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Influence of cationic amphiphilic drugs on the phase-transition temperature of phospholipids with different polar headgroups

Reinhard Hanpft and Klaus Mohr

Department of Pharmacology, University of Kiel, Hospitalstrasse 4–6, D-2300 Kiel (F.R.G.)

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The influence of a number of cationic amphiphilic drugs on the phase-transition temperature (T_t) of liposomes prepared of dipalmitoylphosphatidic acid (DPPA) and of dipalmitoylphosphatidylglycerol (DPPG) was investigated applying the method of differential scanning calorimetry. Under control conditions, the transition from the gel to the liquid crystalline state occurred in DPPA liposomes at $T_t \approx 63°C$ and in DPPG liposomes at $T_t \approx 42°C$, the difference of 20 deg. C reflecting the specific influence of the phospholipid polar headgroups on T_t. Addition of a drug to DPPA liposomes induced a second transition signal at a much lower temperature, indicating the presence of drug-containing DPPA domains. The temperature of the drug-induced transition was almost independent of the amount of drug added but differed according to the drug under investigation. In DPPG liposomes, the temperature of the control transition declined gradually depending on the amount of a drug added until all DPPG molecules were uniformly affected by drug molecules. The temperature of this drug-induced transition differed depending on the drug tested. A comparison revealed that the T_t values induced by a given drug were similar in DPPA, DPPG and also DPPC liposomes in spite of the widely different T_t values of these phospholipids under control conditions. It is concluded that the intercalation of drug molecules between the polar headgroups of the phospholipids almost eliminates the specific influence of the headgroups on the T_t of the investigated phospholipids.

Introduction

Cationic amphiphilic drugs, e.g., local anesthetic, β-receptor blocking or neuroleptic compounds, are characterized by a common molecular structure, i.e., a hydrophobic aromatic ring system with a short hydrophilic alkyl sidechain containing a protonized amino group [1].

Due to their physicochemical properties, cationic amphiphilic drugs bind to the polar headgroup regions of phospholipid membranes in such a way that the protonized amino groups interact with the negatively charged phosphate groups of the phospholipid molecules and the aromatic rings are directed towards the hydrophobic interior of the phospholipid membrane [2–4]. The intercalation of drug molecules induces a depression of the temperature at which phospholipid membranes undergo the transition from the gel to the liquid crystalline state [5–12]. The phase-transition temperature of liposomes consisting of a single phospholipid species is characteris-

Abbreviations: DPPA, dipalmitoylphosphatidic acid; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DPPG, dipalmitoylphosphatidylglycerol; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulfonic acid.

tic for that particular phospholipid and depends on the molecular structure of the fatty acid chains and of the polar headgroups of the phospholipid molecules [13–16].

In a previous study, the influence of a number of cationic amphiphilic drugs on the phase-transition temperature (T_t) of dipalmitoylphosphatidylcholine (DPPC) liposomes was compared applying the method of differential scanning calorimetry. It was found that the various drugs reduced T_t to different levels, i.e., the drugs possessed a different potency to depress T_t [17]. The present study was performed in order to investigate to what extent the depressant action of the cationic amphiphilic drugs on T_t is influenced by the structure of the polar headgroup region of a phospholipid membrane. The effect of cationic amphiphilic drugs on T_t was measured in liposomes prepared of phospholipids, which differed only in the structure of their polar headgroups but had identical fatty acid chains. The lipids chosen were DPPA and DPPG. In contrast to the zwitterionic DPPC, both DPPA and DPPG contain negatively charged headgroups. Due to the different headgroups, the phase-transition in DPPA liposomes ($T_t \approx 63°C$) occurred at a temperature which was 20°C higher than the phase-transition temperature of DPPG liposomes ($T_t \approx 42°C$).

The difference in potency of various cationic amphiphilic drugs to depress T_t was observed with DPPA as well as with DPPG liposomes. Comparison of the present results and those obtained with DPPC liposomes showed that the intercalation of catamphiphilic drug molecules almost completely eliminated the specific influence of the polar headgroup on the phase-transition temperature of the investigated phospholipid membranes. Instead, the drug-induced T_t was determined by the individual potency of a drug to depress T_t and was nearly independent of the nature of the polar headgroup of a phospholipid species.

A preliminary account of this work has been reported previously [18].

Materials and Methods

The sodium salt of 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA, purity about 99%) and the ammonium salt of 1,2-dipalmitoyl-*sn*-glycero-phosphoryl-1′-*sn*-glycerol (DPPG, purity about 99%) were obtained from Sigma (München, F.R.G.) and used without further purification. The drugs were used in form of their hydrochloride salts. The other chemicals were purchased from Merck (Darmstadt, F.R.G.) and were of analytical grade.

Liposomes were prepared in a similar way as described previously for DPPC liposomes [17]. In a glass vial, appropriate amounts of drug dissolved in chloroform or in 15 : 2, v/v chloroform/methanol were added to 5 mg phospholipids (DPPA as dry substance, DPPG dissolved in a mixture of 15 : 2, v/v chloroform/methanol). The solvent was evaporated at room temperature under a stream of nitrogen over a period of 4 h. The resulting precipitate was further dried under vacuum overnight (approx. 12 h). On the following day, 100 µl of a 14 mM Tes/histidine buffer adjusted with HCl to pH 6 were added to the dry drug/lipid mixture. The resulting pH values amounted to pH 6.0–6.7. Since this pH range differed largely from the pK_a values of the investigated phospholipids and drugs, the influence of this small scatter of pH values on the degree of protonation of lipids and drugs appeared to be neglectable. The stoppered glass vials were placed for 2 h in a water-bath, set at a temperature above T_t of the respective phospholipid, i.e., 70°C or 50°C in case of DPPA or DPPG, respectively. Every 30 min, each vial was vigorously shaken on a bench vibrator for about 10 s. This procedure yielded a milky suspension containing multilamellar liposomes of different size, the larger ones being visible by means of light microscopy. The phase-transition temperature of the liposomes was determined applying the method of differential scanning calorimetry using a DSC-2C/intracooler II equipment (Perkin Elmer, Überlingen, F.R.G.).

10 µl of the liposome suspension were enclosed in an aluminium capsule (Perkin Elmer) and measured against a reference sample containing only 10 µl of buffer. Both samples were heated at a constant rate of 5 deg. C/min from 12 to 72°C in case of DPPA and from 7 to 52°C in case of DPPG. The temperature of the samples was recorded on the abscissa of the thermogram. The difference between the energies taken up by both samples was registered at a sensitivity range set-

ting of 0.5 mcal/s on the ordinate. At the endothermic phase transition, the phospholipid-containing sample absorbed an additional amount of energy; this additional heat flow was expressed by the transition signal.

In order to determine the temperature of the phase-transition, a line perpendicular to the abscissa was drawn through the intersection between a straight line fitted to the upward deflection of the transition peak and the baseline. The intersection of the perpendicular line with the temperature scale indicated T_t. The temperature scale was calibrated using cyclohexan and indium. Repeated scans of the same sample showed good reproducibility of the results.

Liposome-free drug solutions below and above their critical micellar concentrations did not reveal DSC signals.

Results

DPPA liposomes

The influence of the anorectic drug chlorphentermine on T_t of DPPA liposomes is illustrated in Fig. 1 by original recordings. Under control conditions, the phase transition of DPPA liposomes occurred at a temperature of $T_t \approx 63°C$ ($\bar{x}, n = 13$). This value matched the T_t of 63°C reported by Jacobson and Papahadjopoulos [19] for liposomes composed of monovalent negatively charged DPPA molecules and agrees with the data communicated by other groups [12,16]. A pretransition endotherm was not observed (see also Ref. 19).

The quantity of drug in the assay was expressed as the molar ratio between the amount of drug added and the amount of phospholipid present. In order to measure the amount of a drug bound and its actual free concentration, liposome samples of DPPA and DPPG were centrifuged and the drug concentration in the supernatant was determined spectrophotometrically. In case of chlorpromazine, promazine, propranolol, chlorphentermine and dibucaine the free drug concentrations were found to be below the sensitivity of the spectrophotometrical assay, which lay in a range of $1 \cdot 10^{-4}$–$1 \cdot 10^{-3}$ M. Accordingly, the indicated molar ratios provide an estimate of the amount of drug bound to the phospholipids. A small amount of chlorphentermine induced an additional transition endotherm

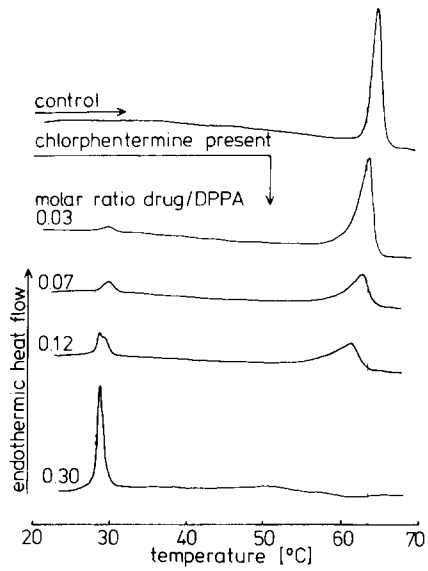

Fig. 1. Calorimetric heating curves of DPPA liposomes recorded under control conditions and in the presence of the cationic amphiphilic drug chlorphentermine. Abscissa: temperature of the sample; ordinate: energy uptake of the sample. Heating rate 5 deg. C/min. The amount of chlorphentermine present is indicated by the molar ratio of drug/DPPA (mol/mol).

at a temperature of about 28°C (Fig. 1), indicating that a fraction of the DPPA molecules melted at this temperature. With increasing molar ratio chlorphentermine/DPPA, the temperature of the drug-induced transition remained unchanged, but the transition peak grew, while the control transition signal declined in size, broadened and started at lower temperatures. Eventually, only the drug-induced transition endotherm was visible, suggesting that all DPPA molecules were more or less uniformly affected by the intercalated chlorphentermine-molecules.

The transition curves depicted in Fig. 1 essentially resemble the differential scanning calorimetry thermograms which Phillips et al. [20] obtained using liposomes composed of a mixture of two phosphatidylcholine lipids with different fatty acid chains and thus different T_t. Therefore, the present findings can be interpreted in analogy to the explanation given by Phillips et al. [20]. Thus, it is supposed that in the gel phase below a temperature of 28°C two kinds of domains within the DPPA membranes coexist, namely clusters of pure

DPPA molecules and clusters containing drug molecules. At the temperature of 28°C, the chlorphentermine-holding domains undergo the transition to the liquid crystalline state, so that fluid drug-containing DPPA regions exist together with rigid drug-free regions being in the gel state. The existence of fluid domains affects the arrangement of the rigid domains and induces melting of the drug-free domains at reduced temperatures.

Above this temperature, the fluid domains probably undergo complete mixing by the accelerated lateral diffusion of the phospholipid molecules; presumably the drug molecules are homogeneously distributed over the DPPA membranes. Upon cooling, however, the clusters separate again.

The fact that the absolute value of the drug-induced T_t was almost independent of the amount of drug present suggests that the concentration of drug molecules within the drug-containing domains remained constant; with increasing amounts of drug added, the drug-holding domains grew at the expense of the drug-free clusters. Accordingly, the temperature, at which the phase-transition occurred in chlorphentermine-containing DPPA regions could be determined from the drug-induced T_t at any amount of chlorphentermine tested.

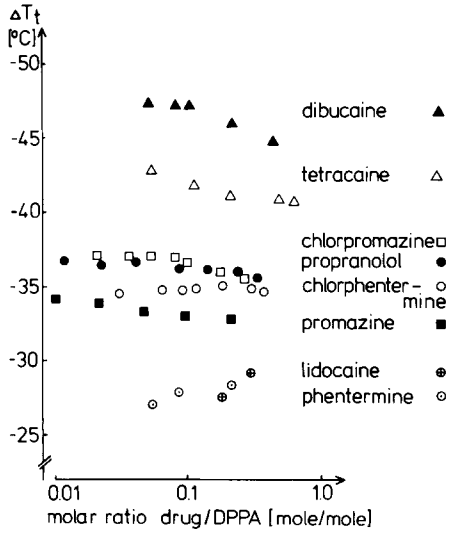

Fig. 2. Influence of various cationic amphiphilic drugs on the phase-transition temperature of DPPA liposomes. Ordinate: temperature range by which the drug-induced transition signal differed from T_t of the control signal (°C). Abscissa: amount of drug present given as the molar ratio drug/DPPA.

As described for chlorphentermine, each investigated cationic amphiphilic compound caused an additional drug-induced transition signal occurring at a distinct temperature; this is shown in Fig. 2, where the differences between the control T_t and the drug-induced T_t were plotted versus the molar ratio drug/DPPA. In case of some drugs like propranolol and promazine, ΔT_t was also nearly constant; in case of other drugs like dibucaine and tetracaine, the value of ΔT_t declined somewhat over the range of molar ratios studied, but compared with the absolute depression of T_t by about 45°C this shift of about 2 deg. C was only slight. On the whole, the drug-induced depression of T_t was more or less independent of the amount of drug added, but the various drugs reduced T_t to distinct levels characteristic for the individual drug; this suggests that the drugs possess a different potency to depress the phase-transition temperature of the drug-containing DPPA domains.

DPPA precipitated at molar ratios exceeding those indicated in Fig. 2, so that drugs which had to be applied in higher amounts, e.g., lidocaine or phentermine, could be investigated in a limited range of molar ratios only. However, since the drug-induced T_t was rather independent of the amount of drug added, the intrinsic potency to depress T_t could be estimated also in case of these drugs.

DPPG liposomes

The thermograms obtained with DPPG liposomes under control conditions exhibited a main transitional endotherm at a $T_t \approx 42$°C (x, $n = 16$), which was preceded by a minor pretransition signal occurring at 36°C. These data are in agreement with values reported by other groups [7,19].

Fig. 3 illustrates the influence of the neuroleptic chlorpromazine on the transition of DPPG liposomes. Even at low molar ratios, the pretransition endotherm disappeared. With increasing molar ratio, the onset temperature of the main transition signal declined. The transition signal broadened, revealed two distinct peaks and then narrowed again at a higher molar ratio, resembling the form of a control transition signal. This narrow signal suggests that the DPPG molecules were homogeneously affected by the incorporated chlorpromazine molecules; accordingly, the onset temperature

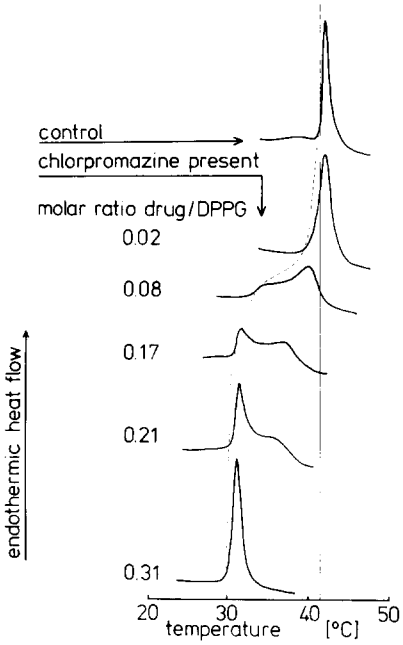

Fig. 3. Calorimetric heating curves of DPPG liposomes recorded under control conditions and in the presence of the cationic amphiphilic drug chlorpromazine. Abscissa: temperature of the sample; ordinate: energy uptake by the sample. Heating rate 5 deg. C/min. The amount of chlorpromazine present is indicated by the molar ratio of drug/DPPG (mol/mol).

of this signal indicated the T_t of the drug-containing DPPG membrane.

The double peak suggests the existence of two domains with distinct T_t values within the DPPG membranes; in analogy to the interpretation given for DPPA liposomes, these domains could consist of drug-containing and drug-free clusters, respectively. Due to the comparably small difference of about 12 deg. C between the drug-induced T_t and the control T_t, a mutual influence of the domains might occur thus explaining the variability and the confluence of the two signals. Alternatively, the separation in drug-containing and drug-free domains could be less clear-cut than in case of DPPA. It could be speculated, whether a homogeneous mixing between drug and lipid might occur at low molar ratios, thus explaining the absence of an additional drug-induced peak and indicating that the drug concentration within the lipid membrane might change. In any case, in the context of the present investigation its important that the narrow signal occurring at high molar ratios indicated the T_t of the membranes when being homogeneously affected by the drug molecules.

At higher dose-ratios of chlorpromazine and of other drugs, the peak broadened again, the transition temperature declined further, and the suspension became clear. Probably, high drug concentrations disrupted the lamellar structure of the DPPG membranes, the drugs thus acting as a detergent. In general, the effects of the investigated drugs on the transition endotherm of DPPG liposomes resemble those observed in case of DPPC liposomes [17]. Plotting the depression of T_t induced by chlorpromazine, chlorphentermine, propranolol, lidocaine and phentermine versus the respective molar ratios (Fig. 4) revealed the dose-dependent depression of T_t, which finally approached a plateau. The plateau corresponded to the narrow transition signal, thus reflecting the T_t of the drug-containing DPPG membranes. Since the different drugs attained different reductions of T_t at their respective plateaus, it has to be concluded that the various drugs differ with respect to their intrinsic potency to depress T_t of DPPG membranes.

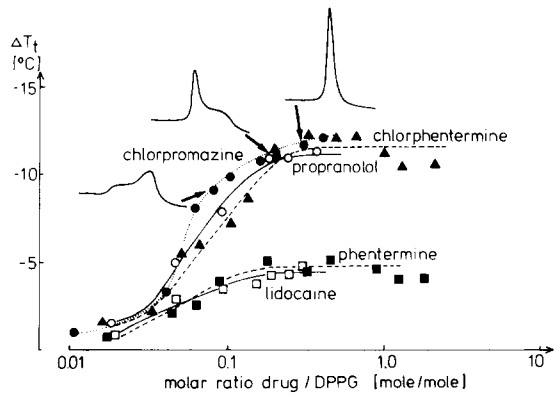

Fig. 4. Influence of the cationic amphiphilic drugs chlorpromazine (●), chlorphentermine (▲), propranolol (○), phentermine (■) and lidocaine (□) on the phase-transition temperature of DPPG liposomes. Ordinate: temperature range by which the onset temperature of the transition signal was depressed compared with the control T_t; abscissa: molar ratio drug/DPPG.

Discussion

Below the phase-transition temperature, the phospholipid molecules in the gel phase are packed in a highly ordered array with the hydrocarbon chains of the fatty acids in an all-*trans* conformation with a strongly restricted motional freedom. At the transition to the liquid crystalline state, the hydrocarbon chains gain more motional freedom, some C-C bonds adopt *gauche* conformations, the thickness of the bilayer decreases and the bilayer expands, since each hydrocarbon chain requires additional lateral space. Accordingly, the packing density in the region of the polar headgroups is thought to be loosened (for review see Ref. 21). In bilayers of phospholipids with given fatty acid chains, the phase-transition temperature can vary, depending on modifications of the structure of the polar headgroup region: Casal and Mantsch [22] reported for instance that the transition temperature of aqueous dispersions of methylated derivatives of dipalmitoylphosphatidylethanolamine (DPPE) declined with increasing methylation (Me) of the amino group, i.e., in the order DPPE > DPMePE > $DPMe_2PE$ > DPPC; furthermore, the transition temperature of phospholipid membranes was shown to be affected by the binding of divalent cations to the surface of the bilayer [7,19,23]. Since the region of the polar headgroups provides a favorable environment for cationic amphiphilic molecules, they intercalate and thus influence the phase-transition temperature of phospholipid bilayers. The results obtained in the previous study on the influence of cationic amphiphilic compounds on the T_t of DPPC liposomes led to the conclusion that the various drugs had a different potency to depress the transition temperature of the DPPC membrane [17]. Similarly, the transition temperature of DPPA liposomes and DPPG liposomes was affected by the cationic amphiphilic drugs to a different extent. In order to compare the effectiveness of the drugs in the various phospholipid membranes, the effects of five cationic amphiphilic drugs on the T_t are compiled in Table I. In spite of the fact that T_t of DPPA liposomes under control conditions was 20°C higher than in DPPG and DPPC liposomes, the corresponding drug-induced T_t values attained similar levels in DPPA, DPPG and DPPC. Some drugs (chlorpromazine, propranolol, chlorphentermine) exerted a stronger, some (phentermine, lidocaine) a weaker depressant action; this rank order of potency was independent of the phospholipid under investigation.

TABLE I

EFFECT OF DIFFERENT CATIONIC AMPHIPHILIC DRUGS ON THE PHASE-TRANSITION TEMPERATURE T_t OF LIPOSOMES FROM DPPA, DPPG and DPPC

The drug-induced T_t values correspond to the data shown for DPPA in Fig. 2, for DPPG in Fig. 4 and for DPPC in Ref. 17. Note that the drug-induced T_t values were similar irrespective of the phospholipid, while the control T_t value of DPPA was about 20°C higher than the T_t values of DPPG and DPPC.

	Phase-transition temperature (°C)		
	DPPA	DPPG	DPPC
Control	63	42	41
Phentermine	36	37	36
Lidocaine	35	38	36
Chlorphentermine	29	31	32
Propranolol	27	31	32
Chlorpromazine	27	30	29

The drug-induced T_t values attained in DPPA differed by a few degrees C from those in DPPG and DPPC; however, in view of the impressive difference between the control T_t values of DPPA and the T_t of DPPG and DPPC, these differences in T_t appear strikingly small. So one is tempted to conclude that the intercalation of drug molecules nearly eliminated the specific influence of the DPPA headgroups, this influence being responsible for the high control T_t of this phospholipid. In the three investigated phospholipids, the drug-induced T_t values were similarly dependent on the species of the added drug. Recent experiments indicate that the different drug-induced T_t values results from a different intrinsic potency of the drugs to depress T_t; i.e., at a given concentration within the phospholipid membrane the various drugs reduce T_t to different levels [24].

Results of Blume and Eibl [16] provide another example that the specific influence of the polar headgroups on T_t of a phospholipid membrane can be eliminated by a modification of the headgroup region. They measured T_t of liposomes prepared from 1,2-dipentadecylmethylidene phospholipids with various headgroups. At neutral pH, T_t values

declined in the other of phosphatidic acid headgroup > phosphatidylethanolamine headgroup > phosphatidylcholine headgroup; at pH 12, however, all phospholipids had a very similar T_t regardless of the different polar headgroups. The authors conclude that phospholipids with identical hydrocarbon chains have similar T_t when no intermolecular hydrogen bonding is possible. If the action of cationic amphiphilic drugs on T_t of phospholipid membranes is also interpreted in this way, it may be concluded that the intercalated drug molecules may interrupt hydrogen-bonds spanned between adjacent headgroups and thus destroy the specific structural arrangement of a specific polar headgroup region. Accordingly, the cationic amphiphilic drugs equalize the influence of different polar headgroups on the temperature at which the array of phospholipid molecules is loosened and the membrane undergoes the gel to liquid-crystalline transition.

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