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DIFFERENTIAL INFLUENCE OF ANIONIC AND CATIONIC CHARGE ON THE ABILITY OF AMPHIPHILIC DRUGS TO INTERACT WITH DPPC-LIPOSOMES

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Abstract—The compounds clofibric acid and chlorphentermine have identical aromatic ring systems, but when charged their side chains are anionic or cationic, respectively. The drugs were applied as tools to investigate whether the interaction of amphiphilic drugs with the zwitterionic dipalmitoylphosphatidylcholine (DPPC) depends on the charge of the polar side chain. In suspensions of DPPC-liposomes, the drug-effect on the phase-transition temperature (T_t) was evaluated by means of differential scanning calorimetry. The drug-binding was determined spectrophotometrically. The clofibric acid anion had a much weaker depressing effect on T_t than the chlorphentermine-cation and a considerably lower ability to bind to the DPPC-liposomes. Furthermore, a plot of the effect versus the binding suggested that the clofibric acid-anion had a lower intrinsic activity to reduce T_t compared with the chlorphentermine-cation. In contrast, when the dissociation-equilibrium was shifted towards the uncharged state both drugs were indistinguishable with respect to effect and binding, suggesting that the differences observed with the charged forms could indeed be attributed to the opposite charges. The findings are tentatively explained to result from a different ability of the anionic and the cationic form to reach the hydrophobic interior of the DPPC-bilayer.

Some of the effects of cationic amphiphilic drugs have been related to their ability to interact with phospholipid-bilayers, e.g. a local anaesthetic effect [1–3], an antiarrhythmic action [4], and the induction of lysosomal phospholipid storage [5]. Catamphiphilic compounds are characterized by an aromatic ring system contributing lipophilia and a side chain with a protonized amino group providing cationic hydrophilia. One of the means to study the interaction of these drugs with artificial phospholipid bilayers is to measure the drug-induced reduction of the temperature (T_t) at which phospholipid-bilayers undergo the transition from the gel to the liquid crystalline state. This is often investigated in liposomes of phosphatidylcholine (lecithine) [e.g. 6–9]. With its cationic quaternary ammonium and its anionic phosphate the phosphatidylcholine-headgroup is zwitterionic. The question arises whether the ability to reduce T_t is affected, if an amphiphilic drug carries an anionic instead of a cationic charge.

In an attempt to clarify this point, a comparative investigation was done applying as tools clofibric acid and chlorphentermine. Both drugs have an almost identical structure but carry opposite charges at the appropriate pH. Clofibric acid (2-[4-chlorophenoxy]-2,2-dimethyl-ethanoic acid) contains a carboxylic group (pK: 3–5 [10]) and is anionic amphiphilic in the dissociated form. Chlorphentermine (2-[4-chlorophenyl]-1,1-dimethyl-ethylamine) carries an amino group (pK: 10 [11]) and is a typical representative of the catamphiphilic compounds. In liposomes of dipalmitoylphosphatidylcholine (DPPC) the ability of both drugs to reduce T_t was recorded with the method of differential scanning calorimetry. For an estimation of the intrinsic activities to affect T_t, drug-binding to the liposomes was determined in parallel and binding–effect curves were evaluated. Effect and binding were also measured under conditions shifting the dissociation equilibrium towards the uncharged forms of the drugs by adjusting the pH of the liposome suspensions. DPPC offers the advantage that the transition temperature is almost independent of the proton concentration at pH > 4 [12]. Part of the results was previously presented in abstract form [13].

METHODS

The preparation of liposomes and the calorimetric determination of the phase-transition temperature was essentially performed as described previously [8, 9].

Preparation of liposomes. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC, purity >99%, the Sigma Chemical Co., München, F.R.G.) was used in an amount of 5 mg per 100 μL of buffer solution. Depending on the amount required per sample, the phospholipid was applied either dissolved in chloroform or directly weighed into the vial. Appropriate amounts of clofibric acid (the Sigma Chemical Co., München, F.R.G.) or chlorphentermine–HCl (Tropon-Werke, Köln, F.R.G.), depending on the solubility, were either added dissolved in chloroform or dissolved in the incubation buffer. When chloroform was used as the solvent, pipetting steps were performed at 6°. Thereafter, the solvent was evaporated at room temperature under a stream of nitrogen for 4 hr. The resulting

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precipitate was further dried under vacuum overnight.

For liposome preparation, aqueous buffer was given at a volume of 100 μL per 5 mg DPPC into the vials, which contained the dry DPPC or DPPC/drug-mixture, respectively (see above). For the acidic pH values 14 mM TES*/histidine was used as the buffer solution. It was adjusted with HCl either to pH 6 in order to obtain chlorphentermine as the cation or to pH 4.5–5 in case of clofibric acid to shift its dissociation equilibrium towards the uncharged form. For the alkaline pH values a boric acid/KCl/NaOH-buffer (Standard Buffer pH 11, Merck, Darmstadt, F.R.G.) was used. In case of clofibric acid even at the highest applied amounts the pH did not fall below pH 9, i.e. the drug was almost entirely present in the anionic form. In case of chlorphentermine–HCl appropriate amounts of NaOH were added to the buffer solution to yield a pH ≥10, i.e. the dissociation equilibrium of chlorphentermine was shifted in favour of the uncharged form. The indicated pH values were determined at room temperature after the liposome suspension had been prepared (contact electrode 403-M8, Ingold, Steinbach, F.R.G.).

The stoppered vials were heated in a water bath at 50°, i.e. above the transition temperature of DPPC. Every 15 min, the vials were vigorously shaken for 10 sec on a Vortex Genie 2™-mixer (Bender & Hobein, Zurich, Switzerland). After 2 hr of incubation, the samples were further processed for determination of the transition temperature or for measurement of the drug-binding.

Determination of the transition temperature. The transition temperature was determined by means of differential scanning calorimetry applying a DSC-2C/intracooler II equipment (Perkin Elmer, Überlingen, F.R.G.). Ten microlitres of the suspension were encapsulated in an aluminium pan (Perkin Elmer) and measured against a reference containing 10 μL of buffer. The samples were heated from 17° to 47° at a rate of 5°/min. The sensitivity range was set to 0.5 mcal/sec. The temperature scale was calibrated using as standards cyclohexane and indium; the calibration was checked every day. Calibration was repeated, when the actual transition temperature deviated from the control values by 0.3° and/or when the interval between the two transition temperatures deviated by more than 0.5° from the true value. The transition temperature T_t was evaluated as follows: a straight line was fitted to the upward deflection of the transition signal; the intersection of this line with the (extrapolated) baseline was connected by a perpendicular with the temperature axis, thus indicating the onset temperature of the transition.

Determination of drug-binding to the liposomes. Three hundred microlitres of the liposome-suspension prepared as described above were given in polycarbonate centrifugation tubes (Beckman) and centrifuged at 356,000 *g* (rotor TL100.1 at 100,000 rpm in a Beckman TL100-centrifuge) for 1 hr at 20°. From the clear supernatant, an aliquot was taken for the determination of the free drug concentration by spectrophotometry (model 3600, Beckman). The spectrophotometry was performed at the wavelength yielding maximum absorption (for clofibric acid 278 nm, for chlorphentermine 266 nm). The amount of drug bound was calculated as the difference between the total drug concentration introduced into the sample and the free concentration found in the supernatant. In order to check the recovery of the applied drug, DPPC-free samples were run in parallel; the drug-concentration found after centrifugation corresponded to the initially introduced concentration. For each amount of drug added, three separate samples were prepared and assayed. Indicated are the mean values of the triplicate determinations; the deviation of a single value from the mean value amounted to less than 10% of the mean.

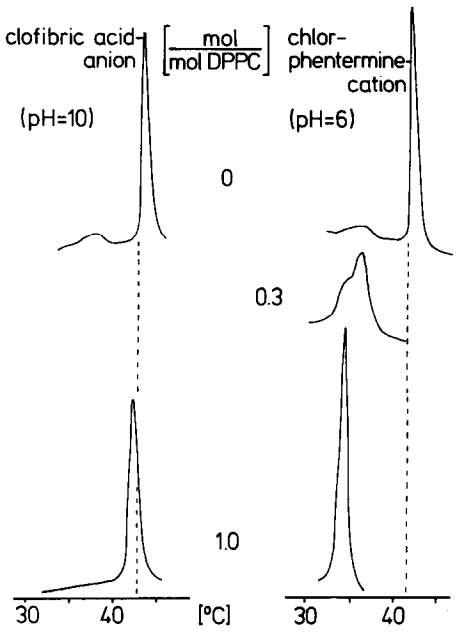

Fig. 1. Differential scanning calorimetry recordings of the effect of the clofibric acid-anion and the chlorphentermine-cation on the phase-transition temperature T_t of DPPC-liposomes. Ordinate: endothermic heat flow, abscissa: temperature of the samples. The strippled line indicates the onset temperature of the main transition signal. Under control conditions this signal is preceded by the small pretransition signal; in the presence of the drugs the pretransition signal had vanished. The added amount of drug is quantified as mol of drug per mol of DPPC.

RESULTS

Effect on the phase-transition temperature

Differential scanning calorimetry recordings obtained in experiments with the clofibric acid-anion and the chlorphentermine-cation are depicted in Fig. 1. In the absence of a drug, the onset temperature of the main transition signal occurred at T_t ~42° at the applied pH ranging from pH 4.5 to 12. This value corresponds to data reported in the literature [6–9, 12, 14].

* TES = *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid.

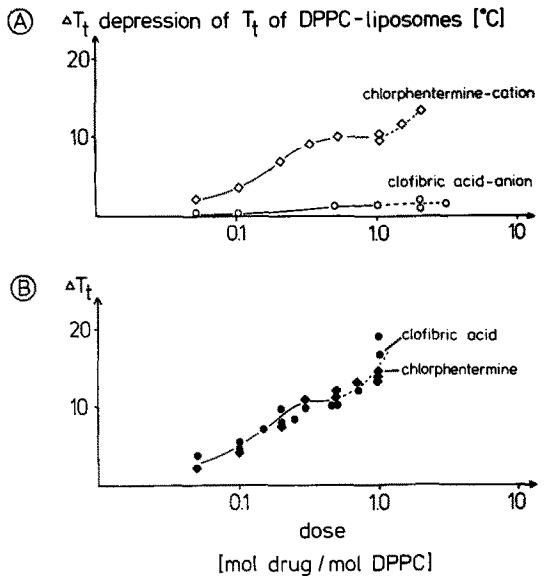

Fig. 2. Dependence of the drug induced reduction of the transition temperature ΔT_t (ordinate) on the amount of drug added (abscissa). ΔT_t is the difference between the T_t under control conditions and T_t obtained in the presence of drug. The dissociation-equilibrium of the drugs was shifted to the indicated forms by adjusting the pH of the liposome-suspension. (A) Dose effect curves of the charged forms (pH = 6 for chlorphentermine-cation, pH ≥ 9 for clofibric acid-anion). (B) Dose effect curves of the uncharged forms (pH ≥10 for chlorphentermine, pH 4.5–5 for clofibric acid). The points represent the results of single determinations. The stippled lines indicate the drug amounts at which morphological alterations occurred in the liposome-suspensions.

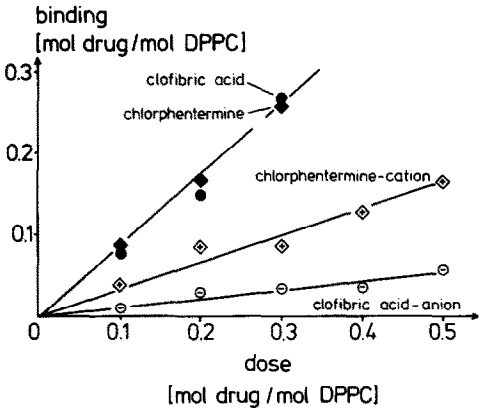

Fig. 3. Liposomal binding of the drugs (ordinate) in dependence on the total amount of drug added to the liposome suspension (abscissa). Indicated are the mean values of triplicate determinations. The maximum deviation of a single value from the mean was less than 10% of the mean.

The clofibric acid-anion, even at an amount of 1 mol per mol of DPPC, induced only a marginal reduction of the T_t amounting to $\Delta T_t \sim 1°$ (Fig. 1). The respective dose effect curve is depicted in Fig. 2A. At molar ratios exceeding 1 mol of drug per mol of DPPC, precipitation of the drug occurred. The chlorphentermine-cation reduced the main transition to a considerable extent (Figs 1 and 2A). The accompanying characteristic alteration of the transition signal has been described and discussed in detail previously [8]. The dose effect attained a plateau between 0.5 and 1.0 mol/mol indicating a reduction of T_t by $\Delta T_t \sim 10°$. This value corresponded to the value found in the previous investigation [8]. At higher molar ratios than 1 mol/mol, the drug exerted a detergent effect and dissolved the liposomes.

When the pH of the liposome suspension was set as to shift the equilibrium towards the uncharged form of the drugs, clofibric acid revealed a strong effect on the phase-transition; the dose–response curve for the reduction of T_t is depicted in Fig. 2B. It matched the dose–effect curve obtained with the uncharged chlorphentermine. Between 0.3 and 0.5 mol/mol, both curves reveal a plateau, at which the reduction of T_t amounted to $\Delta T_t \sim 10°$. At molar ratios exceeding 0.5 mol/mol, precipitation occurred.

A comparison of the dose–effect curves shown in Fig. 2A and B indicates that the potency of chlorphentermine was almost independent of whether it was cationic or uncharged. In contrast, in case of clofibric acid, a negligible effect of the anionic form turned into a pronounced effect, undiscernible from that of chlorphentermine, when the drug attained the uncharged state.

Binding of the drugs

Under conditions as applied in the calorimetric measurements, the binding of the drugs to the liposomes was investigated up to the molar ratios, at which the plateaus had occurred in the dose–effect curves (Fig. 3). Within this dose range, binding was proportional to the dose or the concentration, respectively.

The highest binding was obtained under the conditions to yield the uncharged drugs: both, clofibric acid and chlorphentermine were bound to about 80% of the amount of drug added to the assay. When the pH was set to shift the drugs to the charged state, the ability to bind was affected to a different extent. In case of the clofibric acid-anion the amount of bound drug fell to only about 10% of the added amount. In case of the chlorphentermine-cation binding declined to about 30% of the added amount.

DISCUSSION

When present in the charged forms, clofibric acid had a much lower ability to reduce the phase-transition temperature of DPPC-liposomes than chlorphentermine. Both drugs possess an identical aromatic ring system and a very similar side chain; the main difference lies in the anionic carboxylic group of clofibric acid and the cationic amino group of chlorphentermine. Under conditions to yield the uncharged forms, however, both drugs reduced T_t to the same extent. Thus it can be concluded that in case of the charged forms not the different substituent

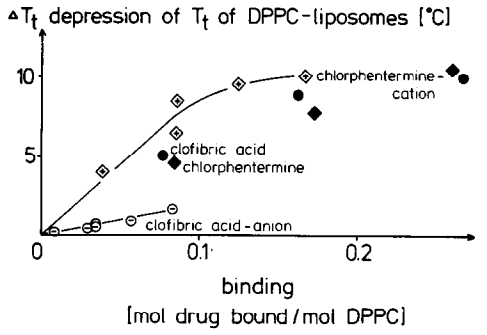

Fig. 4. Reduction of the transition temperature (ordinate) in dependence on the liposomal drug binding (abscissa).

(carboxylic vs amino group) but the opposite charge accounts for the different activities to affect T_t.

The extent by which the transition temperature is reduced by a drug may depend on the concentration of drug within the phospholipid-bilayer, i.e. the extent of binding [8, 15], and/or on the intrinsic ability of the intercalated drug molecules to perturb the arrangement of the adjacent phospholipids [4, 9]. In order to discriminate between these two possibilities, the binding of clofibric acid and chlorphentermine was determined.

The highest ability to bind to the DPPC-liposomes was seen with the uncharged drugs. This finding is in keeping with results reported for the amphiphilic drugs tetracaine and dibucaine, the uncharged forms of which have a higher ability to bind to phosphatidylcholines than the charged, cationic amphiphilic forms [16–18]. The reason may be that neither the uncharged nor the charged form is attracted electrostatically by the zwitterionic phosphatidylcholine, whereas the explusion from the water into the lipid membrane is much higher with the non-polar uncharged forms. In the present context, it should be noted that the equal binding of the uncharged forms of clofibric acid and of chlorphentermine supports the notion that the zwitterionic DPPC cannot discriminate between the molecular structures of the two drugs as long as they are uncharged. However, with the occurrence of the opposite charges binding declines to a different extent; the binding ability of the clofibrate acid-anion then amounts to only one third of the binding ability of the chlorphentermine-cation.

The different ability of the clofibric acid-anion and the chlorphentermine-cation to reduce T_t may thus be attributed to a different binding ability. Yet, an additional factor has to be considered, as is obvious from a plot of the reduction of T_t versus the amount of drug bound to the DPPC (Fig. 4). The binding-effect curve of the clofibric acid-anion lies much lower than the respective curve for the chlorphentermine-cation; at a given level of binding, the clofibric acid-anion induced a smaller reduction of the phase-transition temperature. This finding suggests that a clofibric acid-anion possesses a weaker intrinsic activity to reduce T_t than a chlorphentermine-cation. This result supports the hypothesis put forward previously [4, 9] that amphiphilic drugs bound to phospholipid-bilayers may differ with respect to their intrinsic activity to perturb the structural arrangement of the bilayer. Furthermore, it may be concluded from Fig. 4 that the intrinsic activities of the uncharged forms of clofibric acid and chlorphentermine are identical and close to the intrinsic activity of the chlorphentermine-cation.

The finding that the ability to bind to DPPC and to depress its transition-temperature strongly depends on the charge of an amphiphilic compound may be tentatively explained considering the location of the drug within the phospholipid-headgroup region. The location of a charged drug will be governed by the electrostatic interaction with the charged groups of the phosphatidylcholine headgroups. The cationic amino group of cat-amphiphilic drugs is known to lie next to the anionic phosphate group of the phospholipid [19–22]. The anionic carboxylic group will be repelled by the phosphate and attracted by the cationic choline group of the phospholipid. The phosphate group can be assumed to be closer to the hydrophobic interior than the choline amino group. Accordingly, the clofibric acid-anion will be kept further from the fatty acid region than the chlorphentermine-cation. This location would explain its lower intrinsic activity to reduce T_t [4, 9]. This location could also result in a weaker hydrophobic interaction than in case of the chlorphentermine-cation and would thus explain the lower binding ability of the clofibric acid anion. With the loss of the negative charge, clofibric acid would no longer be restrained in the outer regions of the lipid headgroup and could reach a location similar to that of the uncharged chlorphentermine.

Whether or not this hypothesis is true, the results of the study indicate that a negatively charged amphiphilic drug may have a considerably lower potency to interact with the zwitterionic phosphatidylcholine than a similarly structured, but positively charged amphiphilic drug.

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