Ole_2PtdEtn$ dispersion. The L_β-L_α phase transition temperature of $T_m = -4.9°C$ with an enthalpy for the transition of $\Delta H = 5.5$ kcal · mol^{-1} are in agreement with published values [32]. Flunarizine slightly depresses the T_m of $Ole_2PtdEtn$, the effect increasing with increasing flunarizine mole ratios (see Fig. 4b). There is only a very slight reduction in the ΔH for this transition at higher mole ratios of flunarizine. Flunarizine does, however, have a striking effect on the L_α-H_{II} phase transition of $Ole_2PtdEtn$. Control values for this transition are a transition temperature of $T_{mH} = 17.8°C$ and an enthalpy for the transition of $\Delta H_H = 0.35$ kcal · mol^{-1}. The value for the transition enthalpy is in good agreement with that published by Epand [34] but the transition temperature reported here is somewhat higher than his value. Epand noted, however, that the transition is highly dependent upon scan rate. We can therefore presume that our higher transition temperature is due to the fact that we have employed a faster scan rate. Data in Fig. 4b indicate that flunarizine induces a strong downward shift in the L_α-H_{II} phase transition temperature proportional to its mole ratio. At the highest flunarizine mole ratio ($x = 0.1$) that were examined the L_α-H_{II} phase transition was no longer detectable by DSC.

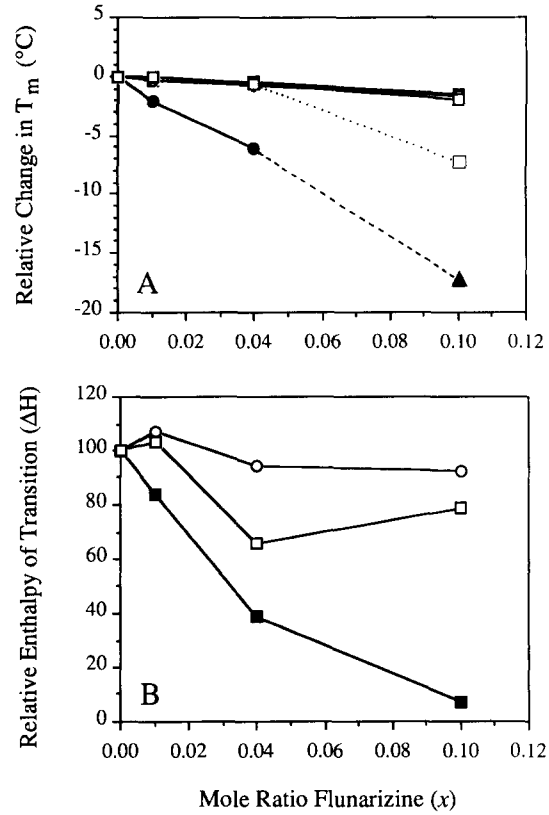

Fig. 5. (A) A DSC study of the effect of increasing flunarizine mole ratios on the gel to liquid-crystalline phase transition temperatures of dioleoylphospholipid dispersions (phosphatidylcholine (■), phosphatidylethanolamine (○) and phosphatidylserine (□)) and on the liquid-crystalline to hexagonal II phase transition temperature of dioleylphosphatidylethanolamine (●,▲). Value at highest mole ratio (▲) was estimated using ^{31}P-NMR. (B) The effect of flunarizine on the gel to liquid-crystalline phase transition enthalpies of dioleoylphospholipid dispersions: phosphatidylcholine (■), phosphatidylethanolamine (○) and phosphatidylserine (□). Values represent the average of four determinations.

Fig. 4c shows a typical DSC thermogram of a $Ole_2PtdSer$ dispersion. Control values of $T_m = -9.3°C$ for the L_β-L_α phase transition temperature and $\Delta H = 6.7$ kcal · mol^{-1} for the enthalpy of the transition are in good agreement with values published by Van Dijck [32], but do differ slightly from those of Demel et al. [36]. The presence of flunarizine caused a slight downward shift in T_m and a substantial reduction of almost 40% of the ΔH for the L_β-L_α phase transition of $Ole_2PtdSer$ at quite modest mole ratios ($x = 0.04$). At higher mole ratios ($x = 0.1$) the profile of the thermogram becomes greatly altered revealing two separate melting peaks. This is indicative of a lateral phase separation within the drug/phospholipid dispersion. Such phase separations have been interpreted as representation of drug-rich and drug-poor phospholipid domains [9]. The T_m of the lower melting peak, which presumably represents a phase of high flunarizine–phospholipid interaction is quite markedly shifted by 7.4°C. It is interesting to note that after this appearance of two transition peaks the combined enthalpy of the two peaks is greater than that of the preceding lower flunarizine mole ratio. This is presumably because the phase separation of the mixture has reduced the total number of PtdSer molecules in close contact with flunarizine molecules.

Fig. 5A illustrates the influence of flunarizine on the phase transition temperatures of the dioleoylphospholipids. It shows that flunarizine once again has a dramatic effect on the L_α-H_{II} phase transition of the PtdEtn. ^{31}P-NMR was again used to confirm this effect (results not shown) and indicated that the $x = 0.1$ flunarizine/$Ole_2PtdEtn$ sample was in the H_{II} phase at 0°C. This corresponds to a shift in the phase transition temperature of over −17°C. These results are represented in Fig. 5A and indicate a strong linear reduction in T_{mH} with increasing flunarizine mole ratio. The influence of flunarizine on the temperature of the L_β-L_α phase transitions is much less marked. However, whilst flunarizine has a somewhat modest influence on the T_m values of $Ole_2PtdCho$ and $Ole_2PtdEtn$ it does have a large effect on a proportion of the PtdSer in the flunarizine/PtdSer dispersion at the highest mole ratio ($x = 0.1$). It is interesting to note that the effect of flunarizine on the remainder of the PtdSer is the same as that observed with the two other phospholipid classes.

The influence of flunarizine on the relative enthalpies of the L_β-L_α phase transitions of the dioleoylphospholipids that have been examined are presented in Fig. 5B: the values for the L_α-H_{II} phase transition of the PtdEtn have again been omitted for the abovesaid reasons. We can observe that increasing mole ratios of flunarizine have no effect on the enthalpy of the L_β-L_α phase transition of the PtdEtn whilst having a significant effect on that of the PtdSer, reducing it by over 35% at one point, and a dramatic effect on that of the PtdCho virtually diminishing the enthalpy of the phase transition almost to zero at $x = 0.1$.

It is, of course, of great interest to compare these results with those obtained with the Eld_2-phospholipids (summarized in Figs. 2A and 2B). Whilst it is immediately apparent how different the effect of flunarizine is on the relative enthalpies of the two PtdCho's it should be stressed how similar the effects of flunarizine are, in general, on the corresponding molecular species of the different phospholipid classes. Excepting this effect on the ΔH values of the PtdCho's, which will be examined in further detail, what we observe for both molecular species are moderate effects on the T_m values of the PhdCho's and PtdEtn's, a more significant depression of the T_m values of the PtdSer's and a dramatic depression of the T_{mH} values of the PtdEtn's (Figs. 2A and 5A). We also see virtually no effect on the ΔH values of the PtdEtn's but significant reductions in those of the PtdSer's (Figs. 2B and 5B).

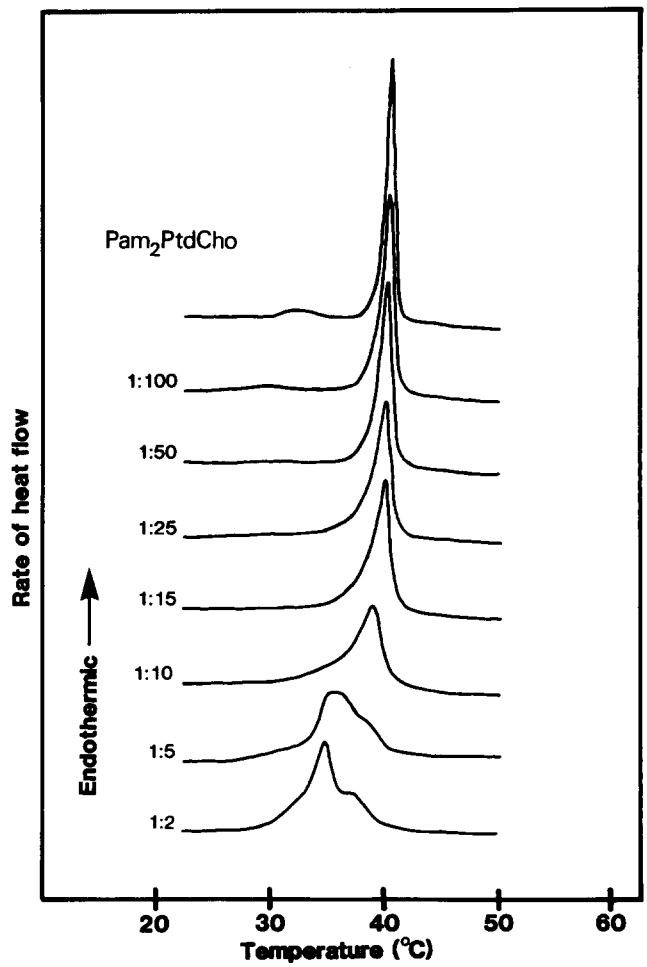

Fig. 6. Typical DSC thermograms showing the effect of flunarizine on the phase behaviour of dipalmitoylphosphatidylcholine dispersions. Numbers on the figure represent flunarizine to phospholipid mole ratios.

The effect of flunarizine on phosphatidylcholines

In order to gain further insight into the large differences that were observed in the effect of flunarizine on Eld_2- and Ole_2PtdCho the study was extended to examine the effect of flunarizine on the phase behaviour of dipalmitoylphosphatidylcholine (Pam_2PtdCho).

Typical thermograms of Pam_2PtdCho in the absence of and in the presence of increasing mole ratios of flunarizine are presented in Fig. 6. The control thermogram for Pam_2PtdCho reveals a pretransition at 31.8°C and a L_β-L_α phase transition at $T_m = 40.6$°C with an enthalpy of the main transition of $\Delta H = 7.7$ kcal · mol^{-1}. These values are in good agreement with previously reported results (for example, see Ref. 33). In the presence of flunarizine the pretransition is no longer observed at mole ratios above $x \geqq 0.02$. Increasing the mole ratio of flunarizine causes a depression of T_m and a broadening of the phase transition profile whilst having no effect on the ΔH of the transition. These results effectively show no discernable difference to those obtained with Eld_2PtdCho. Therefore the study was extended to include higher mole ratios of flunarizine for both Pam_2- and Eld_2PtdCho. It is clear that at higher mole ratios of flunarizine ($x > 0.1$) a difference in its effects on the two PtdCho's becomes apparent. The relative effects of flunarizine on the three different PtdCho's that have been examined in this study are presented in Figs. 7A and 7B. The effect of flunarizine on the transition temperatures of the three PtdCho's is given in Fig. 7A. Whilst it is clear that there are differences in the effects of flunarizine with each of these phospholipids it can be reasonably concluded that they are not particularly significant for the range of flunarizine: phospholipid mole ratios that has been examined for all three PtdCho's, especially when one considers the considerable differences in the transition temperatures of these phospholipids (−18°C, 11°C and 41°C). However, what is quite remarkable is the effect of flunarizine on the enthalpies of these phase transitions (Fig. 7B). From these data we can clearly see a mole ratio dependent effect of flunarizine on two of the PtdCho's. Whereas Ole_2PtdCho is very sensitive to the presence of flunarizine, Eld_2PtdCho is only sensitive to the presence of high mole ratios of flunarizine and Pam_2PtdCho is quite insensitive to the presence of flunarizine.

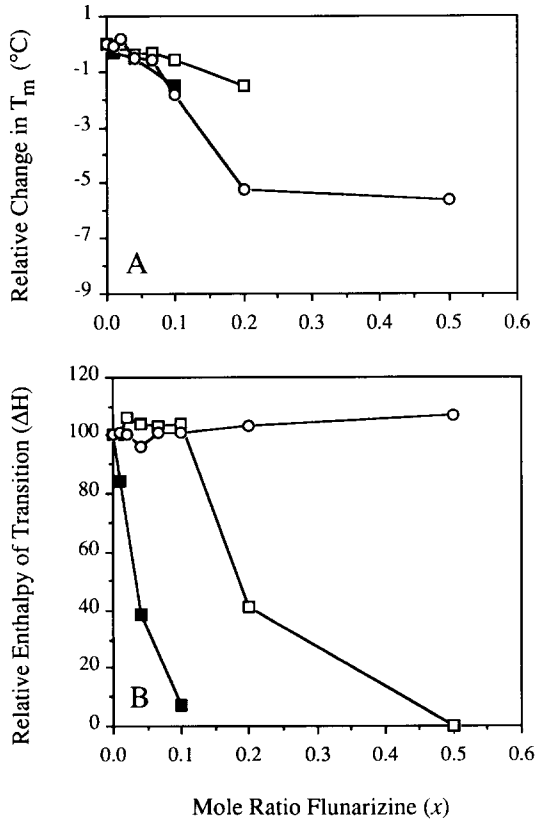

Fig. 7. The effect of increasing flunarizine mole ratios on (A) the gel to liquid-crystalline phase transition temperatures and (B) the phase transition enthalpies of phosphatidylcholine dispersions: dipalmitoyl (○), dielaidoyl (□) and dioleoyl (■).

Discussion

The gel to liquid-crystalline (L_β-L_α) phase transition of a phospholipid corresponds to an order to disorder transition and represents a change from a state in which the phospholipid acyl chains are rigid and in close contact with their neighbouring phospholipid acyl chains to a state in which relatively free rotational movement around carbon-carbon bonds is permitted for the acyl chains [37]. This is the so-called *trans-gauche* isomerization of the acyl chains. The temperature at which the endothermic L_β-L_α phase transition of a phospholipid occurs is dependent upon chain length [38], unsaturation and upon headgroup structure (see Ref. 39).

The profile of a DSC thermogram of a phospholipid phase transition is determined by the enthalpy of the transition and the degree of cooperativity: the more cooperative the transition the higher and narrower the peak (for transitions of similar enthalpy). The concept of cooperativity and its implications for the L_β-L_α phospholipid phase transition has been extensively discussed by Biltonen and Freire [40]. There are several means of mathematically defining cooperativity and the size of cooperative units [9,10,40], but to all intents and purposes these remain somewhat arbitrary definitions relating peak height to width. It is, however, tenable to assume that as a transition becomes less cooperative the peak profile will be gradually transformed from a tall narrow peak to a shorter, broader peak indicating that the now less cooperative transition occurs over a wider temperature range. This change in the profile of the DSC thermogram of the phospholipid phase transition is of the essence to the results presented here.

Mountcastle et al. [41] have demonstrated that the presence of the gaseous anesthetics halothane and enflurane decreased the transition temperature and increased the width of the transition without affecting the enthalpy for the L_β-L_α phase transition of $Pam_2PtdCho$. They concluded that the gaseous anesthetics not only decrease T_m but also influence the degree of cooperative interaction between phospholipid molecules, decreasing the degree of cooperativity, resulting in the clusters of phospholipid molecules involved in the phase transition becoming smaller in size and thus more numerous in the region of the phase transition temperature. In this way, we can envisage how the presence of a 'foreign' molecule or additive in phospholipid bilayers could interfere with phospholipid–phospholipid interactions and thus reduce cluster size, thereby increasing cluster number and consequently decreasing cooperativity.

Flunarizine clearly has the ability to significantly influence phospholipid phase transitions too, as is highlighted in the study with the dielaidylphospholipids. The depression of the onset of the L_β-L_α phase transition of $Eld_2PtdCho$ indicates a destabilization of the L_β phase (Fig. 3A). This broadening of the phase transition indicates a loss of cooperativity. That T_m, the phase transition end-point and ΔH are not altered implies that despite this loss of cooperativity the thermodynamic characteristics of the phase transition have not been greatly altered. The lowering of the onset of the L_β-L_α phase transition and thereby the broadening of that transition indicate that the PtdCho molecules exist in more numerous smaller clusters. However, the lack of effect on the other parameters indicate interestingly that this has had little bearing on the nature of the phase transition.

This is even more apparent if we examine the effect of flunarizine on the L_β-L_α phase transition of $Eld_2PtdEtn$. In this case flunarizine has only a small effect on the thermodynamic parameters of this phase transition (Figs. 2A,2B and 3B). The fact that flunarizine does strongly effect the phase behaviour of this phospolipid is, however, immediately apparent from its dramatic effects on the L_α-H_{II} phase transition. It is now widely accepted that the non-bilayer hexagonal II phase behaviour of molecules such as PtdEtn is governed by their molecular shape [42,43], these molecules possessing a so-called 'inverted cone' shape. A strong induction (stabilization) of the H_{II} phase of PtdEtn, as witnessed here, can be caused in several ways including increasing acyl chain unsaturation [43], headgroup dehydration [44,45] and expansion of the hydrophobic domain of the bilayer [44,46,47]. All of these are factors which accentuate the cone shape of the PtdEtn molecule. Early studies by Hornby and Cullis [46] and later, more detailed studies by Epand [47] have shown that the L_α-H_{II} phase transition of PtdEtn is extremely sensitive to the presence of hydrophobic molecules which, by expanding the hydrophobic phase of the bilayer, cause a large downward shift of the temperature of this phase transition whilst having only a marginal effect on the L_β-L_α phase transition. It would seem that flunarizine has the same effect on the phase behaviour of PtdEtn as that which a molecule such as eicosane does due to the similarity between their effects ; that is to say that flunarizine depresses T_{mH} by expanding the hydrophobic domain of $Eld_2PtdEtn$. These results are indicative of a location for flunarizine deep in the hydrophobic core of the phospholipid bilayer with this phospholipid.

The somewhat limited effects of flunarizine on the L_β-L_α phase transitions of $Eld_2PtdCho$ and $Eld_2PtdEtn$ contrast strongly with its effects on that of $Eld_2PtdSer$ (Fig. 3C). Flunarizine quite clearly has a greater influence on the L_β-L_α phase transition of the PtdSer than it does on those of the corresponding PtdCho and PtdEtn reducing the T_m values by 3.9, 0.6 and 1.2°C, respectively. The drug also reduces the ΔH of this transition by over 50% whilst not affecting those of the two other phospholipid classes. There is also a downward shift in the onset of the phase transition and a corresponding downward shift in the transition endpoint (the temperature at which 100% conversion of the phospholipid to the L_α phase occurs) and therefore no broadening of the phase transition. This indicates that flunarizine both destabilizes the L_β phase and stabilizes the L_α phase of $Eld_2PtdSer$ which contrasts with its effect on the PtdCho where only destabilization of the L_β phase was apparent. Charge-influenced headgroup interactions play an important role in the phase transition characteristics of anionic lipids such as PtdSer [39]. Flunarizine presumably reduces phospholipid–phospholipid interactions of the PtdSer thus encouraging the formation of the fluid L_α phase. These results suggest a more specific flunarizine–PtdSer interaction, probably a charge-influenced drug–phospholipid headgroup interaction.

When charged, cationic amphiphilic molecules are considered to be located in the so-called interface region of the phospholipid bilayer; the location in the plane of the membrane which matches their dual hydrophilic and hydrophobic nature with that of the phospholipids. Several recent studies have confirmed this to be the case for quite a large variety of pharmacologically unrelated drugs which possess this amphiphilic nature, including tetracaine [48], dibucaine [49], propanalol [50], chlorpromazine [51], nimodipine [52], *n*-alkanals [5]) and adriamycin and ethidium bromide [54]. It has been proposed in most of these cases that there is an interaction between a positively charged quaternary nitrogen of the drug molecule and the negatively charged phosphate moiety of the phospholipid headgroup for each of these molecules.

We can envisage a similar interaction between flunarizine and the PtdSer headgroup. Such an interaction would reduce phospholipid–phospholipid interactions and could be expected to result in a reduction of ΔH and a shift in T_m of Eld_2PtdSer as witnessed in the experiments reported in this paper. However, a location of flunarizine at the interface region of PtdSer bilayers is in conflict with its apparent location in the hydrophobic phase of PtdEtn bilayers discussed above. Hornby and Cullis [46] have observed that chlorpromazine, a molecule which in their studies was situated at the hydrophilic/hydrophobic membrane interface, stabilizes the bilayer L_α phase causing an upward shift in T_{mH} of PtdEtn. The results presented here demonstrate that this is clearly not the case for flunarizine which, on the contrary, induced the formation of the H_{II} phase further supporting the notion of a location deeper in the hydrophobic domain of the PtdEtn bilayer. This leads us to the inevitable conclusion that flunarizine probably has a different location in bilayers of each of these phospholipids.

These different locations for flunarizine in the membrane can be reconciled by consideration of its charged state. Clearly flunarizine must be charged when interacting with the headgroup of PtdSer and uncharged when able to induce the formation of the H_{II} phase in PtdEtn. Cationic amphiphilic drugs are often assumed to be fully positively charged under physiological conditions and, of course, this is usually the case, most of these compounds having a pK of between 8.5 and 10.0. In contrast, flunarizine has a dissociation constant in water of $pK = 7.7$ (Janssen Pharmaceutica, Analytical Department, Beerse, Belgium) and should be approximately 75% in its charged form at the experimental pH used here (pH 7.2–7.3). However, it has been demonstrated by Fernández and Fromherz that the pK of an amphiphilic molecule is sensitive to its environment; a molecule can have a different 'apparent' pK in charged and uncharged micelles from its pK in aqueous solution [55]. They showed that the 'apparent' pK of a cationic amphiphile is lower in neutral micelles and higher in negatively charged micelles when compared to the measured pK in water. Similar behaviour has also been demonstrated for alkyl amines incorporated into PtdCho vesicles [56], the 'apparent' pK being 1–2 units lower in the PtdCho vesicles than the pK in water.

The influence of the interfacial region on the pK of cationic amphiphilic drugs has also been clearly demonstrated by examining the pK of tetracaine in cationic, neutral and anionic micelles [57] and in PtdCho [58,59] and PtdCho/PtdSer vesicles [58]. The pK of tetracaine is shown to decrease by approximately 1 pK unit from the value in aqueous solution upon incorporation into a neutral membrane. It has also been demonstrated using deuterium NMR that tetracaine sits higher in the membrane when it is charged and penetrates more deeply when uncharged [60]. Taking these facts into account we can conceive that, under the experimental conditions used here, flunarizine is charged in Eld_2PtdSer bilayers and as a result sits high in the membrane reducing intermolecular headgroup interactions causing alterations in the L_β-L_α phase transition parameters. In the neutral Eld_2PtdCho and Eld_2PtdEtn bilayers flunarizine can be expected to be mostly uncharged due to the influence of those phospholipids on its ionization and consequently located deeper in the hydrocarbon region of the bilayer. Here it will exert less influence on the L_β-L_α phase transition of these phospholipids but as a result of this location have a dramatic effect on the L_α-H_{II} phase transition of the PtdEtn.

Experiments were also carried out to examine the effect of phospholipid acyl chain composition on flunarizine–phospholipid interactions by examining its effects on dioleoyl phospholipids. Once again, flunarizine has had a far greater influence on the T_m of the L_β-L_α phase transition of the Ole_2PtdSer than on those of the corresponding PtdCho and PtdEtn. There is also a large effect on the ΔH of the PtdSer and no effect on that of the PtdEtn. The effects on Ole_2PtdEtn and Ole_2PtdSer are very similar to those on the corresponding dielaidylphospholipids. The influence of flunarizine on the phase behaviour of Ole_2PtdEtn mirrors its effects on Eld_2PtdEtn. We again observe little effect on thermodynamic parameters of the L_β-L_α phase transition and a strong promotion of the H_{II} phase. Comparison of Figs. 2 and 5 clearly shows that these effects are essentially identical for the two different PtdEtn species. The influence of flunarizine on Ole_2PtdSer is similar but not identical to that on Eld_2PtdSer. These effects are the same at lower mole ratios but are somewhat different at the highest mole ratio examined ($x = 0.1$). At this mole ratio an apparent lateral phase separation is observed with Ole_2PtdSer which was not observed with Eld_2PtdSer. However, despite this difference the effects of flunarizine on phase transition parameters are quite similar for the two PtdSer species and the results with Ole_2PtdSer reinforce the idea of a more specific interaction of flunarizine with this phospholipid class. Therefore, these changes in acyl chain composition do not seem to have effected the interaction of flunarizine with these two phospholipid classes to any great extent. Interestingly this is not the case with PtdCho.

Experiments carried out with Eld_2PtdCho, Ole_2PtdCho and with Pam_2PtdCho demonstrate a remarkable influence of acyl chain composition on the interaction of flunarizine with this phospholipid class. Although there is no clear acyl chain-dependent effect on the T_m of the L_β-L_α phase transition of these lipids (Fig. 7A), there is a dramatic acyl chain-dependent effect upon the ΔH of the transition (Fig. 7B). This dependence is inversely proportional to acyl chain intermolecular interactions. Flunarizine has no effect at all

on the ΔH of the L_β-L_α phase transition of Pam_2PtdCho. This phospholipid with its saturated acyl chains will undergo a large degree of acyl chain-acyl chain interactions whereas Ole_2PtdCho, which has a highly disordering *cis* double bond in its acyl chains consequently displays diminished acyl chain-acyl chain interaction and is more sensitive to the presence of flunarizine as a result. Eld_2PtdCho, containing *trans* double bonds, will have a higher degree of inter acyl chain interaction than Ole_2- but lower than that of Pam_2PtdCho hence the intermediate effect of flunarizine on that phospholipid. The similar effects on the T_m values of the PtdCho's tested is indicative of a common location for flunarizine in these bilayers, the different effects on the ΔH values that phospholipid–phospholipid interactions themselves have a large bearing on how flunarizine interacts with those phospholipids. The differences in the acyl chain dependency of the effects of flunarizine with the different phospholipid classes probably reflects the degree to which the acyl chains themselves influence the phase transitions of those different phospholipid classes.

The results presented here demonstrate the complexity of drug–phospholipid interactions, not to mention phospholipid–phospholipid interactions, and emphasize that drug interactions with the different phospholipids, particularly the different phospholipid classes, must be studied as individual cases. Flunarizine binding to the different phospholipid classes, their influence on its ionization state and the effect of factors such as pH on these interactions are currently being investigated.

Acknowledgements

P.G.T. gratefully acknowledges the financial support of Janssen Pharmaceutica B.V. (Tilburg, The Netherlands) and the Janssen Research Foundation (Neuss, F.R.G.). We would like to thank Professors H. de Gier and B. de Kruijff for the use of their laboratory facilities and W. Geurts van Kessel for instruction in the art of phospholipid synthesis. This paper is dedicated to Jacqueline Mandersloot (deceased 1988) whose help, enthusiasm and friendship made differential scanning calorimetry a most enjoyable pastime.

References

1 Spedding, M. and Cavero, I. (1984) Life Sci. 35, 575–587.
2 Godfraind, T.G. (1987) Am. J. Cardiol. 59, 11B-23B.
3 Hosey, M.M. and Lazdunski, M. (1988) J. Membr. Biol. 104, 81–105.
4 Janis, R.A. and Scriabine, A. (1983) Biochem. Pharmacol. 32, 3499–3507.
5 Holmes, B., Brogden, R.N., Heel, R.C., Speight, T.M. and Avery, C.S. (1984) Drugs 27, 6–44.
6 Thomas, P.G., Zimmermann, A.G. and Verkleij, A.J. (1988) Biochim. Biophys. Acta 946, 439–444.
7 Lüllmann, H., Plosch, H. and Ziegler, A. (1980) Biochem. Pharmacol. 29, 2969–2974.
8 Cullis, P.R. and Verkleij, A.J. (1979) Biochim. Biophys. Acta 552, 546–551.
9 Jain, M.K. and Wu, N.M. (1977) J. Membr. Biol. 34, 157–201.
10 Bach, D. (1986) in Topics in Molecular and Structural Biology, Vol. 4, Biomembrane Structure and Function (Chapman, D., ed.), pp. 1–41, Macmillan Press, London.
11 Frenzel, J., Arnold, K. and Nuhn, P. (1978) Biochim. Biophys. Acta 507, 185–197.
12 Mountcastle, D.B., Biltonen, R.L. and Halsey, M.J. (1978) Proc. Natl. Acad. Sci. USA 75, 4906–4910.
13 Bruggeman, E.P. and Melchior, D.L. (1983) J. Biol. Chem. 258, 8298–8303.
14 Kursch, B., Lüllmann, H. and Mohr, K. (1983) Biochem. Pharmacol. 32, 2589–2594.
15 Constantinides, P.P., Inouchi, N., Tritton, T.R., Sartorelli, A.C. and Sturtevant, J.M. (1986) J. Biol. Chem. 261, 10196–10203.
16 Hanpft, R. and Mohr, K. (1985) Biochim. Biophys. Acta 814, 156–162.
17 Papahadjopoulos, D., Jacobson, D., Poste, G. and Shepherd, G. (1975) Biochim. Biophys. Acta 394, 504–519.
18 Post, J.A., Langer, G.A., Op den Kamp, J.A.F. and Verkleij, A.J. (1988) Biochim. Biophys. Acta 943, 256–266.
19 Thomas, P.G. and Verkleij, A.J. (1987) Biochem. Soc. Trans. 15, 1062.
20 Baer, E. and Buchnea, D. (1959) Can. J. Biochem. Physiol. 37, 953–959.
21 Hanahan, D.J. and Brockerhoff, H. (1960) Arch. Biochem. Biophys. 91, 326–328.
22 Van Deenen, L.L.M. and De Haas, G.H. (1964) Adv. Lipid Res. 2, 168–229.
23 Geurts van Kessel, W.S.M., Tieman, M. and Demel, R.A. (1981) Lipids 16, 58–63.
24 Dekker, C.J., Geurts van Kessel, W.S.M., Klomp, J.P.G., Pieters, J. and De Kruijff, B. (1983) Chem. Phys. Lipids 33, 93–106.
25 Davidson, F.M. and Long, C. (1958) Biochem. J. 16, 458–466.
26 Cullis, P.R. and De Kruijff, B. (1976) Biochim. Biophys. Acta 436, 523–540.
27 Comfurius, P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta 488, 36–42.
28 Carvalho, C.M., Oliveira, C.R., Lima, M.P., Leysen, J.E. and Carvalho, A.P. (1989) Biochem. Pharmacol. 38, 2121–2127.
29 Scheufler, E., Vogelgesang, R., Wilffert, B., Pegram, B.L., Hunter, J.B., Wermelskirchen, D. and Peters, T. (1990) J. Pharmacol. Exp. Therapeutics 252, 333–338.
30 Böttcher, C.J.F., Van Gent, C.M. and Priest, C. (1961) Anal. Chim. Acta 24, 203– 204.
31 Sen, A., Brain, A.P.R., Quinn, P.J. and Williams, W.P. (1982) Biochim. Biophys. Acta 686, 215–224.
32 Van Dijck, P.W.M. (1979) Biochim. Biophys. Acta 555, 89–101.
33 Van Dijck, P.W.M., De Kruijff, B., Van Deenen, L.L.M., De Gier, J. and Demel, R.A. (1976) Biochim. Biophys. Acta 455, 576–587.
34 Epand, R.M. (1985) Chem. Phys. Lipids 36, 387–393.
35 Cullis, P.R. and De Kruijff, B. (1978) Biochim. Biophys. Acta 513, 31–42.
36 Demel, R.A., Paltauf, F. and Hauser, H. (1987) Biochemistry 26, 8659–8665.
37 Chapman, D. (1958) J. Chem. Soc. 784–789.
38 Chapman, D., Williams, B.M. and Ladbrooke, B.D. (1967) Chem. Phys. Lipids 1, 445–475.
39 Boggs, J.M. (1980) Can. J. Biochem. 58, 755–770.
40 Biltonen, R.L. and Freire, E. (1978) CRC Crit. Rev. Biochem. 5, 85–124.
41 Mountcastle, D.B., Biltonen, R.L. and Halsey, M.J. (1978) Proc. Natl. Acad. Sci. USA 75, 4906–4910.
42 Israelachvili, J.N. and Mitchell, D.J. (1975) Biochim. Biophys. Acta 389, 13–19.

43 Cullis, P.R. and De Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207–218.
44 Seddon, J.M., Cevc, G. and Marsh, D. (1983) Biochemistry 22, 1280–1289.
45 Epand, R.M. and Bryszewska, M. (1988) Biochemistry 27, 8776–8779.
46 Hornby, A.P. and Cullis, P.R. (1981) Biochim. Biophys. Acta 647, 285–292.
47 Epand, R.M. (1985) Biochemistry 24, 7092–7095.
48 Boulanger, Y., Schreier, S. and Smith, I.C.P. (1981) Biochemistry 20, 6824–6830.
49 Browning, J.L. and Akutsu, H. (1982) Biochim. Biophys. Acta 684, 172–178.
50 Herbette, L.G., Katz, A.M. and Sturtevant, J.M. (1983) Mol. Pharmacol. 24, 259–269.
51 Kuroda, Y. and Kitamura, K. (1984) J. Am. Chem. Soc. 106, 1–6.
52 Herbette, L.G., Chester, D.W. and Rhodes, D.G. (1986) Biophys. J. 49, 91–93.
53 Westerman, P.W., Pope, J.M., Phonphok, N., Doane, J.W. and Dubro, D.W. (1988) Biochim. Biophys. Acta 939, 64–78.
54 Nicolay, K., Sauterau, A.-M., Tocanne, J.-F., Brasseur, R., Huart, P., Ruysschaert, J.-M. and De Kruijff, B. (1988) Biochim. Biophys. Acta 940, 197–208.
55 Fernández, M.S. and Fromherz, P. (1977) J. Phys. Chem. 81, 1755–1761.
56 Ptak, M., Egret-Charlier, M., Sanson, A. and Bouloussa, O. (1980) Biochim. Biophys. Acta 600, 387–397.
57 García-Soto, J. and Fernández, M.S. (1983) Biochim. Biophys. Acta 731, 275–281.
58 Westman, J., Boulanger, Y., Ehrenberg, A. and Smith, I.C.P. (1982) Biochim. Biophys. Acta 685, 315–328.
59 Schreier, S., Frezzatti, W.A., Araujo P.S., Chaimovich, H. and Cuccovia, I.M. (1984) Biochim. Biophys. Acta 769, 231–237.
60 Kelusky, E.C. and Smith, I.C.P. (1984) Can. J. Biochem. Cell Biol. 62, 178–184.