ELSEVIER

Biochimica et Biophysica Acta 1234 (1995) 225–234

BBA Biochimica et Biophysica Acta

Capsaicin affects the structure and phase organization of phospholipid membranes

Francisco J. Aranda *, José Villalaín, Juan C. Gómez-Fernández

Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Murcia, Apdo. 4021, E-30080, Murcia, Spain

Received 29 June 1994; revised 18 October 1994; accepted 24 November 1994

Abstract

Capsaicin is a natural compound with pharmacological and toxicological effects, which given its hydrophobicity, can influence the structure of membranes. The interaction of capsaicin with model membranes of dipalmitoylphosphatidylcholine and dielaidoylphosphatidylethanolamine has been studied by using differential scanning calorimetry, fluorescent probe spectroscopy and ^{31}P-nuclear magnetic resonance. Capsaicin remarkably affects the phase transition of dipalmitoylphosphatidylcholine, shifting the transition temperature to lower values, and giving rise, at relatively high capsaicin concentrations, to the appearance of two peaks in the thermogram. These peaks may correspond to separated phases as indicated by the partial phase diagram. Whereas capsaicin did not affect the fluorescence polarization of the probes diphenylhexatriene and trimethylammonium-diphenylhexatriene, it clearly affected that of the probe 2-anthroyloxystearic acid, indicating that the perturbation produced by capsaicin on the membrane would be mainly at the position where this fluorophore is located. On the other hand, capsaicin, at relatively low concentrations, gives rise to immiscible phases in the presence of dielaidoylphoshatidylethanolamine and decrease the temperature of the lamellar to hexagonal H_{II} phase transition. At concentrations of capsaicin higher than 0.3 mol fraction, isotropic phases were detected. The possible implications of the effects of capsaicin on biological membranes are discussed.

Keywords: Capsaicin; Model membrane; DSC; NMR, ^{31}P-; Lipid polymorphism

1. Introduction

Capsaicin is the pungent ingredient in a wide variety of red peppers of the genus *Capsicum* (Fig. 1). A large number of studies [1–3] have established the molecule of capsaicin as an important probe for sensory neuron mechanism, but the mechanism underlying the neuronal effects of capsaicin is not completely clear. The available evidence indicates that capsaicin interacts with a specific recognition site (receptor) on the cell membrane [4,5], leading to activation of a cation channel and inducing depolarization and Ca^{2+} influx. It has been also suggested that the unique action of capsaicin on a particular population of sensory neurons is most likely due to the functional make up of their plasma membrane structure. Capsaicin, a lipophilic molecule, will dissolve in lipid structures and affect membrane fluidity and/or ion permeability of the plasma membrane, resulting in Ca^{2+} (and possibly other cations) to stream across the membrane [3,6]. Since the activity of capsaicin takes place at the membrane level, it might involve an initial interaction of the molecule with the membrane phospholipids, even if these phospholipids are not the final 'target' of capsaicin.

Moreover, capsaicin exerts other cell non-selective actions on non-sensory neurons and non-neuronal cells. These non-selective effects include inhibition of cardiac muscle excitability [7], inhibition of visceral smooth muscle activity [8], and inhibition of platelet aggregation [9]. In addi-

Abbreviations: 2-AS, 2-anthroyloxystearic acid; DEPE, dielaidoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; DPH, 1,6-diphenyl-1,3,5-hexatriene; ^{31}P-NMR, ^{31}P-nuclear magnetic resonance; T_c, main gel to liquid-crystalline phase transition temperature; TMA-DPH, 1-(4-(trimethylammonium)phenyl)-6-phenyl-1,3,5-hexatriene.

* Corresponding author. Fax: +34 68 364147.

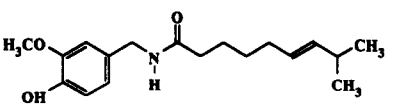

Fig. 1. Structure of capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide).

SSDI 0005-2736(94)00293-2

tion, capsaicin has been reported to influence a variety of enzymatic activities [10,11] and other cell and tissue functions [12]. The molecular mechanism of capsaicin's non-selective effects remain to be disclosed but do not seem to be mediated by a specific receptor [13], instead they may be due to a direct interaction of capsaicin with the cell membrane and thereby influencing the structure and function of the membrane. Recently it has been reported that capsaicin inhibits the aggregation of platelet in response to different stimuli [14] and that capsaicin has effects on physical properties (fluidity) of non-neuronal cell plasma membranes [15].

In addition to the extremely valuable use of capsaicin as a tool for investigating the role of sensory neurons in biological functions, it has been speculated on the implication for human therapeutics as capsaicin and its analogues (vanilloids) comprise a class of potential non-narcotic, non-steroidal analgesic-antiinflammatory agents [16]. Topical capsaicin creams are already in use, but as yet their efficacy is inconclusive [17,18]. In the therapeutic action of capsaicin, the interaction with the lipid membrane might be important.

From this point of view, the knowledge of the interaction between capsaicin and membrane lipids may help to get insight into the mechanism of action of the drug, particularly in its non specific effects. The aim of this work is to study the interaction of the capsaicin molecule with phospholipid model systems of different composition. Membrane model systems are widely used to study the mechanism of interaction of biologically active lipophilic molecules with membranes [19–21]. It is known that dispersion of individual or mixtures of phospholipids of biological origin or synthetic ones can adopt several structures, including the micellar phase, the familiar bilayer phase, the hexagonal H_{II} phase and lipidic particles [22]. The ability of different lipids to adopt these different liquid-crystalline structures is known as 'lipid-polymorphism'. These non-bilayer structures can greatly affect the functional behavior of the membrane [23]. For example, the energetics and kinetics of transport across membranes may in principle be modulated by the internal stresses across the lipid bilayer, which in turn is a direct function of the tendency of the lipid components to adopt non-lamellar phases [24]. On the other hand, there is strong evidence that the function of membrane proteins can be affected by the modification of the properties of the lipid matrix in which they are embedded [25]. Since lipid polymorphism has such potentially biological importance, in this report we check also whether capsaicin may modulate it. We chose dielaidoylphosphatidylethanolamine (DEPE) as a phospholipid model system, since phosphatidylethanolamine is the major class of phospholipid in eukaryotic systems which spontaneously adopts hexagonal H_{II} phases in the presence of excess aqueous buffer at physiological temperatures. At the same time, we present a detailed study on the interaction of capsaicin with dipalmitoylphosphatidylcholine (DPPC), as a model of the most important class of membrane phospholipids, the phosphatidylcholines.

The interaction of capsaicin with phosphatidylcholine and phosphatidylethanolamine was studied by using differential scanning calorimetry (DSC) and ^{31}P-nuclear magnetic resonance (^{31}P-NMR). DSC has been used to assess the influence of capsaicin on the phase behavior of the phospholipids. Calorimetric data were analyzed by constructing partial phase diagrams and the effect of capsaicin on the fluidity of the membrane has been also investigated. We found that capsaicin promotes hexagonal H_{II} phases formation in DEPE system. The modulation of DPPC thermotropic properties and DEPE polymorphic behavior by capsaicin is discussed in the light of the possible mechanism of action of its non-selective effects.

2. Materials and methods

2.1. Materials

Dioleoylphosphatidylcholine, palmitoyllysophosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC) and dielaidoylphosphatidylethanolamine (DEPE) were obtained from Avanti Polar Lipids, (Birmingham, AL, USA). Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained form Sigma (Poole, Dorset, UK). 2-Anthroyloxystearic acid (2-AS) was obtained from P-L Biochemicals (St. Goar, Germany). 1-(4-(Trimethylammonium)phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was obtained from Molecular Probes (Junction City, OR, USA). Organic solvents were obtained from Merck (Darmstadt, Germany). Twice distilled and deionized water was used.

2.2. Differential scanning calorimetry

The lipid mixtures for calorimetry measurements were prepared by combination of chloroform/methanol (1:1) solutions containing 5 μmol of the phospholipid and the appropriate amount of capsaicin when indicated. The organic solvents were evaporated under a stream of dry N_2, free of O_2, and the last traces of solvents were removed by a further 1–2 h evaporation under high vacuum. After the addition of 1 ml of 0.1 mM EDTA, 100 mM NaCl, 10 mM Hepes, pH 7.4 buffer, multilamellar liposomes were formed by mixing, using a bench-vibrator, always keeping the samples at a temperature above the gel to liquid-crystalline phase transition temperature of the lipid. The suspensions were centrifuged at 10000 rpm in a bench microfuge and the pellets were collected and placed into small aluminum pans. Pans were sealed and scanned in a Perkin-Elmer DSC-4 calorimeter, using a reference pan containing buffer. The heating and cooling rates were 4° C/min in all the experiments. The DSC instrument was set at a sensitivity

of 1 mcal/s. Peak areas were measured by weighing paper cut-outs of the peaks For the determination of the total phospholipid contained in a pan, this was carefully opened, the lipid was dissolved with chloroform/methanol (1:1) and the phosphorus contents were determined using the method of Bötcher et al. [26]. The instrument was calibrated using indium as standard.

In order to obtain the phase diagrams, the solidus and fluidus points were determined from the beginning of the heating and cooling thermograms (i.e., onset temperatures) respectively. In our type of calorimeter the point at which the thermogram returns to the baseline (i.e., completion temperature) is partially determined by instrumental factors. By that reason Philips et al. [27] introduced the procedure that we are using here. Heating and cooling thermograms were very similar. However, in the case of the $H_{II} \rightarrow L_{\alpha}$ phase transition of DEPE a hysteresis of 5° C was observed with respect to the $L_{\alpha} \rightarrow H_{II}$ transition. This hysteretic behavior of the $L_{\alpha} \leftrightarrow H_{II}$ transition has been previously observed via calorimetry and ^{31}P-NMR in several phosphatidylethanolamine systems [28–30] and it has been explained by the slower water transport in the H_{II} phase than in the L_{α} phase [31]. For the sake of simplicity the fluidus line for the $L_{\alpha} \leftrightarrow H_{II}$ phase transition in Fig. 7 was corrected for this hysteresis, allowing a direct observation of the effect of capsaicin on the transition.

In order to check the incorporation of capsaicin into the membranes, capsaicin was extracted from vesicles using n-pentane and its concentration was determined using an $\varepsilon_{281} = 2500\ M^{-1}$ in methanol. It was found that, even for the most concentrated samples, more than 90% of the added capsaicin was incorporated into the membrane.

2.3. Fluorescence spectroscopy

Samples for fluorescence polarization measurements were prepared by mixing organic solutions of lipids, capsaicin and the appropriate amounts of fluorescent probes (200:1, mol/mol) and multilamellar liposomes were formed as described above. Measurements were carried out using a Shimadzu RF-540 spectrofluorimeter. Excitation and emission wavelengths were 360 and 430 nm for DPH and TMA-DPH and 341 and 446 nm for 2-AS, respectively. To ensure that depolarization due to light scattering did not occur, the value of polarization was measured before and after diluting the sample. In cases where dilution gave an increase in polarization, the samples were diluted again until the value of polarization had reached a maximum and was no longer concentration dependent.

2.4. ^{31}P-Nuclear magnetic resonance

The samples for ^{31}P-NMR were prepared by combination of organic solutions containing 50 mg of phospholipid and the appropriate amount of capsaicin, evaporation of the solvents and formation of multilamellar vesicles as described above. The suspensions were centrifuged at 10000 rpm in a bench microfuge and pellets were placed into conventional 5 mm NMR tubes and ^{31}P-NMR spectra were obtained in the Fourier Transform mode in a Varian Unity 300 spectrometer. All chemical shift values are quoted in parts per million (ppm) with reference to pure lysophosphatidylcholine micelles (0 ppm), positive values referring to low-field shifts. All spectra were obtained in the presence of a gated-broad band decoupling (5 W input power during acquisition time) and accumulated free inductive decays were obtained from up to 600 scans. A spectral width of 25000 Hz, a memory of 8 K data points, a 1.3 s interpulse time and a 80° radio frequency pulse were used. Prior to Fourier transformation, an exponential multiplication was applied resulting in a 100 Hz line broadening.

3. Results

3.1. Interaction between capsaicin and phosphatidylcholine systems

We first studied the effect of capsaicin on the thermotropic phase transition of DPPC. The DSC profiles obtained for pure DPPC and mixtures of DPPC with capsaicin are shown in Fig. 2. For the pure phospholipid the main gel to liquid-crystalline phase transition tempera-

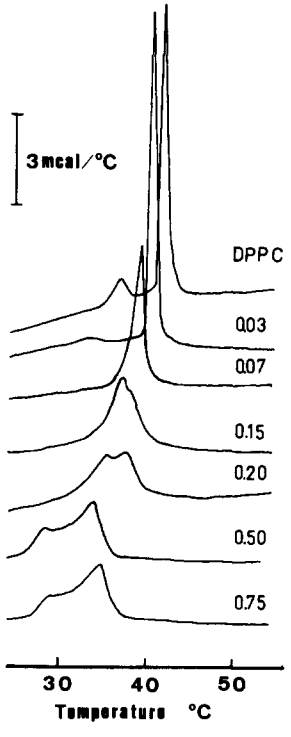

Fig. 2. DSC thermograms for mixtures of DPPC/capsaicin. The concentration of capsaicin in the membrane (mol fraction) is expressed on the curves. The profiles correspond to heating scans.

Table 1
The enthalpy changes (ΔH, kcal/mol) for the gel to liquid-crystalline phase transition of mixtures of DPPC/capsaicin and DEPE/capsaicin at different capsaicin mol fractions

Capsaicin mol fraction	ΔH (kcal/mol)	
	DPPC	DEPE
0	8.5	8.3
0.01	8.8	7.0
0.03	7.5	7.2
0.05	8.1	7.1
0.07	8.4	7.0
0.10	8.4	6.9
0.15	8.7	7.0
0.20	7.5	7.2
0.30	7.9	7.7
0.50	8.2	7.8
0.75	8.9	8.1

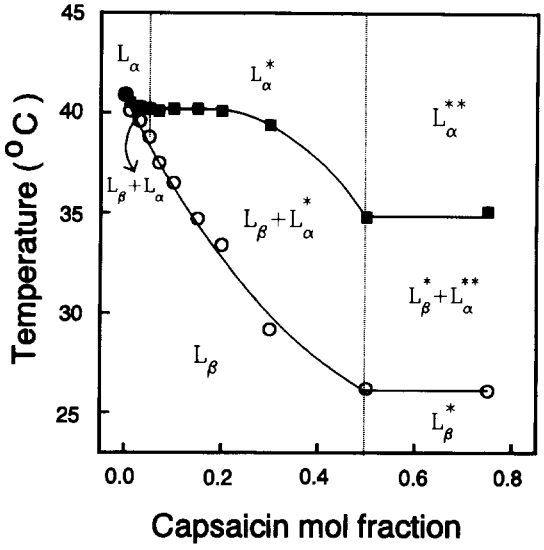

Fig. 3. Partial phase diagram for the mixture of DPPC/capsaicin. Open circles are obtained from DSC heating scans and black squares from DSC cooling scans. L_β indicates a gel phase and L_α a liquid-crystalline phase. $L_\beta{}^*$ indicates that different gel phases coexist and $L_\alpha{}^*$ and $L_\alpha{}^{**}$ indicate that different fluid phases coexist. Dashed lines separate different regions of the diagrams.

ture (T_c) appears at 41° C, this value being in good agreement with those reported before [32]. The thermotropic pretransition of DPPC is abolished at low concentration of capsaicin and can no longer be detected at mol fractions higher than 0.03. The presence of low concentration of capsaicin makes the main transition to broaden and shift to lower temperatures. Increasing the concentration of capsaicin produces a further broadening and shift of the transition peak to lower temperatures and induces the appearance of two well resolved peaks in the thermogram. Both peaks, already observed at a capsaicin mol fraction of 0.15, are shifted to lower temperatures as the content of capsaicin is increased. Despite this significant effect on the thermogram, capsaicin does not significantly affect the enthalpy change of the gel to liquid-crystalline phase transition of DPPC (Table 1).

Using the DSC data from heating and cooling experiments, the partial phase diagram corresponding to the DPPC/capsaicin system was constructed and it is shown in Fig. 3. The solidus line displays a near ideal behavior at low concentrations of capsaicin, the temperature decreasing as the capsaicin mol fraction increases. Only at the highest capsaicin mol fractions tested (0.5 to 0.75) a gel phase immiscibility is observed, since the solidus line keeps horizontal at a temperature value of 26° C. The fluidus line shows clear differences with the solidus line. At very low capsaicin concentrations (up to 0.03–0.05 mol fraction) the fluidus line displays a near ideal behavior. A fluid phase immiscibility is observed, since the fluidus line keeps horizontal at a temperature value of 40.2° C, in a concentration range from 0.05 to 0.2 mol fraction. Increas-

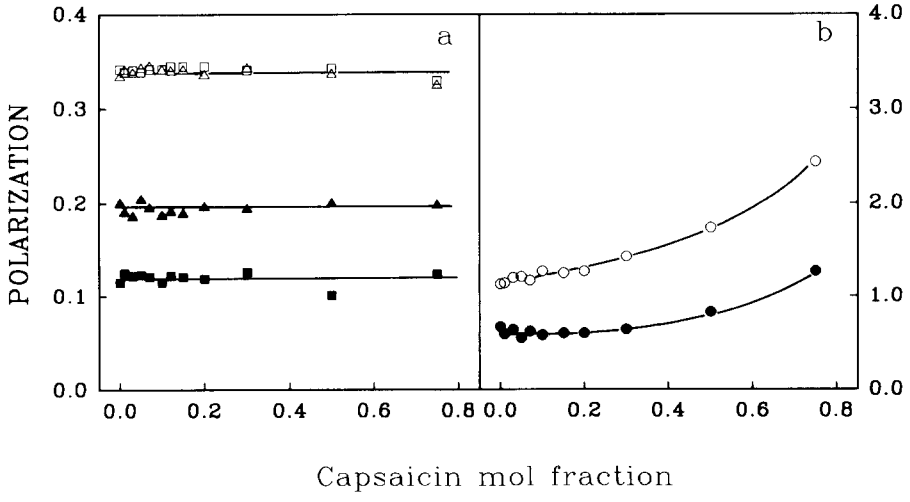

Fig. 4. Polarization values for (a) DPH (squares) and TMA-DPH (triangles) and (b) 2-AS (circles), incorporated into DPPC containing different capsaicin mol fractions. Open symbols correspond to 25° C and black symbols to 55° C.

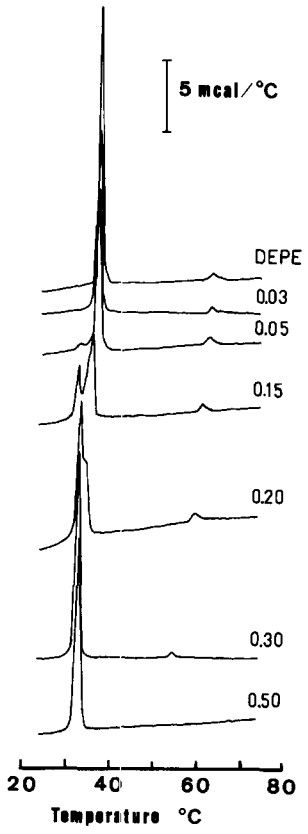

Fig. 5. DSC thermograms for the mixture DEPE/capsaicin. The concentration of capsaicin in the membrane (mol fraction) is expressed on the curves. The profiles correspond to heating scans.

ing the capsaicin content produces a dramatic decline of the fluidus line which leads to a new fluid immiscibility at the highest capsaicin mol fraction mixtures (0.5 to 0.75).

The possible effect of capsaicin on phosphatidylcholine lipid polymorphism was investigated by means of ^{31}P-NMR on dioleoylphosphatidylcholine and on lysophosphatidylcholine systems. Incorporation of capsaicin into dioleoylphosphatidylcholine bilayers does not change the phospholipid phase (data not shown). Pure lysophosphatidylcholine forms micelles in aqueous solution, which due to fast tumbling and lateral diffusion of the lipids give rise to a narrow isotropic signal [33]. The presence of capsaicin does not change the lineshape of the signal (data not shown).

To investigate the effect of capsaicin on the phosphatidylcholine membrane fluidity, fluorescence polarization measurements were carried out on DPPC/capsaicin systems using different fluorescent probes. Fig. 4A shows that the presence of capsaicin does not affect the fluorescence polarization of either DPH or TMA-DPH below and above the main gel to liquid-crystalline phase transition of DPPC. However, the presence of capsaicin at molar fractions higher than 0.2 produces and increase in the fluorescence polarization of 2-anthroyloxystearic acid (2-AS), both below and above T_c (Fig. 4B).

3.2. Interaction between capsaicin and phosphatidylethanolamine systems

The effect of capsaicin on the thermotropic phase transitions of DEPE is shown in Fig. 5. Aqueous dispersions of DEPE can undergo a gel to liquid-crystalline phase transition in the lamellar phase and in addition a lamellar to hexagonal H_{II} phase transition [34]. This is shown in the thermogram corresponding to DEPE dispersed in buffer (Fig. 5, upper part). The gel to liquid-crystalline phase transition occurs around 37° C and the bilayer to hexagonal H_{II} phase transition occurs around 63° C in agreement with previous data [34]. The latter has a much smaller transition enthalpy due to the fluid character of both the lamellar and the hexagonal H_{II} phase [35]. In the presence of capsaicin a new transition occurs at the low-temperature side of the gel to liquid-crystalline phase transition. The size of this new peak increases at the expense of the main peak when the amount of capsaicin is increased. Only the peak located at low temperature is present in the samples containing 0.5 and 0.75 mol fractions of capsaicin. However, the effect of capsaicin on the bilayer to hexagonal H_{II} phase transition is different. Incorporation of increasing amounts of the molecule results in a shift of the transition to lower temperatures so that at a capsaicin mol fraction of 0.5 the transition can no longer be observed. The presence of low concentrations of capsaicin (mol fraction 0.01) produces a slight decrease in the enthalpy change of the gel to liquid-crystalline phase transition of DEPE, but increasing capsaicin concentrations does not cause further changes (Table 1).

The effect of capsaicin on the thermotropic phase transitions of DEPE was further investigated by means of ^{31}P-NMR (Fig. 6). DEPE when organized in bilayer structures gives rise to a characteristic asymmetrical ^{31}P-NMR line-shape, with a high-field peak and a low-field shoulder [36]. The chemical shift anisotropy (measured as 3 times the chemical shift difference between the high-field peak and the position of isotropically-moving lipid molecules) is approx. 40 ppm in the liquid-crystalline state, in agreement with previous data [36], characteristic of an axially symmetrical shift tensor (Fig. 6A). In the gel state (at 25° C), as shown in Fig. 6A, the lineshape is broadened, possibly due to increased (^{1}H-^{31}P) dipolar interactions [37]. In the hexagonal H_{II} phase (Fig. 6A, 70° C), due to rapid lateral diffusion of the phospholipids around the tubes of which this phase is composed, the chemical shift anisotropy is further averaged, resulting in a line-shape with a reversed asymmetry, i.e., a high-field shoulder and a low-field peak with two-fold reduction in absolute value of chemical shift anisotropy [22,38]. Incorporation of capsaicin at a 0.1 mol fraction produces a small effect on the $L_\alpha \leftrightarrow H_{II}$ transition (Fig. 6B) resulting in the appearance of the characteristic spectrum corresponding to the hexagonal H_{II} phase at temperatures slightly lower than those at which this is observed in the pure phospholipid. At 61° C a hexagonal

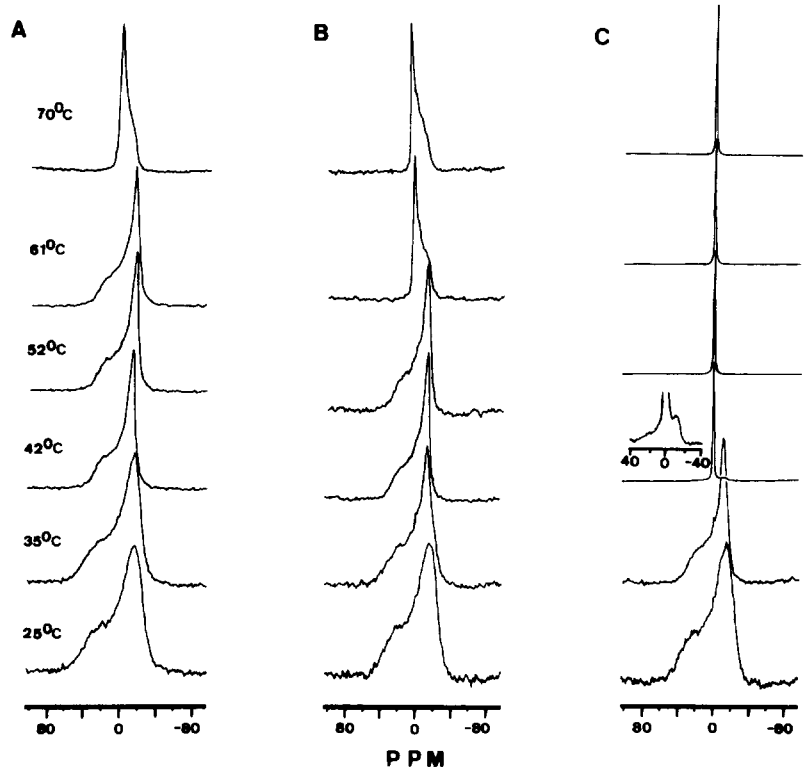

Fig. 6. ^{31}P-NMR spectra at different temperatures corresponding to pure DEPE (A); DEPE/capsaicin, 0.1 mol fraction (B) and DEPE/capsaicin, 0.5 mol fraction (C). The inset in part C corresponds to the amplification of the spectrum at 42° C.

H_{II} structure is present, whereas the pure phospholipid is still organized in extended bilayer structures (Fig. 6A). It is also observed that at 35° C where the pure phospholipid is organized in a gel lamellar phase (Fig. 6A), the presence of capsaicin (0.1 mol fraction) induces the appearance of fluid lamellar structures. The presence of higher concentration of capsaicin (0.5 mol fraction, Fig. 6C) give rise to the appearance of a spectral component with resonance position at 0 ppm (at temperatures of 35° C and higher) indicating that the spectrum originated form DEPE is progressively replaced by a spectrum characteristic of phospholipid molecules undergoing a rapid motion that leads to a nearly complete averaging of the chemical shift anisotropy. Increasing the temperature produces a progressive enhancement of the isotropic component at the expenses of the lamellar one. Above 42° C, the isotropic peak is the only one which is observed (Fig. 6C).

From the DSC data a partial phase diagram was constructed. In order to get more insight into the polymorphic behavior of DEPE/capsaicin systems, the information about the structural organization of the phospholipid obtained from ^{31}P-NMR experiments has been incorporated into the phase diagram as well. For a capsaicin concentration range from 0 to 0.3 mol fractions, the system displays a near-ideal behavior, the temperature for both the gel to liquid-crystalline and the bilayer to hexagonal H_{II} phase transitions decreasing as the concentration of capsaicin increases, except for the appearance of a solid-immiscibility at a capsaicin molar fraction of 0.05, as the solidus-line keeps horizontal from that concentration on. From a given capsaicin concentration within this range (0 to 0.3 mol fraction), increasing the temperature gives rise to the consecutive transition from L_β (gel phase) to L_α (liquid-crystalline phase) to H_{II} (hexagonal H_{II} phase), the region of co-existence of $L_\alpha + H_{II}$ phases being particularly very narrow which indicates the high degree of cooperativity of this transition. At capsaicin concentration higher than 0.3 mol fraction, the bilayer to hexagonal H_{II} phase transition cannot be further observed and there is a solid and fluid immiscibilities in the gel to liquid-crystalline transition as both the solidus and the fluidus lines remain horizontal. Inclusion of the ^{31}P-NMR data make the phase diagram more complex. For a 0.5 mol fraction of capsaicin, when the temperature is raised from 35° C to 42° C a bilayer and a isotropic signal are found superimposed in the spectrum (Fig. 6C) indicating a region of co-existence of both phases. Above 42° C only isotropic phases are observed.

4. Discussion

The molecule of capsaicin consists of three main sections, a lipophilic alkyl chain at one end, connected via a acyl-amide linkage to the vanillyl group bearing the polar hydroxyl group at the other end of the molecule (Fig. 1).

This amphiphilic nature make biological membranes one of the most likely sites of capsaicin action.

The aim of this work is to provide information on the interaction of capsaicin with lipid vesicles formed by two different classes of phospholipids of major relevance, phosphatidylcholine and phosphatidylethanolamine. We have performed the study of the interaction of capsaicin with these model membranes by using different physical techniques such as DSC, ^{31}P-NMR and fluorescence spectroscopy.

We will discuss first the interaction of capsaicin with DPPC bilayers. We used DSC in order to characterize the influence of capsaicin on the thermotropic properties of the phospholipids. The profile of a DSC thermogram of a phospholipid phase transition is determined by the transition temperature and the enthalpy change. Determining the temperatures of the transitions allows the construction of phase diagrams, which provide information regarding the equilibrium between gel and liquid-crystalline phases.

For DPPC/capsaicin systems we observe a progressive broadening of the transition peak and a shift of the T_c to lower temperatures (Fig. 2). These results are indicative of the establishment of a molecular interaction between the phospholipid acyl chains and the capsaicin molecule, perturbing the cooperative behavior of the phospholipid which can be explained by the intercalation of the capsaicin molecule between the DPPC molecules. Therefore these observations are compatible with part of the capsaicin molecule (the nine carbon alkyl chain) aligning itself principally with the prevailing directions of the phospholipid acyl chains. The hydroxyl and the amide groups of capsaicin would be placed near the lipid/water interface where they could form hydrogen bonding with water and may also establish other types of interactions with the polar part of the phospholipids. These polar interactions will keep the capsaicin molecule in the upper part of the phospholipid palisade. Such a location would perturb the phospholipid acyl chains, and consequently we found that capsaicin significantly decreased the onset temperature for the gel to liquid-crystalline phase transition. However, we have found that capsaicin failed to significantly perturb the ΔH of the transition (Table 1). At concentration of 0.15 and higher mol fractions of capsaicin a second peak appears in the thermogram. According to the phase diagram (Fig. 3) this might be due to a lateral phase separation of a capsaicin-rich domain. Nevertheless, enough capsaicin seems to remain in the bulk phase so that there is a further shift of T_c. It has been described that the free energy of the lipid bilayer may be modulated not only by the interaction between hydrocarbon chains but also between the head groups [39] and that the headgroups and interfacial interaction markedly influence the lipid-phase behavior [40]. The location of capsaicin allows the interaction of the molecule with the interfacial region of the bilayer and probably the perturbation exerted by capsaicin at this level may partially compensate the effects at the level of the acyl chains, in terms of changes of ΔH. It seems that the presence of capsaicin results in that the packing of some phospholipids can be perturbed at lower temperatures but that these modified molecules (which probably give rise to the low temperature endotherm in the thermogram at high capsaicin content) undergo the transition with a ΔH similar to that of the unperturbed phospholipids. Similar effects on the gel to liquid-crystalline phase transition of DPPC, i.e., a decrease of T_c with no change in ΔH, have been previously reported for C_5-C_{10} alcohols and C_7-C_{10} fatty acids [41].

In the partial phase diagram corresponding to DPPC/capsaicin (Fig. 3) the fluidus line keeps horizontal in a concentration range from 0.05 to 0.2 mol fraction, indicating a fluid phase immiscibility. This is an interesting observation which has been reported for unsaturated fatty acids [42], diacylglycerols [43] and retinol [20]. This type of immiscibility was predicted on the basis of theoretical calculations for mixtures of DPPC and anaesthetics [44], where a relatively strong interaction between the anaesthetic was supposed, so that clusters were formed. Our results can also be explained by a process of formation of capsaicin-rich domains when capsaicin is incorporated into DPPC membranes. In the L_α^* region (L_α^* indicating that different fluid phases coexist), DPPC and capsaicin rich fluid domains should be present. When the temperature decreases below 40° C the system enters a region of coexistence of fluid and solid phases ($L_\beta + L_\alpha^*$ region). When the temperature is further decreased below the solidus line, which displays near-ideal behavior, the system enters the L_β region, where only one solid phase is present. At the highest capsaicin mol fractions tested the system presents gel and fluid immiscibilities. From the temperatures of the two peaks observed in the thermogram of these concentrated samples (Fig. 2) it seems that two different capsaicin-phospholipid domains should be present, one richer in capsaicin than the other. These two capsaicin domains are present both in the fluid phase above 35° C (region L_α^{**}, where L_α^{**} represents another region presenting fluid coexisting phases different from L_α^*) and in the gel phase below 26° C (region L_β^*, where L_β^* indicates that different gel phases coexist), with a region of coexistence of fluid and solid phases (region $L_\beta^* + L_\alpha^{**}$).

When we studied the effect of capsaicin on the fluidity properties of DPPC using TMA-DPH, (localized near the phospholipid headgroup because of its cationic headgroup) and DPH (which reports on the conditions nearer the core of the bilayer) we found that capsaicin does not affect the fluidity of the bilayer neither below nor above the gel to liquid-crystalline phase transition of DPPC. This is in agreement with the data reported in [14] for dimiristoylphosphatidylcholine/cholesterol systems where no effect of capsaicin on the DPH fluorescence anisotropy was found. However, when we used a different fluorescent probe, a fatty acid with a anthroyloxy group located near

the lipid/water interface, 2-AS, we found that high concentrations of capsaicin produced a rigidification of the membrane both below and above the transition temperature of the phospholipid. 2-AS, due to its structure and location in the membrane, seems to be a better reporter of the effect of capsaicin, and this result indicates that at high concentrations of capsaicin, when capsaicin rich domains segregate in the membrane, the lipids are in a more ordered state. It is interesting to note that high concentrations of capsaicin were found to significantly reduce the fluidity of red cell and peritoneal mast cell membranes [15].

We will now consider the effect of capsaicin on DEPE system. From our calorimetric experiments it can be concluded that the effect of capsaicin on the gel to liquid-crystalline phases transition of DEPE is different to that observed on DPPC in that increasing concentrations of capsaicin in DEPE induces lateral phase separation. At difference with DPPC systems, at very high capsaicin concentrations only one phase exists. The effect of capsaicin on the bilayer to hexagonal H_{II} phase transition is different, since incorporation of increasing concentration of capsaicin results in a progressive decrease of the transition temperature. It can be suggested that upon capsaicin incorporation, part of the DEPE molecules and most likely those interacting with capsaicin, give rise to a broad bilayer to hexagonal H_{II} phase transition which is shifted to lower temperatures and which cannot be detected in the thermogram due to its increased width and low energy content. The remainder of the DEPE molecules still show a slightly perturbed transition. Upon increasing the capsaicin content, the fraction of unperturbed DEPE molecules decreases, so that the enthalpy of the detectable transition decreases, and eventually at the highest capsaicin concentration the transition is not observed. This interpretation was confirmed by our ^{31}P-NMR experiments which showed that capsaicin is able to promote hexagonal H_{II} structures at lower temperatures than those observed for pure DEPE. At a capsaicin concentration of 0.1 mol fraction this effect is small as the transition to H_{II} phase is only shifted down by about 3° C (Fig. 6B). At the highest capsaicin concentrations (Fig. 6C) we also found the presence of an isotropic signal in the spectra. It should be noted that the origin of an isotropic resonance in the ^{31}P-NMR spectra is not known with certainty since it may correspond to regions of the bilayer surface with a relative high curvature or it could be also originated by a separate phase, either cubic or an isotropic melt. It has been previously described that *N*-methyldioleoylphosphatidylethanolamine can form structures which give rise to isotropic signals at temperatures well bellow those at which a hexagonal H_{II} phase is first observed [45,46], and something similar has been also reported for mixtures of unsaturated phosphatidylethanolamines and phosphatidylcholines [47–51]. The kinetic model of the lamellar to hexagonal H_{II} phase transition mechanism suggested by Siegel and coworkers [31,52,53]

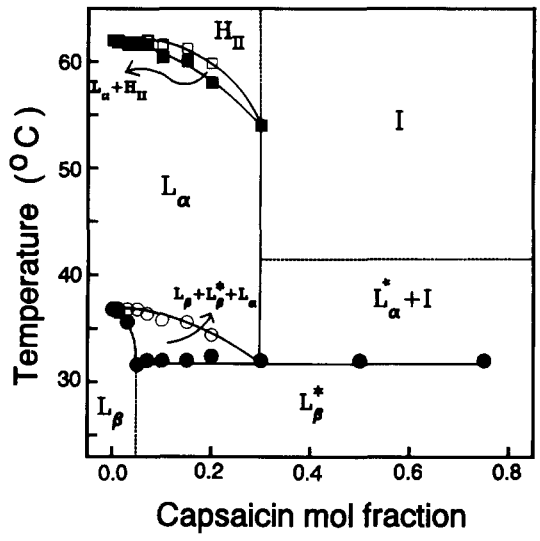

Fig. 7. Partial phase diagram for the system DEPE/capsaicin. Black symbols are obtained from DSC heating scans and open symbols from DSC cooling scans. Circles correspond to the gel to liquid-crystalline phase transition and squares to the bilayer to hexagonal-H_{II} phase transition. L_β indicates a gel phase, L_β * indicates that different gel phases coexist, L_α indicates a liquid-crystalline phase, L_α * indicates that different liquid-crystalline phases coexist, H_{II} indicates a hexagonal-H_{II} phase and I indicates an organization of lipids undergoing isotropic motion.

includes the formation of inverted micellar intermediates and interlamellar attachment structures which will produce isotropic spectral resonances signals in ^{31}P-NMR. The appearance of isotropic signals in DEPE has been previously reported for mixtures of this lipid with tocopherol [19] and retinoids [21]. From the phase diagram (Fig. 7), it can be concluded that at very low capsaicin concentrations the system behaves as nearly ideal but increasing the concentration produces, first, a gel immiscibility, and then a gel and fluid immiscibilities. At high temperatures the system evolves to a phase composed of isotropically moving phospholipid present in capsaicin-lipid domains.

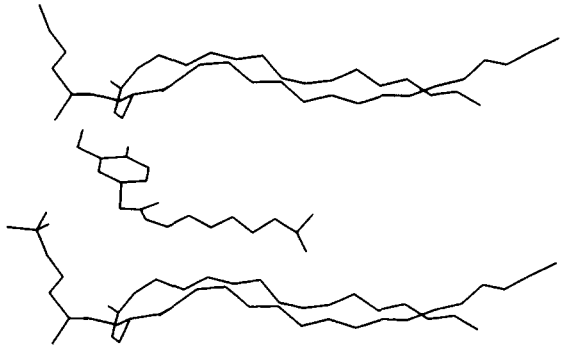

Fig. 8. A theoretical representation of the association between capsaicin and phospholipids in membranes. DEPE is presented at the top, capsaicin is at the center and DPPC is presented at the bottom. Protons have been removed for clarity.

In Fig. 8 a representation of the possible location of capsaicin in phospholipid membranes is presented. In this figure, the nine carbon alkyl chain of the capsaicin molecule is aligned along the phospholipid acyl chains, whereas the hydroxyl and the amide groups of capsaicin are located closer to the lipid/water interface. This location at the upper part of the phospholipid palisade could explain the effect observed on the DPPC membranes. This location would enable the polar part of the molecule of capsaicin to establish hydrogen bonding with the polar head of the phospholipids, which in the case of DEPE membranes, could lead to an effective reduction of the headgroup area of the phospholipid, explaining the facilitating effect of capsaicin on the formation of hexagonal H_{II} structures.

In summary, capsaicin is able to be incorporated in phospholipid membranes, probably aligning itself with the phospholipid acyl chains, perturbing the packing of the lipids and affecting their thermotropic properties. When considering the possible action of capsaicin in biological membranes, it is interesting to note that, as shown above, the system may present immiscibilities, which could give rise to the formation of domains, where the concentration of capsaicin could be specially high. The formation of domains would facilitate the activity of capsaicin even when its concentration is very low. It should be noted also that capsaicin shows fluid-phase immiscibility, a remarkable effect, given the normal fluid condition of biological membranes. On the other hand capsaicin is able to affect lipid polymorphism on phosphatidylethanolamine systems, promoting the hexagonal H_{II}-phase. The results of this work clearly show that capsaicin far for being an inert molecule, drastically affects the structural properties of the most abundant natural phospholipids in biological membranes, i.e., phosphatidylcholines and phosphatidylethanolamines. Interestingly, capsaicin can promote the formation of non-lamellar phases and these non-bilayers structures can greatly affect the functional behavior of the membrane [23].

We believe that the results presented here could be useful in order to get insight into the interaction of capsaicin with membranes and to the understanding of the non-specific effects of the drug, opening the possibility, which needs to be further explored, that some of the non-specific effects of capsaicin might be exerted through the alteration of membrane function produced by the interaction of this molecule with the lipidic component of the membrane.

Acknowledgements

This work was supported by Grants PB 92-0987 from Comisión Interministerial de Ciencia y Tecnología, Spain, and PCT 93/22 from Dirección General de Educación y Universidad, Comunidad Autónoma de Murcia, Spain.

References

[1] Jancsó, N. (1968) in Pharmacology of Pain (Lim, R.K.S., ed.), pp. 33–55, Pergamon Press, Oxford.
[2] Szolcsányi, J. (1984) in Antidromic Vasodilatation and Neurogenic Inflamation (Chahl, L.A., Szolcsányi, J. and Lembeck, F., eds.), pp. 7–25, Akadémiai Kiadó, Budapest.
[3] Buck, S.H. and Burks, T.F. (1986) Pharmacol. Rev. 38, 179–226.
[4] Szallasi, A. and Blumberg, P.M. (1990) Life Sci. 47, 1399–1408.
[5] Dray, A., Campbell, E.A., Hughes, G.A., Patel, I.A., Perkins, M.N., Rang, H.P., Rueff, A., Seno, N., Urban, L. and Walpole, C.S.J. (1991) Br. J. Pharmacol. 102, 78P.
[6] Govindarajan, V.S. and Sathyanarayana, M.N. (1991) Crit. Rev. Food Sci. Nutr. 29, 435–475.
[7] Franco-Cereceda, A. and Lundberg, J.M. (1988) Naunyn Schmiedebergs Arch. Pharmacol. 377, 649–655.
[8] Holzer, P. and Lembeck, F. (1979) Naunyn Schmiedebergs Arch. Pharmacol. 310, 169–174.
[9] Wang, J.P., Hsu, M.F., Hsu, T.P. and Teng, C.M. (1985) Thromb. Res. 37, 669–679.
[10] Srinivasan, M.R. and Sathyanarayana, M.N. (1989) Ind. J. Exp. Biol. 27, 910–912.
[11] Yagi, T. (1990) Arch. Biochem. Biophys. 281, 305–311.
[12] Matucci-Cerini, M., Marabini, S., Jantsch, S., Cagnoni, M. and Partsch, G. (1990) Ann. Rheum. Dis. 49, 598–602.
[13] Holzer, P. (1991) Pharmacol. Rev. 43, 143–201.
[14] Hogaboam, C.M. and Wallace, J.L. (1991) Eur. J. Pharmacol. 202, 129–131.
[15] Meddings, J.B., Hogaboam, C.M., Reynolds, J.D. and Wallace, J.C. (1991) Biochim. Biophys. Acta 1070, 43–50.
[16] Maggi, C.A. and Meli, A. (1988) Gen. Pharmacol. 19, 1–43.
[17] Carte, R.B. (1991) Drug Dev. Res. 22, 109–123.
[18] Rumsfield, J.A. and West, D.P. (1991) DICP Ann. Pharmacother. 25, 381–387.
[19] Micol, V., Aranda, F.J., Villalaín, J. and Gómez-Fernández, J.C. (1990) Biochim. Biophys. Acta 1022, 194–202.
[20] Ortiz, A., Aranda, F.J. and Gómez-Fernández, J.C. (1992) Biochim. Biophys. Acta 1106, 282–290.
[21] Ortiz, A., Aranda, F.J., Villalaín, J. and Gómez-Fernández, J.C. (1992) Biochim. Biophys. Acta 1112, 226–234.
[22] Cullis, P.R., Hope, M.J., De Kruijff, B., Verkleij, A.J. and Tilcock, C.P.S. (1985) in Phospholipid and Celullar Regulation (Kuo, J.F.,ed.), Vol. I, pp. 1–60, CRC Press, Boca Raton, FL.
[23] De Kruijff, B., Cullis, P.R., Verkleij, A.J., Hope, M.J., Van Echteld, C.J.A. and Taraschi, T.F. (1985) in The Enzymes of Biological Membranes (2nd Edn.) (Martonosi, A.N., ed.), Vol. I, pp. 131–204, Plenum Press, New York.
[24] Seddom, J.M. (1990) Biochim. Biophys. Acta 1031, 1–69.
[25] Navarro, J., Toivio-Kinnucan, M. and Racker, E. (1984) Biochemistry 26, 5439–5447.
[26] Bötcher, C.F.J., Van Gent, C.M. and Priest, C. (1961) Anal. Chim. Acta 24, 203–204.
[27] Phillips, M.C., Ladbrooke, B.D. and Chapman, D. (1970) Biochim. Biophys. Acta 196, 35–44.
[28] Rand, R.P. and Fuller, N.L. (1985) Biophys. J. 47 (2.Pt.2), 423a. (Abstr.)
[29] Yager, P. and Chang, E.L. (1983) Biochim. Biophys. Acta 731, 491–494.
[30] Tilcock, C.P.S. and Cullis, P.R. (1982) Biochim. Biophys. Acta 684, 212–219.
[31] Siegel, D. (1986) Biophys. J. 49, 1155–1170.
[32] Chapman, D., Williams, R. and Ladbrooke, B.D. (1967) Chem. Phys. Lipids 1, 445–461.
[33] Killian, J.A., De Kruijff, B., Van Echteld, C.J.A., Verkleij, A.J., Leunnissen-Bijvelt, J. and De Gier, J. (1983) Biochim. Biophys. Acta 728, 141–144.
[34] Gallay, J. and De Kruijff, B. (1984) Eur. J. Biochem. 142, 105–112.

[35] Cullis, P.J. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420.
[36] Van Echteld, C.J.A., Van Stigt, R., De Kruijff, B., Leunnissen-Bijvelt, J., Verkleij, A.J. and De Gier, J. (1981) Biochim. Biophys. Acta 648, 287–291.
[37] Seelig, J. and Gallay, H.U. (1976) Biochemistry 15, 5199–51204.
[38] Seelig, J. (1978) Biochim. Biophys. Acta 507, 207–218.
[39] Israelachvili, J.N., Mitchell, D.J. and Ninham, B.W. (1977) Biochim. Biophys. Acta 470, 185–201.
[40] Cevc, G. and Marsh, D. (1987) Phospholipid Bilayers, Wiley-Interscience, New York.
[41] Jain, M.K. and Wu, N.M. (1977) J. Membr. Biol. 34, 157–201.
[42] Ortiz, A. and Gómez-Fernández, J.C. (1987) Chem. Phys. Lipids 45, 75–91.
[43] Ortiz, A., Villalaín, J. and Gómez-Fernández, J.C. (1988) Biochemistry 27, 9030–9036.
[44] De Verteuil, F., Pink, D.A., Vadas, E.B. and Zuckerman, A. (1981) Biochim. Biophys. Acta 640, 207–222.
[45] Gagné, J., Stamatatos, L., Diacovo, T., Hui, S.W., Yeagle, P.L. and Silvius, J.R. (1985) Biochemistry 24, 4400–4408.
[46] Gruner, S.M., Tate, M.W., Kirk, G.L., So, P.T.C., Turner, D.C., Keane, D.T., Tilcock, C.P.S. and Cullis, P.R. (1988) Biochemistry 27, 2853–2866.
[47] Cullis, P.R. and De Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207–218.
[48] Hui, S.W., Stewart, T.P., Yeagle, P.L. and Albert, A.D. (1981) Arch. Biochem. Biophys. 207, 227–240.
[49] Tilcock, C.P.S., Bally, M.B., Farren, S.B. and Cullis, P.R. (1982) Biochemistry 21, 4596–4601.
[50] Verkleij, A.J. (1984) Biochim. Biophys. Acta 779, 43–63.
[51] Ellens, H., Siegel, D.P., Alford, D., Yeagle, P.L., Boni, L., Lis, L.J., Quinn, P.J. and Bentz, J. (1989) Biochemistry 28, 3692–3703.
[52] Siegel, D. (1986) Biophys. J. 49, 1171–1183.
[53] Siegel, D. (1986) Chem. Phys. Lipids 42, 279–301.