Eur Biophys J (2002) 31: 554–558
DOI 10.1007/s00249-002-0244-0

BIOPHYSICS LETTER

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Effects of membranotropic agents on mono- and multilayer structures of dipalmitoylphosphatidylcholine

Received: 1 December 2001 / Revised: 31 May 2002 / Accepted: 1 July 2002 / Published online: 22 August 2002

Abstract We have studied the action of some membranotropic agents (MTAs) on the parameters of mono- and multilayers of dipalmitoylphosphatidylcholine (DPPC). The MTAs used included an antimicrobial drug, decamethoxinum, the model amphiphilic agent stearoyl-L-α-alanine, and cholesterol as a reference substance. Using differential scanning calorimetry and the Langmuir monolayer technique, we measured the temperature and enthalpy of the main phase transition of DPPC, the mean molecular area, the collapse pressure and the free energy of the mixed monolayers of DPPC and MTA. A good correlation has been obtained between the structure of the MTA used and changes in the parameters of both mono- and multilayers. Thus, for cholesterol, its well-known condensing effect in the L_α phase correlates with its behavior in the mixed monolayers. The disturbing action of decamethoxinum (depression of the phase transition in DPPC multilayers and relatively high free energy of mixing in monolayers) is presumably connected with interaction of its charged ammonium moieties with polar phospholipid heads. At the same time, stearoyl-L-α-alanine condensed the lipid layers and increased the melting point of DPPC, owing to its interaction with both polar and non-polar lipid moieties. One can conclude that the three MTAs used can really be considered as representative examples of three different types of behavior in mono- and multilayers.

Keywords Dipalmitoylphosphatidylcholine · Membranotropic agents · Differential scanning calorimetry · Langmuir monolayers

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Abbreviations *DSC*: differential scanning calorimetry · *DPPC*: 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine · *MTA*: membranotropic agent

Introduction

It is commonly known that cell membranes possess some extremely important features resulting from the supramolecular ordering of their lipids, or the phase state of lipid bilayers (Jones 1979; Kelker and Hatz 1980; Seddon and Templer 1995; Goodby 1998). This phase state depends upon a great number of factors such as media temperature and pH, degree of lipid hydration, lipid composition, presence of exogenic molecules, etc. The phase state of the bilayers defines, in turn, such properties of membranes as microviscosity, non-specific permeability, conformation of membrane proteins, etc. (Kagawa 1978). So, it was of interest to pay more attention to the ordering effects in membranes related to their phase state and molecular packing.

There have been a considerable number of investigations concerning effects of various agents on lipid phase state. These studies have been principally concerned with lipid-protein interactions (e.g. Ben-Tal et al. 1996; Epand 1998; Gil et al. 1998; Marsh and Horvath 1998; Sabra and Mouritsen 1998; White and Wimley 1998; Mozsolits et al. 2001) and lipid-lipid interactions (e.g. Seddon 1996; Lemmich et al. 1997; Bhattacharya and Saubhik 2000; Campbell et al. 2001) as well as drug action (e.g. Reig et al. 1989; Albertini et al. 1992; Betrencourt et al. 1999; Micol et al. 2001). In many cases, it becomes possible to establish the true membranotropic mechanism of a given molecule, and a huge amount of interesting effects has been found. As a general conclusion to be drawn from these data, the action of a given molecule is defined by its geometrical shape, variation of hydrophobicity along the molecule, dipole moment, localization of charged moieties, etc., rather than its detailed chemical structure. Important examples are antimicrobial peptides that possess

membranotropic properties similar to those of non-peptide drugs (e.g. Epand and Vogel 1999; Sitaram and Nagaraj 1999). However, as far as we know, no general correlations involving different chemical classes have been made yet. In the present paper, an attempt has been made to view the data on membranotropic activity properties just from the "structural" point of view.

In this work we studied the influence of different substances with model membranes (membranotropic agents, MTAs) on their liquid crystalline supramolecular ordering, as well as on their structural and functional properties. As a base for our model membranes, we have chosen one of the most widely used lipids, dipalmitoylphosphatidylcholine. The foundations for our choice of membranotropic agents will be given in the next section.

Experimental

As a base for model membranes, we used 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC), a neutral phospholipid with hydrophobic chains of medium length, purchased from Biolek (Ukraine). The substances used as membranotropic agents (cholesterol, decamethoxinum and stearoyl-L-α-alanine) were purposely selected as bearers of certain molecular structure features, with which different effects on multi- and monolayer behavior could be expected. Cholesterol was selected as a "reference substance", with its behavior as a phospholipid membrane component described in a number of papers (Hinz and Sturtevant 1972; Ivkov and Berestovskii 1981; Krull et al. 1985; Ipsen et al. 1990; Bhattacharya and Saubhik 2000). Decamethoxinum, with its molecules consisting of two polar moieties, the linked hydrocarbon chain and two side radicals (Fig. 1), is known as an antimicrobial drug (Mashkovskii 1978), with the mechanism of its pharmacological action presumably related to its interaction with cell membranes. Stearoyl-L-α-alanine, the ester of L-α-alanine and stearic acid, has been synthesized as a model substance, providing localization of the amino acid residue in the hydrophilic part of the lipid monolayer with its hydrocarbon chain located in the hydrophobic part.

Monolayer studies were carried out using a Langmuir trough produced by NIOPIK (Russia). The trough contained two independent Teflon sections, of 2000 cm^3 volume each. The surface tension was registered by a Wilhelmy balance. The set was controlled by a computer, yielding digital data of surface pressure π (0.1% precision) and working surface area A (0.1% precision).

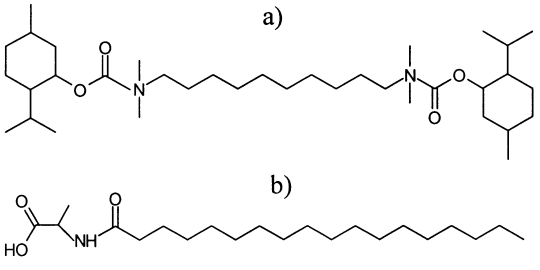

Fig. 1 Molecular structures of substances used: (a) decamethoxinum, a molecule containing a flexible hydrocarbon chain linking two polar ammonium groups and two cyclic hydrocarbon side groups; (b) stearoyl-L-α-alanine, the ester of alanine and stearic acid. Its structure provides localization of the amino acid residue in the hydrophilic part of the lipid monolayer, whereas its hydrocarbon chain sets into the hydrophobic part

As a subphase, distilled and deionized water was used at pH 6.5. In order to form monolayers, the systems investigated were dissolved in chloroform (5×10^{-4} M). The surface layer deposited on the subphase was compressed (3.2×10^{-3} m s^{-1}) at 20 °C.

The phase transitions parameters of DPPC multilayers containing MTA were obtained by differential scanning calorimetry (DSC) using a Mettler TA 3000 thermoanalytical system. To obtain mulitlayer lamellar composition, all compounds were dissolved in chloroform and mixed in the appropriate amounts. The solvent was removed using rotational evaporation under nitrogen flow, and the necessary amount of water was added. Full saturation with water was reached after keeping for 4–5 days at 0–5 °C. Samples of 15–25 mg were placed in aluminum pans and repeatedly heated and cooled within the range 15–50 °C.

Results

In Table 1 the collapse pressure for each of the pure monolayers is given, as well as the value of mean molecular area, $<A>$. It can be concluded from the comparatively high value of the collapse pressure that each of the MTAs, as well as DPPC itself, forms rather stable pure monolayers at the air/water surface.

The results for mixed DPPC/MTA monolayers are given in Table 2. Intermolecular phospholipid/MTA interactions caused the $<A>$ values for the mixed monolayers not to correspond to the concentration-weighted sums of appropriate $<A>$ values for the pure monolayers. To show this difference more clearly, the third data column of Table 2 is given. It can be concluded from these data that neither stearoylalanine nor cholesterol exhibits significant interaction with a lipid monolayer, in contrast to decamethoxinum which causes substantial deviations.

It should be noted that non-linear behavior of mean molecular area $<A>$ of the mixed monolayers was observed not only as a function of concentration, but also as a function of surface pressure. We obtained significant deviation from additive values, $\delta A(\pi)$, for all the monolayers investigated, calculated as:

$$\delta A(\pi) = A_{\mathrm{LD}}(\pi) - cA_{\mathrm{D}}(\pi) - (1-c)A_{\mathrm{L}}(\pi) \qquad (1)$$

where A_{L} and A_{D} are the mean molecular areas in the pure lipid and MTA monolayers, A_{LD} is the mean molecular area in the mixed monolayer, and c is the molar concentration of MTA in a mixed monolayer.

The $\delta A(\pi)$ dependence obtained (Fig. 2) proved to be largely determined by the MTA structure and the nature of the lipid/MTA interactions. Thus, certain small deviations observed with cholesterol at low-pressure values

Table 1 Parameters for pure Langmuir monolayers

Substance	$<A>$ (nm^2)	Collapse pressure (mN m^{-1})
DPPC	0.68	38
Cholesterol	0.50	43
Stearoyl-L-α-alanine	0.46	50
Decamethoxinum	0.55	24

Table 2 Parameters for mixed Langmuir monolayers

Composition	$< A >$ (nm^2)	Collapse pressure, ($mN\ m^{-1}$)	Mean deviation of $< A >$ from linearity by concentration (%)
DPPC/cholesterol	0.64	38	–2.9
DPPC/stearoyl-L-α-alanine	0.59	43	–10.2
DPPC/decamethoxinum	0.77	35	+15.5

disappear when the surface pressure is increased. The condensing effect of cholesterol is widely found in the literature (Krull et al. 1985; Reig et al. 1989; Serfis et al. 2001) and results from hydrophobic interactions between the steroid molecule and the hydrophobic phospholipid core. For stearoylalanine, on the contrary, the mean molecular area in mixed monolayers decreases with pressure as compared with the corresponding value for a mixture of non-interacting molecules.

We suppose that, owing to the monolayer architecture, the main part of the stearoylalanine/DPPC interaction takes place in the polar moieties, while the stearoyl residue of stearoylalanine seems to be not affecting the liquid crystalline ordering of the phospholipid alkyl chains. So, this effect is likely to result from formation of H-bonds between the alanine residues and polar lipid head groups.

A common feature of all the samples studied is approximately linear pressure dependence of δA over a wide range of π values:

$$\delta A(\pi) \approx \delta A(0) + k\pi \qquad (2)$$

where $\delta A(0)$ is the deviation in the absence of the surface pressure, and k is a coefficient determined by the average slope of the $\delta A(\pi)$ plot.

The values of $\delta A(\pi)$ depend sensitively on the structure of MTA. Thus, for monolayers containing cholesterol and stearoylalanine, k is rather small (–3.3 and –8.5 $nm^2\ N^{-1}\ m^{-1}$, respectively) and $\delta A(0)$ ranges between 0 and 0.1 nm^2. Taking into account the sign of δA for each of the substances, one can see that, for decamethoxinum, δA decreases with pressure, while for stearoylalanine it increases.

In the presence of decamethoxinum (Fig. 2, curve 3), $\delta A(0)$ is much larger [an approximation to zero gives $\delta A(0) = 0.80\ nm^2$], and $k = -29.7\ nm^2\ N^{-1}\ m^{-1}$. From the physical point of view, it implies a significant compressibility of the monolayer combined with its low density. Such behavior may be due to peculiar features of the decamethoxinum molecule (see Fig. 1): two ammonium atoms are linked with a flexible hydrocarbon chain. Under low pressure, the chain is maximally extended, and the distance between the two positively charged moieties is the largest. In such a conformation the molecule possesses a large hydrate shell that could push apart the neighboring lecithin molecules, leading to a significant increase in the molecular cross-area value. It seems obvious that under pressure the linking chain becomes bent and the charged moieties approach each other, so δA decreases (the limit value is $\sim 0.05\ nm^2$). This value is determined by interaction of the ammonium atoms with phospholipid headgroups and the two charged parts.

Based on the π-A diagrams obtained, values were calculated for the free energy of mixing in the monolayers. The free energy of mixing, $\Delta G_{\mathrm{mix}}(\pi)$, can be defined as (Labauff and Zack 1971):

$$\Delta G_{\mathrm{mix}}(\pi) = \int_0^{\pi} \delta A(\pi)\mathrm{d}\pi = \int_0^{\pi} (A_{\mathrm{LD}} - (1-c)A_{\mathrm{L}} - cA_{\mathrm{D}})\mathrm{d}\pi \qquad (3)$$

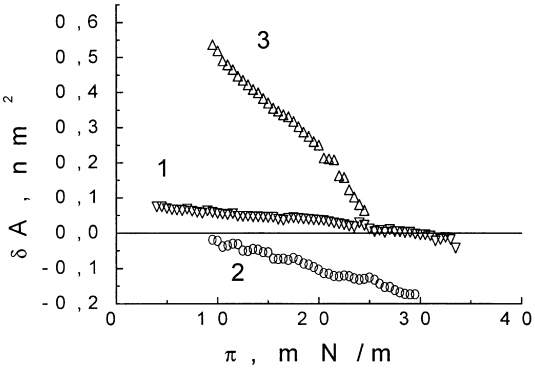

Fig. 2 Deviations of mean molecular area from additive value in mixed monolayers as a function of surface pressure, calculated by Eq. 1 (see text). *1*: DPPC/cholesterol (84:16 mol/mol); *2*: DPPC/stearoyl-L-α-alanine (89:11 mol/mol); *3*: DPPC/decamethoxinum (78:22 mol/mol). The *horizontal line* marks the zero pressure value. For monolayers *1* and *3*, the deviation almost disappears under pressure, and the systems transform into "mechanical mixtures"; for monolayer *2*, a condensing effect with pressure is observed

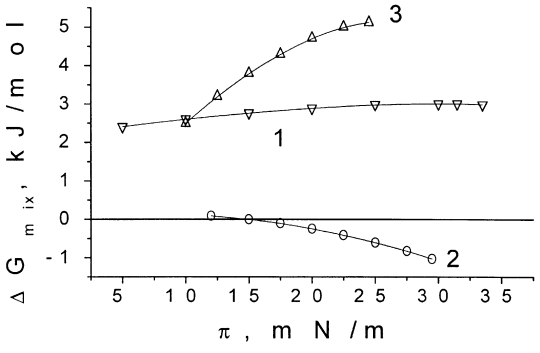

Fig. 3 Free energy of mixing in DPPC monolayers containing: *1*, cholesterol (0.16 M); *2*, stearoyl-L-α-alanine (0.11 M); *3*, decamethoxinum (0.22 M), calculated according to Eq. 3 (see text). The zero value of the free energy is marked by the *horizontal line*. As is clear from the figure, DPPC mixing with cholesterol and decamethoxinum is not energetically favorable, while DPPC and stearoyl-L-α-alanine are miscible with some energy gains

Table 3 Temperatures and enthalpies of the phase transitions in hydrated DPPC doped with MTA

Composition of organic component	Transition to L_α phase			Transition $L_{\beta'}$-$P_{\beta'}$	
	T_m (°C)	ΔH_m (kJ kg^{-1})	ΔT_m (°C)	T_∞ (°C)	ΔH_c (kJ kg^{-1})
DPPC	42.3 ± 0.4	50.0 ± 4.7	2.2 ± 0.5	36.5 ± 0.6	4.7 ± 1.7
DPPC/cholesterol	41.1 ± 0.3	20.2 ± 1.6	6.5 ± 0.5	–	–
DPPC/stearoyl-L-α-alanine	44.2 ± 0.2	40.6 ± 2.2	2.9 ± 0.1	–	–
DPPC/decamethoxinum	35.8 ± 0.1	54.0 ± 0.1	2.2 ± 0.1	–	–

where A_L and A_D are the mean molecular areas in the pure lipid and MTA monolayers, A_{LD} is the mean molecular area in a mixed monolayer, and c is the molar concentration of MTA in the mixed monolayer.

Surface pressure dependencies of ΔG_{mix} (Fig. 3) show that for all the samples studied the free energy is increased under pressure. Thus, for decamethoxinum, comparatively high (3–5 kJ mol^{-1}) positive ΔG_{mix} values were obtained, which substantially increased with pressure, corresponding to its strong disturbing effect on the lipid structure. As for stearoyl-L-α-alanine, comparatively low ΔG_{mix} values (0.1–1.0 kJ mol^{-1}) were obtained, and dependence upon pressure was weak. For cholesterol, ΔG_{mix} values at low pressures were comparable to those for decamethoxinum, but in this case ΔG_{mix} practically did not increase with pressure.

It is of interest to compare the monolayer technique results with thermodynamic parameters for water dispersions of DPPC, such as temperatures and enthalpies of pre- and main transition peaks (Table 3). We certainly realize that direct comparison of physical parameters of multi- and monolayers is not strictly correct (though in some cases it was successfully made, e.g. Albrecht 1978), but we assume such comparison would be reasonable to qualitatively assess the effects of MTA introduction. As one can see from Table 3, the three MTAs show significantly different behavior in DPPC multilayers. The only common feature is the disappearance of the pre-transition peak ($\Delta H_p \rightarrow 0$). From comparison with Fig. 3 one can also conclude that the increase in δA values under pressure corresponds to enhancement of the liquid crystalline phase thermal stability and elevation of T_m. Similarly, for the systems with decreasing δA under pressure, a T_m decrease is observed. Another important point is the sign of ΔG_{mix}. For cholesterol, which is known to be incorporated into the non-polar moiety of the lipid layers, $\Delta G_{mix} > 0$, whereas for stearoyl-L-α-alanine, $\Delta G_{mix} < 0$, which is in agreement with its condensing effect resulting from interaction with the polar part of the layer. Thus, the three MTAs can really be considered as representative examples of three different types of behavior in mono- and multilayers.

As has been already noted, the strongest MTA/DPPC interaction is observed for decamethoxinum. Such conclusion is also confirmed by our results of mass spectrometry experiments (Pashinskaya et al. 1999), where non-valent complex formation was observed between one lecithin molecule and 1–3 decamethoxinum molecules.

Conclusions

The use of such well-known classical methods as DSC and the Langmuir monolayer technique allowed us to obtain some interesting results concerning the mechanisms of membranotropic action of the substances investigated. First, the antimicrobial agent decamethoxinum, widely used in the Ukraine as a pharmacological substance, was found to extremely strongly disturb the membrane, which could be one reason for its antimicrobial activity. Another important point of the present investigation is the idea to use a special type of model MTA to study monolayer interactions with hydrophilic molecules. The molecule studied – stearoyl-L-α-alanine, consisting of an L-α-alanine amino acid residue and a saturated alkyl chain – exhibited clear condensing action resulting from interactions between zwitterionic parts of the MTA and DPPC.

Acknowledgements The authors are grateful to Drs. M.V. Kosevich and V.A. Pashinskaya from the B. Verkin Institute for Low Temperatures and Engineering (Kharkov) for providing samples of decamethoxinum and for helpful discussions.

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