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Biochimica et Biophysica Acta 1509 (2000) 346–360

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Effect of angiotensin II non-peptide AT_1 antagonist losartan on phosphatidylethanolamine membranes

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Received 17 March 2000; received in revised form 7 August 2000; accepted 17 August 2000

Abstract

Losartan was found to affect both the thermotropic behavior and molecular mobility of dimyristoyl- and dipalmitoyl-phosphatidylcholine membranes (Theodoropoulou and Marsh, Biochim. Biophys. Acta 1461 (1999) 135–146). At low concentrations, the antagonist is located close to the interfacial region of the phosphatidylcholine bilayer while at high mole fractions it inserts deeper in the bilayers. In the present study, we investigated the interactions of losartan with phosphatidylethanolamine membranes using differential scanning calorimetry (DSC), electron spin resonance (ESR) and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. DSC showed that the antagonist affected the thermotropic transitions of dimyristoyl-, dipalmitoyl- and dielaidoyl-phosphatidylethanolamine membranes (DMPE, DPPE and DEPE, respectively). ESR spectroscopy showed that the interaction of losartan with phosphatidylethanolamine membranes is more superficial than in the case of phosphatidylcholine bilayers. Additionally, losartan increased the spin-spin broadening of 12-PESL spin labels in the gel phase of DMPE and DPPE membranes, while in the case of DEPE membranes the opposite effect was observed. ^{31}P-NMR showed that the antagonist stabilizes the fluid lamellar phase of DEPE membranes relative to the hexagonal H_{II} phase. Our results show that losartan affects the thermotropic behavior of phosphatidylethanolamine membranes, while the molecular mobility of the membranes is not affected greatly. Furthermore, its interactions with phosphatidylethanolamine membranes are more superficial than with phosphatidylcholine bilayers.

Keywords: Angiotensin II; Losartan; Phosphatidylethanolamine membranes; Differential scanning calorimetry; Electron spin resonance spectroscopy; Nuclear magnetic resonance spectroscopy

1. Introduction

Hypertension is one of the most frequently seen cardiovascular diseases with a high factor of mortality. The regulation of blood pressure as well as the fluid and electrolyte balance are importantly affected

Abbreviations: Losartan, 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole potassium salt; ANG II, angiotensin II; RAS, renin–angiotensin system; ACE, angiotensin converting enzyme; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; *n*-PESL, 1-acyl-2-[*n*-(4,4′-dimethyloxazolidine-*N*-oxyl)-stearoyl]-*sn*-glycero-3-phosphoethanolamine; DSC, differential scanning calorimetry; ESR, electron spin resonance; ^{31}P-NMR, ^{31}P-nuclear magnetic resonance

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0005-2736/00/$ – see front matter
PII: S0005-2736(00)00317-5

by angiotensin II (ANG II), the body's most potent vasoconstrictor, produced by the renin–angiotensin system (RAS).

One approach for the development of antihypertensive drugs was the intervention in the RAS at the stage of ANG II's synthesis, using renin or angiotensin converting enzyme (ACE) inhibitors [1,2]. ACE inhibitors were shown to be clinically effective in the treatment of hypertension and congestive heart failure [3]. However, this approach was found to suffer side effects [4,5].

The most selective method for the treatment of hypertension would be the antagonism of ANG II at its receptor. Many peptide analogs of ANG II itself, with varying degrees of agonist and antagonist properties, were synthesized for this purpose. A prototype of this type of receptor antagonists is saralasin (Sar^1Ile^8-ANG II), which was extensively studied preclinically [6] and was shown to lower blood pressure in humans [7]. However, the therapeutic utility of this drug was limited by the need for parenteral administration and the presence of agonist effects in 23% of the patients [8]. The breakthrough came with the discovery of non-peptide ANG II receptor antagonists in 1982 by Takeda Chemical Industries [9]. Novel series of molecules were designed after the original patent publications, which led to the discovery of the prototype compound losartan (DUP 753, MK 954) by Du Pont Merck Pharmaceutical Company [10,11].

Analysis of receptors with single-residue and combined mutations revealed that specific amino acid residues in transmembrane domains III, IV, V, VI and VII are involved in binding of losartan to the mammalian AT_1 receptor. Furthermore, despite their locations on different transmembrane domains, most of these residues are positioned within a small distance of each other within the plasma membrane, suggesting that the antagonist binds to the mammalian AT_1 receptor in a plane that is one or two α-helical turns below the membrane surface [12,13]. A drug may reach a binding site on a membrane-associated receptor protein by direct diffusion through the aqueous surroundings or by partitioning into the lipid bilayer and then laterally diffusing to the active site. The highly ordered structure of the lipid bilayer may restrict lipophilic or amphiphilic drugs to a particular depth of penetration in the bilayer; a drug positioned at the proper depth of penetration for optimal reaction with a receptor site would be an active molecule. The orientation of the drug within the membrane and its conformation when it enters the bilayer are also very important features for the interaction with the active site of the receptor [14]. Therefore, the investigation of losartan's interactions with the phospholipid bilayer component of membranes becomes essential because the major determinants of losartan binding appear to be residues located within the transmembrane regions while there is no contribution from the extracellular domains.

In our previous study, we investigated the interactions of losartan with phosphatidylcholine bilayers, using a combination of differential scanning calorimetry (DSC) and electron spin resonance spectroscopy (ESR) [15]. The phosphatidylcholines used were DMPC and DPPC that are saturated and differ only in the length of their acyl chains. We selected these two phospholipids because they are those most extensively characterized, using DSC and ESR spectroscopy. Specifically, we chose DMPC and DPPC also because they exhibit single, highly cooperative gel to liquid-crystalline phase transitions at 23.8°C and 41.2°C, respectively, temperatures well above the freezing point of water and below the melting point of losartan (267–269°C). It was shown, in this former study, that losartan interacts with phosphatidylcholine membranes by affecting both their thermotropic behavior and their molecular mobility. Particularly striking was the effect of high concentrations of the antagonist on the chain-melting behavior of DMPC and the associated changes in vesicular structure that were induced in this region. At low concentrations, the antagonist seemed to be located close to the interfacial region of the phosphatidylcholine bilayer, while at high mole fractions it inserted deeper into the lipid bilayers. These results opened up the possibility that losartan, besides its antagonist action on the receptor level, could exert some of its effects through its interaction with the lipids of the membrane bilayer. A more limited DSC study published previously [16] was qualitatively consistent with our studies on phosphatidylcholines [15].

The finding that losartan intercalates close to the interfacial region of the phosphatidylcholine bilayer now led us to investigate the mode of losartan asso-

ciation with bilayers formed by zwitterionic phospholipids with a different headgroup. In the present study, we investigated the interactions of losartan with phosphatidylethanolamine membranes. Phosphatidylethanolamine is a major component of both eukaryotic and prokaryotic membranes. A combination of DSC, ESR and ^{31}P-nuclear magnetic resonance (^{31}P-NMR) spectroscopy was used. These biophysical techniques can give complementary information about the thermotropic and dynamic properties of biologically active molecules in membranes [15,17–28].

2. Materials and methods

2.1. Materials

Dimyristoyl- and dipalmitoyl-phosphatidylethanolamine (DMPE and DPPE, respectively) were obtained from Fluka Chemical Co. (Buchs, Switzerland). Dielaidoyl-phosphatidylethanolamine (DEPE) was obtained from Avanti Polar Lipids Inc. (Birmingham, AL, USA). Losartan was kindly provided by Merck's Research Laboratories (MSD Sharp and Dohme GmbH, Haar, Germany). Phosphatidylethanolamine spin labels (*n*-PESL) were synthesized by B. Angerstein as described [29].

2.2. DSC and ESR spectroscopy

Sample preparation, and calorimetric and ESR measurements were performed as described in our previous publication [15]. Briefly, the dried mixtures of phospholipids and losartan were prepared by first codissolving appropriate amounts of the lipid and the antagonist in a dichloromethane/methanol mixture (2:1 v/v). For the preparation of DSC samples, 7 mg of the phospholipid was used, while in the case of ESR spectroscopy, 1 mg of the phospholipid was used and 1 mol% of the spin-labeled lipid in dichloromethane was added. The organic solvent was then evaporated under a stream of oxygen-free N_2, at a temperature above the corresponding phase transition temperature of the phospholipid. The lipid/drug mixtures were then dried under high vacuum overnight. The dried samples were hydrated with double distilled and deionized water (200 μl and 50 μl for DSC calorimetry and ESR spectroscopy, respectively).

Outer hyperfine splittings ($2A_{max}$) were used to characterize the rotational disorder and rotational rates of the spin-labeled lipid chain segments [30]. This quantity is defined on the experimental spectra given subsequently (Fig. 3). In general, the values of $2A_{max}$ will depend both on the order parameter of the spin-labeled chain segment and on rotational correlation time. For fluid-phase membranes, it will depend most strongly on the chain order, but in gel-phase membranes it will also depend strongly on the (slow) rotational rates. For rotational mobility with components in the slow-motional regime [31], the change in this parameter, ΔA_{max}, therefore provides a useful measure of the change in lipid chain mobility induced by losartan. Rapid isotropic rotation reduces A_{max} to $A_0 = 15.0$ G, which is the isotropic average hyperfine coupling constant [24]. Therefore, $\Delta A_{max}/(A_{max} - A_0)$ is the appropriate normalized quantity that characterizes the changes in A_{max}.

2.3. ^{31}P-NMR spectroscopy

^{31}P-NMR spectra of hydrated pure DEPE and mixtures with losartan incorporated at mole fractions of $x = 0.05$ and $x = 0.20$ were obtained. Dried mixtures of phospholipid and losartan were prepared following the same procedure as used for DSC samples [15]. In the case of samples for ^{31}P-NMR spectroscopy, 50 mg of the phospholipid and appropriate amounts of the antagonist were used. After hydration with 2 ml distilled and deionized water, the samples were transferred to 10 mm NMR tubes and centrifuged for 30 min in a benchtop centrifuge at 3000 rpm. The excess water was removed prior to acquisition of the spectra. In this way, the concentrated, fully hydrated lipid could be centered optimally in the NMR coil.

Proton dipolar decoupled ^{31}P-NMR spectra were recorded at 121.5 MHz on a Bruker MSL-300 spectrometer operating in the Fourier transform mode. All data were collected using one pulse acquisition with quadrature phase cycle sequence provided in the Bruker library of pulse programs. The 90° pulse used was 11 μs and the recycle delay 4 s. Prior to acquisition, the samples were held above their phase transition temperature for 10 min to ensure complete

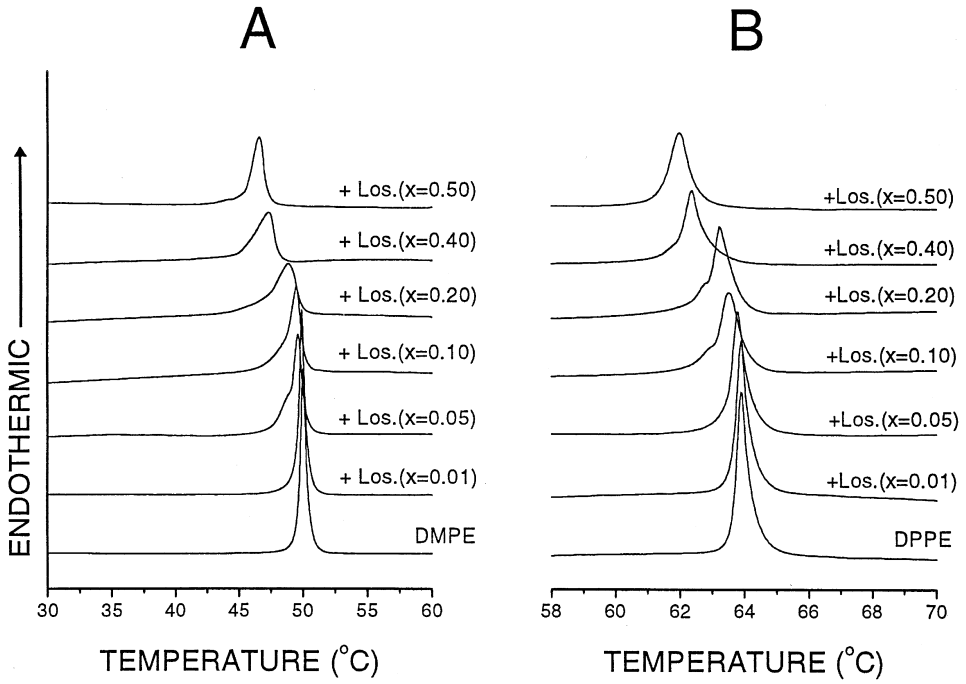

Fig. 1. DSC thermograms of hydrated DMPE (A) or DPPE (B) membranes containing losartan at the different mole fractions, x, indicated. Note that the extent of the temperature scales differs in (A) and (B).

hydration. The spectra were obtained over the temperature range 50–86°C. The samples were held for 15 min at each temperature before recording the spectrum, to ensure thermal equilibration. Temperature was regulated by a thermostated nitrogen gas flow system. Each spectrum was an accumulation of 800 scans. All chemical shift values are referenced to 85% phosphoric acid (0 ppm). Prior to Fourier transformation, a trapezoidal multiplication was performed using Bruker software packages ($TM_1 = 0$ and $TM_2 = 50$).

3. Results

3.1. DSC

The DSC traces of DMPE preparations containing increasing concentrations of losartan are shown in Fig. 1A. The hydrated pure DMPE undergoes a gel to liquid-crystalline phase transition centered at 50.0°C, in agreement with previous reports [32]. The incorporation of increasing concentrations of the antagonist results in a progressive shift of the observed transition to lower temperatures and a broadening of the corresponding peak (Table 1). As shown in Table 1, the presence of losartan does not affect very significantly the total enthalpy change (ΔH) of the observed phase transition. At mole fraction $x = 0.05$, a shoulder at the lower temperature side of the endotherm is formed. As the concentration of the antagonist is increased to $x = 0.50$, this shoulder and the whole transition is shifted to lower temperatures.

The effect of the ANG II antagonist on the thermotropic phase transition of DPPE is also shown in Fig. 1B. DPPE bilayers show a phase transition from the gel to the liquid-crystalline phase at 63.9°C, in agreement with previous reports [32]. Similar effects to those described for DMPE membranes are obtained when the antagonist is incorporated in DPPE membranes. The presence of losartan results in a broadening of the phase transition peak and a shift to lower temperatures (Table 1). The total enthalpy change of the observed phase transition was little affected by the incorporation of the antagonist up to mole fractions $x = 0.40$ (Table 1). Only at $x = 0.50$ is there an increase in ΔH for DPPE, which is not observed for DMPE. Furthermore, at mole fractions $x \geq 0.10$, a low temperature shoulder is ob-

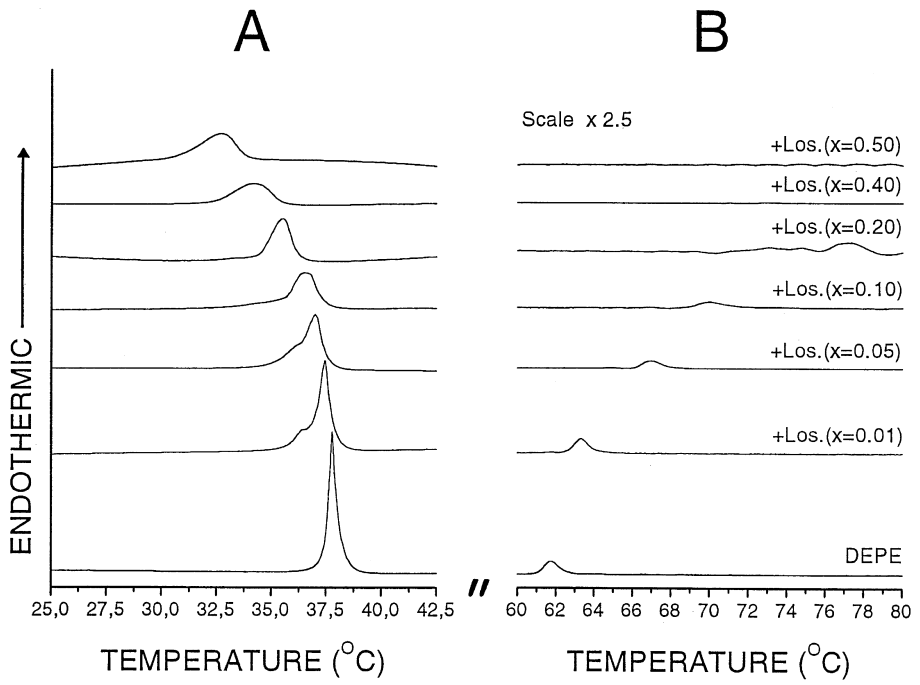

Fig. 2. DSC thermograms of hydrated dispersions of DEPE containing losartan at the different mole fractions, x, indicated.

served. As the drug concentration is increased, the shoulder is shifted to lower temperatures.

The DSC thermograms of hydrated pure DEPE and mixtures with losartan are shown in Fig. 2. The aqueous dispersions of DEPE undergo a gel to liquid-crystalline phase transition in the lamellar phase at 37.8°C (Fig. 2A) and a lamellar to inverted hexagonal H_{II} structural phase transition at 61.8°C (Fig. 2B), in agreement with previous data [33]. Increasing concentration of losartan produces a broadening of the peak that corresponds to the gel to liquid-crystalline phase transition and a progressive shift in the transition to lower temperatures (Table 2). The progressive broadening of the transition, which is greater than that for the saturated DMPE and DPPE lipids at the higher losartan concentration, corresponds to a decreasing cooperativity of the chain melting. At mole fractions $x \geq 0.01$ a shoulder at the lower temperature side of the endotherm is observed, as in the case of the thermograms of DMPE and DPPE membranes containing the antagonist. The lamellar to inverted hexagonal H_{II} phase transition is shifted to higher temperatures as the concentration of losartan increases to $x = 0.20$, while at higher drug mole fractions, this transition is not observed at temperatures up to 80°C (Table 2).

Table 1
Calorimetric peak temperatures (T_c), half-widths ($\Delta T_{c1/2}$) and total enthalpy changes (ΔH) of DMPE or DPPE bilayers containing different concentrations of losartan

Sample	T_c (°C)[a]		ΔH (kcal/mol)[a]		$\Delta T_{c1/2}$ (°C)[a]	
DMPE, DPPE	50.0	63.9	5.1	7.3	0.5	0.4
+losartan ($x = 0.01$)	49.9	63.9	5.3	6.9	0.5	0.4
+losartan ($x = 0.05$)	49.6	63.8	5.4	6.8	0.8	0.5
+losartan ($x = 0.10$)	49.5	63.5	5.2	7.0	1.0	0.7
+losartan ($x = 0.20$)	48.8	63.3	5.4	7.4	1.9	0.6
+losartan ($x = 0.40$)	47.4	62.4	5.1	7.4	1.6	0.6
+losartan ($x = 0.50$)	46.6	62.0	5.6	8.6	1.0	0.7

[a]Values in the left-hand column correspond to DMPE bilayers and those on the right to DPPE bilayers.

3.2. ESR spectroscopy

ESR spectra of phosphatidylethanolamines spin-labeled at the 5-, 12- or 14-position of the *sn*-2 chain (5-PESL, 12-PESL and 14-PESL, respectively) incorporated in DMPE bilayers are compared in Fig. 3 (dotted lines) with the corresponding ones from preparations containing losartan at $x = 0.20$ (solid lines). The spectra were obtained at different temperatures

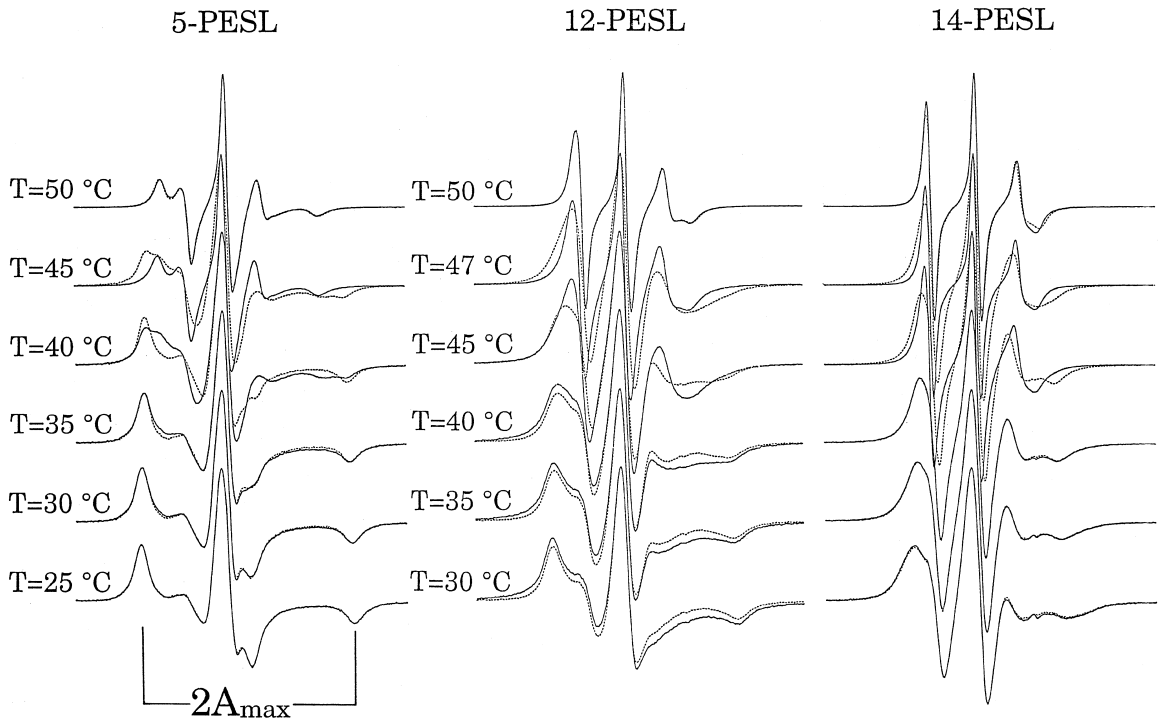

Fig. 3. ESR spectra of 5-, 12- and 14-PESL spin labels incorporated either in DMPE membranes (dotted line) or DMPE membranes containing losartan at mole fraction $x = 0.20$ (solid line) obtained at the temperatures indicated, over the range 25–50°C for 5-PESL and 30–50°C for 12- and 14-PESL spin probes. The outer hyperfine splitting $2A_{max}$ is also indicated. Total scan width = 100 G.

in order to cover different lamellar phases of the phosphatidylethanolamine membranes.

In the case of 5-PESL spin labels, DMPE bilayers exhibit a chain-melting phase transition in the region around 45°C, as is observed from the two-component spectra and the decrease in the outer hyperfine splitting $2A_{max}$ ($\Delta A_{max}/(A_{max}-A_0) = 41.4\%$ between 40 and 50°C). The bilayers containing losartan exhibit this transition at around 40°C ($\Delta A_{max}/(A_{max}-A_0) = 53.8\%$ between 35 and 45°C). The mobility of the spin label is not affected by the antagonist (at $x = 0.20$) either in the gel or in the liquid-crystalline phase, as is concluded from the near identity of the spectra and the observation that the outer hyperfine splitting $2A_{max}$ of the spectra is not affected by the incorporation of losartan into DMPE membranes. As is evident in Fig. 3, the spin-spin broadening of the 12-PESL spin labels in the gel phase of DMPE bilayers is increased somewhat by the presence of losartan in the membranes. Spin-spin broadening arises from mutual interactions between spin labels and only occurs at high local concentrations of the (otherwise dilute) spin-labeled lipids. The increase in spin-spin broadening therefore demonstrates that losartan increases the partial exclusion of the spin-labeled phospholipids from the tightly packed DMPE molecules in the gel phase. The observed phase transition is also shifted to between 40 and 45°C ($\Delta A_{max}/(A_{max}-A_0) = 77.8\%$ between 40 and

Table 2
Calorimetric peak temperatures (T_c), half-widths ($\Delta T_{c1/2}$) and total enthalpy changes (ΔH) of DEPE dispersions containing different concentrations of losartan

Sample	T_c (°C)[a]		ΔH (kcal/mol)[a]		$\Delta T_{c1/2}$ (°C)[a]	
DEPE	37.8	61.8	7.0	0.5	0.3	0.9
+losartan ($x = 0.01$)	37.4	63.4	7.2	0.5	0.6	0.9
+losartan ($x = 0.05$)	37.0	66.9	7.2	0.4	0.9	1.2
+losartan ($x = 0.10$)	36.5	70.1	7.0	0.2	1.3	1.5
+losartan ($x = 0.20$)	35.5	77.1	6.5	0.4	1.3	2.0
+losartan ($x = 0.40$)	34.2	–	6.5	–	1.9	–
+losartan ($x = 0.50$)	32.7	–	9.4	–	2.2	–

[a]Values in the left-hand column correspond to the transition from the lamellar gel to the lamellar liquid-crystalline phase and those on the right to the structural phase transition from the lamellar liquid-crystalline phase to the hexagonal H_{II} phase.

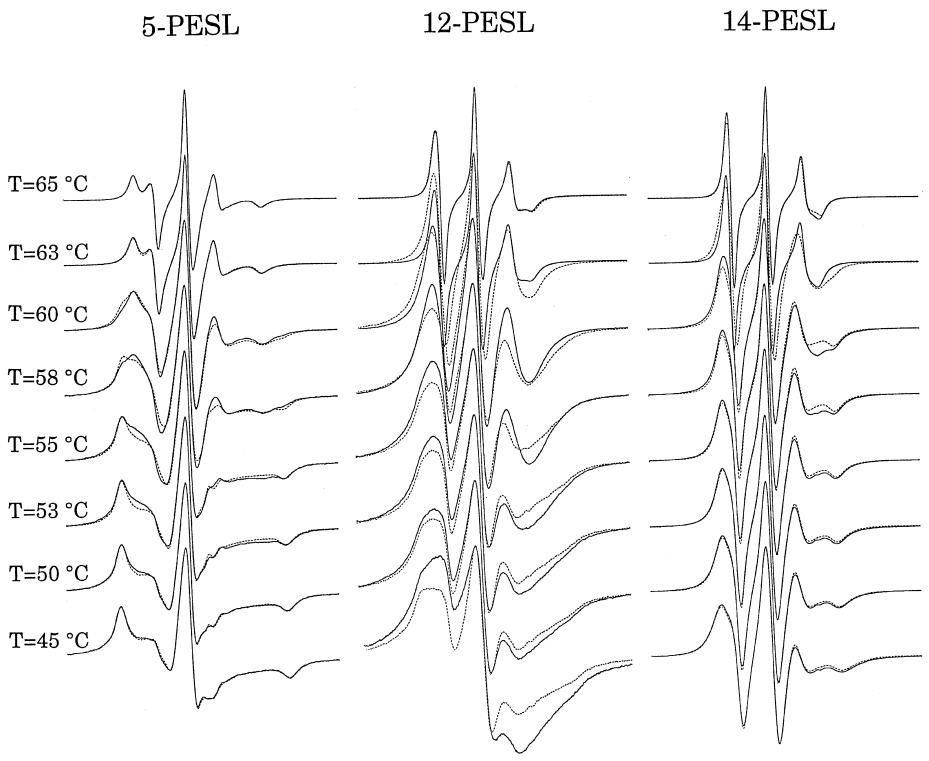

Fig. 4. ESR spectra of 5-, 12- and 14-PESL spin labels incorporated either in DPPE membranes (dotted line) or DPPE membranes containing losartan at mole fraction $x = 0.20$ (solid line) obtained at the temperatures indicated, over the range 45–65°C. Total scan width = 100 G.

45°C) while in pure DMPE this transition occurs at around 47°C ($\Delta A_{max}/(A_{max}-A_0) = 71.8\%$ between 45 and 50°C). ESR spectra of the 14-PESL spin label show that the preparation containing the antagonist undergoes the chain-melting phase transition between 40 and 45°C ($\Delta A_{max}/(A_{max}-A_0) = 74.8\%$ between 40 and 45°C). The corresponding pure lipid preparation undergoes this transition at around 47°C ($\Delta A_{max}/(A_{max}-A_0) = 62.4\%$ between 45 and 50°C). Moreover, in the presence of losartan, the mobility of the 14-PESL spin label is increased in the liquid-crystalline phase relative to in its absence, as is evident from the decrease of the outer hyperfine splitting $2A_{max}$ ($\Delta A_{max}/(A_{max}-A_0) = 39.0\%$ at 50°C).

Losartan was incorporated also into DPPE membranes at a mole fraction $x = 0.20$, and the corresponding ESR spectra of 5-, 12- and 14-PESL spin labels are shown in Fig. 4. Results qualitatively similar to those described for the DMPE preparation containing $x = 0.20$ of losartan are obtained. No effect of the antagonist on the mobility of the 5-PESL spin label is observed either in the gel or in the fluid phase at $x = 0.20$. Losartan shifts the chain-melting transition to between 55 and 58°C ($\Delta A_{max}/(A_{max}-A_0) = 42.8\%$ between 53 and 60°C), while membranes of DPPE alone exhibit this transition between 58 and 60°C ($\Delta A_{max}/(A_{max}-A_0) = 41.7\%$ between 55 and 63°C). An increase of spin-spin broadening on incorporation of the antagonist in DPPE bilayers is observed in the gel phase for the ESR spectra of the 12-PESL spin label. The degree of spin-spin broadening is much greater than for gel-phase DMPE (cf. Fig. 3). This precludes precise determination of the chain-melting event from the 12-PESL spectral lineshapes. Nonetheless, a depression of the chain-melting transition temperature by losartan is evident. In the case of 14-PESL spin labels, a shift of the chain-melting transition due to losartan incorporation is also observed. In the preparation containing the antagonist, this transition occurs at around 60°C ($\Delta A_{max}/(A_{max}-A_0) = 67.7\%$ between 58 and 63°C), while in the pure DPPE preparation it occurs at around 63°C ($\Delta A_{max}/(A_{max}-A_0) = 58.3\%$ between 60 and 65°C). Furthermore, the mobility

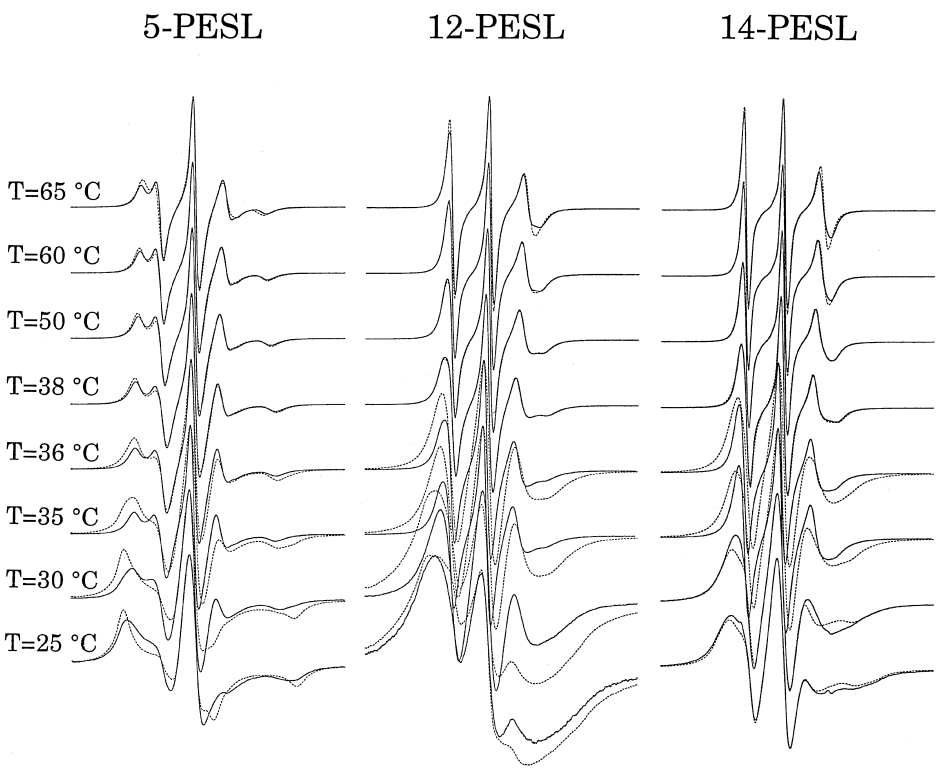

Fig. 5. ESR spectra of 5-, 12- and 14-PESL spin labels incorporated either in DEPE dispersions (dotted line) or DEPE dispersions containing losartan at mole fraction $x = 0.20$ (solid line) obtained at the temperatures indicated, over the range 25–65°C. Total scan width = 100 G.

of the 14-PESL spin label is increased in the liquid-crystalline phase by the presence of losartan ($\Delta A_{max}/(A_{max}-A_0) = 18.6\%$ at 65°C).

The ESR spectra obtained from the 5-, 12- and 14-PESL spin labels incorporated in DEPE dispersions containing losartan at mole fraction $x = 0.20$ (solid lines) are compared with the corresponding ones of DEPE preparation without the antagonist (dotted lines) in Fig. 5. The temperature dependences of the outer hyperfine splittings, $2A_{max}$, from the spectra of 5-PESL spin label incorporated in DEPE dispersions containing losartan at different concentrations ($x = 0.20$ or $x = 0.60$) are given in Fig. 6. As is evident from this figure, DEPE dispersions undergo a chain-melting transition at around 35°C ($\Delta A_{max}/(A_{max}-A_0) = 30.3\%$ between 30 and 36°C). When the antagonist is incorporated in DEPE dispersions at mole fraction $x = 0.20$, this transition is shifted to around 25°C ($\Delta A_{max}/(A_{max}-A_0) = 21.8\%$ between 25 and 30°C). At higher drug concentration, $x = 0.60$, the transition is broadened very considerably. Furthermore, in Fig. 6 a second transition is seen in dispersions of DEPE alone, at around 60°C. This corresponds to the structural phase transition from the lamellar liquid-crystalline to the inverted hexagonal H_{II} phase. The transition is shifted to between 70 and 75°C in the preparation containing losartan at $x = 0.20$, while it is not detected in the preparation containing the antagonist at $x = 0.60$.

At 50°C, DEPE membranes and preparation containing the antagonist at $x = 0.20$ are in the lamellar liquid-crystalline phase, as is seen in the corresponding thermograms (Fig. 2). The mobility of the 5-PESL spin label is increased due to the presence of losartan in the lamellar liquid-crystalline phase as is seen in Fig. 5 ($\Delta A_{max}/(A_{max}-A_0) = 2.4\%$ at 50°C). At 65°C, the mobility of the 5-PESL spin label is, however, decreased in the preparation containing the antagonist ($\Delta A_{max}/(A_{max}-A_0) = 10.3\%$). This switch can be explained by the different membrane organization of the preparations with and without losartan. At 65°C, DEPE membranes are organized in the inverted hexagonal H_{II} phase while the preparation containing losartan at mole fraction $x = 0.20$ is still

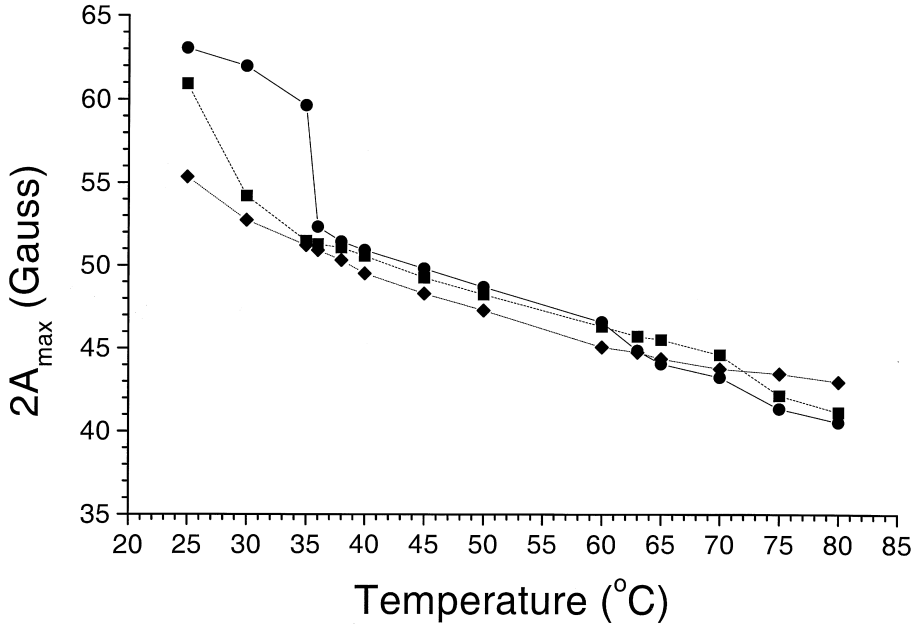

Fig. 6. Temperature dependence of the outer hyperfine splitting ($2A_{max}$) of 5-PESL spin label incorporated in DEPE dispersions containing losartan at different concentrations. (●) hydrated DEPE alone, (■) DEPE dispersions containing losartan at mole fraction $x = 0.20$, and (◆) DEPE dispersions containing losartan at mole fraction $x = 0.60$.

in the lamellar liquid-crystalline phase (Fig. 2). In the case of the 12-PESL spin label, the spin-spin broadening in the gel phase of the DEPE spectra is decreased by addition of losartan at mole fraction $x = 0.20$ (Fig. 5). In the lamellar liquid-crystalline phase, the mobility of the 12-PESL spin label is increased in the preparations containing the antagonist ($\Delta A_{max}/(A_{max} - A_0) = 4.2\%$ at 50°C). At 65°C, as in the case of the 5-PESL, the mobility of the spin label is decreased in the membranes containing losartan ($\Delta A_{max}/(A_{max} - A_0) = 43.8\%$). Similar effects are observed in the spectra of the 14-PESL spin labels from DEPE dispersions (Fig. 5). The mobility of the spin label is increased in the lamellar liquid-crystalline phase of preparations containing the antagonist ($\Delta A_{max}/(A_{max} - A_0) = 3.0\%$ at 50°C). On the other hand, a decrease in the mobility of the 14-PESL spin label is observed at 65°C, when losartan is incorporated into DEPE membranes at mole fraction $x = 0.20$ ($\Delta A_{max}/(A_{max} - A_0) = 78.9\%$).

3.3. ^{31}P-NMR spectroscopy

The effect of losartan on the thermotropic phase transitions of DEPE was further investigated by broad-line ^{31}P-NMR spectroscopy and the corresponding proton decoupled spectra are shown in Fig. 7. The powder line shapes of ^{31}P-NMR spectra from hydrated DEPE are characteristic of the structural organization of the phospholipid dispersions. For bilayers, an asymmetrical line shape with a high-field peak and a low-field shoulder is observed [34]. When DEPE is organized in a hexagonal H_{II} phase, a line shape of reversed asymmetry (high-field shoulder peak and a low-field peak), with a 2-fold reduction in effective chemical shift anisotropy, is obtained [35]. As is shown in Fig. 7A, up to 65°C, the ^{31}P-NMR spectra of hydrated DEPE alone are characteristic of a bilayer membrane organization. At higher temperatures, at least up to 86°C, DEPE is organized in a hexagonal H_{II} phase, as is evident from the powder pattern of reversed asymmetry. When losartan is incorporated into DEPE membranes at mole fraction $x = 0.05$ (Fig. 7B), the characteristic line shape of a bilayer phase is retained up to 68°C. At 70°C, a small signal appears, superimposed on the bilayer line shape. The resonance position of this signal coincides with the chemical shift of the low field peak of the hexagonal H_{II} phase line shape. At higher temperatures, the spectra are char-

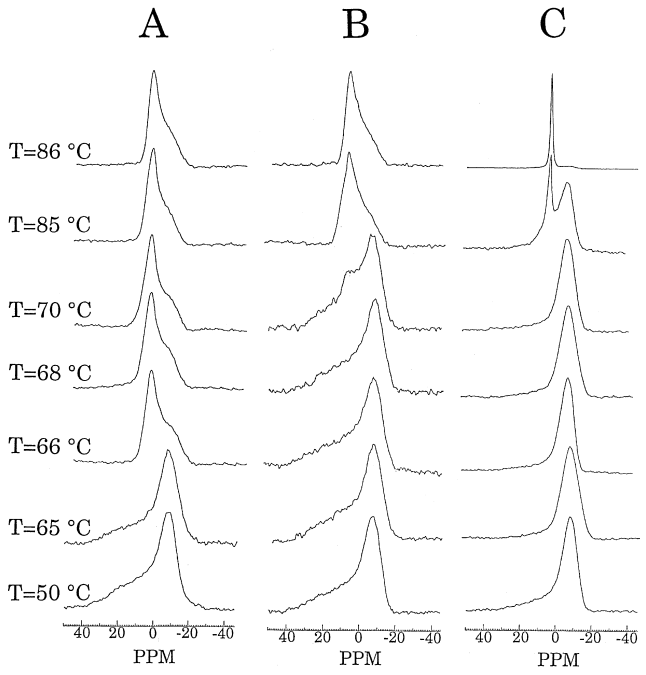

Fig. 7. Proton-decoupled ^{31}P-NMR spectra of DEPE dispersions (A) or DEPE dispersions containing losartan at mole fractions $x = 0.05$ (B) and $x = 0.20$ (C) obtained at the temperatures indicated, over the range 50–86°C.

acteristic of a hexagonal H_{II} phase organization. More pronounced spectral effects are observed when the antagonist is incorporated at the higher mole fraction of $x = 0.20$ (Fig. 7C). At temperatures up to 70°C, the line shape of the spectra is characteristic of a bilayer structure, but with evidence for magnetic alignment of the lipid dispersion (i.e., an increase in intensity of the $\sigma_\perp$ peak at the expense of the $\sigma_\parallel$ shoulder). A composite spectrum, indicative of a mixture of phases, is observed at 85°C, while at 86°C a narrow isotropic peak dominates. The latter is indicative of rapid motional averaging on the timescale of the ^{31}P-NMR chemical shift anisotropy. Such a line shape has been proposed to represent an intermediate phase between the hexagonal H_{II} and the bilayer phase [35]. Isotropic ^{31}P-NMR line shapes are not diagnostic on this point, however.

4. Discussion

The fact that besides its action on AT_1 and AT_2 receptors, ANG II also interacts with the lipid bilayer of biological membranes and the knowledge that the major determinants of losartan binding appear to be residues located within the transmembrane regions III, IV, V, VI and VII of the AT_1 receptor, led us to investigate the interactions of losartan, an AT_1 ANG II antagonist, with the phospholipid bilayer component of membranes [15]. In our first study, we showed that losartan interacts with phosphatidylcholine membranes by affecting both their thermotropic behavior and molecular mobility. Furthermore, it was suggested that, at low concentrations, the antagonist was located close to the interfacial region of the phosphatidylcholine bilayer while at high mole fractions it was inserted deeper in the bilayers. In the present study, we investigated the interactions of losartan with phosphatidylethanolamine membranes using DSC, ESR and ^{31}P-NMR spectroscopy.

Phosphatidylethanolamine is the second most abundant zwitterionic phospholipid in mammalian cell membranes, after phosphatidylcholine. Membranes composed of phosphatidylethanolamine differ from those of phosphatidylcholine, however, in terms of several important physical characteristics that are related to differences in headgroup hydration and hydrogen bonding [36]. Here we contrast the present results on the interactions of losartan with phosphatidylethanolamines with those that we obtained previously on the interactions with phosphatidylcholines. The study was conducted over a wide range of concentrations of the antagonist, extending to values that are well above the likely physiological range. This was done in order to obtain a more complete biophysical characterization of the membrane interactions with losartan. Furthermore, it allows a more detailed comparison with the results obtained previously with phosphatidylcholines over the same wide compositional range. It will be noted, however, that many of the changes induced by losartan increase progressively with increasing concentration, and are simply expressed with greater intensity at the higher concentrations.

Our DSC results show that losartan affects the cooperativity in thermodynamic properties of DMPE, DPPE and DEPE membranes by lowering the transition temperature from the gel to the lamellar liquid phase and broadening the corresponding endothermic peak (Figs. 1 and 2). These effects are formally similar to those observed for phosphatidyl-

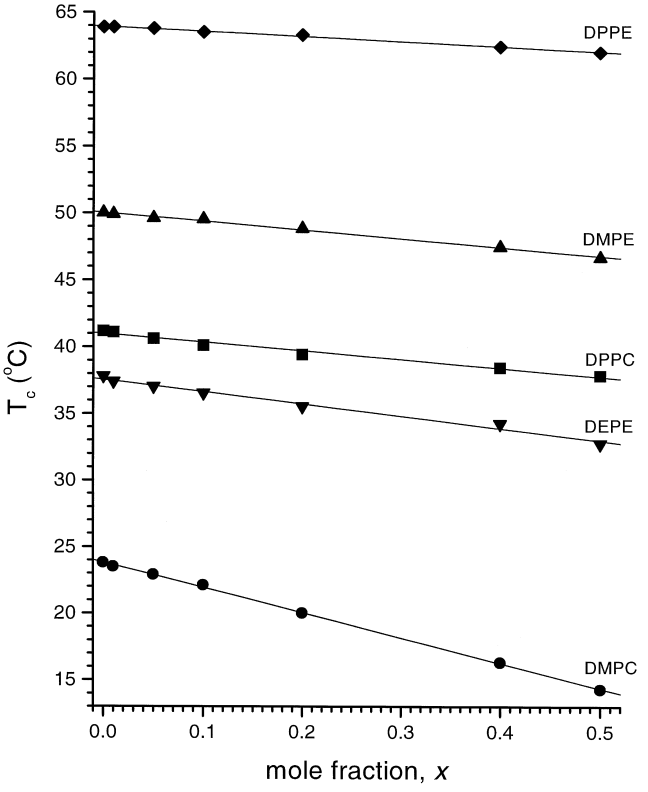

Fig. 8. Dependence of the chain-melting transition temperature, T_c, on mole fraction, x, of losartan in DMPC (●), DPPC (■), DMPE (▲), DPPE (♦) and DEPE (▼) bilayers. Data for DMPC and DPPC are from [15]. In each case, the value of T_c corresponds to the peak of the major calorimetric endotherm. For DMPC, which displays a complex extended chain-melting behavior, this corresponds to the major onset of chain melting. Lines are linear regressions.

choline bilayers [15]. In the case of phosphatidylcholines, the chain-melting behavior of the bilayers was sensitively affected by losartan. This was especially dramatic with DMPC bilayers, for which at high drug concentrations the thermotropic transition region was very extensively broadened and displayed a complex endothermic behavior characterized by three major calorimetric peaks. This behavior was associated with changes in the vesicular structure that were induced in this region [37]. Unlike with DMPC, such a strong effect of losartan on the thermotropic behavior is not observed here with DMPE. The morphological changes accompanying the complex melting behavior observed with DMPC have been related to solvent-dependent interactions [37]. Therefore it is likely that they are not exhibited with DMPE, as compared to DMPC, because phosphatidylethanolamine membranes are less highly hydrated than those of phosphatidylcholines [36,38]. It is not known with certainty whether the appearance of a shoulder on the main calorimetric endotherms of DMPE, DPPE and DEPE at losartan concentrations higher than $x=0.05$, $x=0.10$ and $x=0.01$, respectively (Figs. 1 and 2) is a manifestation of effects similar to those discussed for phosphatidylcholine bilayers [15]. Interestingly, the shoulder appears on the low-temperature side of the main endotherm rather than on the high-temperature side, as was observed with DMPC. This might correlate with the different gel-phase structures that are known for phosphatidylethanolamines compared with phosphatidylcholines.

The dependence of the peak chain-melting transition temperature, T_c, on mole fraction, x, of losartan incorporated is given in Fig. 8 for fully hydrated bilayers of the various phosphatidylcholine [15] and phosphatidylethanolamine species. In each case, the peak transition temperature decreases linearly with increasing mole fraction of losartan. This is in accordance with thermodynamic expectations for the freezing point depression by a single solute component. The gradient of the decrease in transition temperature with mole fraction of losartan differs between the different lipids. In the general case of solute partitioning between the gel and fluid lipid states, the depression in T_c is given by [39]:

$$T_c = T_c^o - \frac{2RT_c^{o^2}}{\Delta H_t^o}\frac{1-K}{1+K}\cdot x \qquad (1)$$

where R is the ideal gas constant, T_c^o and ΔH_t^o are the transition temperature and transition enthalpy, respectively, in the absence of solute, and K is the distribution coefficient of the solute between gel and fluid lipid phases. For the extreme case that the solute partitions completely into the fluid phase, then $K=0$ and the depression in transition temperature has its maximum value.

Values of the gradient in transition temperature, dT_c/dx, with mole fraction of losartan, obtained from linear regression are given in Table 3. All values are considerably smaller than the theoretical maximum, i.e., $-2RT_c^{o^2}/\Delta H_t$, values of which are also given in Table 3. These are calculated from values of T_c^o obtained from the linear regressions and of

Table 3
Dependence of chain-melting transition temperature, T_c, on mole fraction, x, of losartan in various lipid bilayers, deduced from linear regression[a]

Lipid	dT_c/dx (K)	$2RT_c^{o2}/\Delta H_t$ (K)	K
DMPC	−18.92 ± 0.21	66.10 ± 1.26	0.555 ± 0.003
DPPC	–6.60 ± 0.43	51.58 ± 0.70	0.773 ± 0.010
DMPE	–6.67 ± 0.21	81.35 ± 1.61	0.848 ± 0.002
DPPE	–3.84 ± 0.14	61.84 ± 0.85	0.883 ± 0.003
DEPE	–10.07 ± 0.65	54.75 ± 0.81	0.689 ± 0.013

[a] $dT_c/dx = -(2RT_c^{o2})/\Delta H_t$, if losartan is confined to the high temperature phase. K is the partition coefficient of losartan between gel and fluid phases.

ΔH_t^o from calorimetry as described above. Clearly, however, losartan partitions into the fluid phase in each of the phospholipid systems. Independent evidence for this, in several cases, comes from perturbation of the spin-labeled lipid ESR spectra in the fluid phase. The values of the distribution coefficient K, deduced from Eq. 1 and the data given in Fig. 8, are also presented in Table 3. A value of $K<1$ represents preferential partitioning into the fluid phase, i.e., a lowering of the transition temperature. A value of $K>1$ correspondingly represents a preferential partitioning into the gel phase, i.e., an increase in transition temperature. A direct correlation is found between the values of K and the chain-melting transition temperature, T_c^o, for all lipids. Those with a lower transition temperature favor partitioning into the fluid lipid phase in the region of phase coexistence at T_c. The most strongly enhanced effect is for DMPC, which explains why the chain-melting transition of this lipid is so effectively perturbed by losartan, as compared with both phosphatidylethanolamines and longer-chain phosphatidylcholines.

Losartan also affected the structural phase transition from the lamellar fluid phase to the inverted hexagonal H_{II} phase of DEPE, which was broadened and shifted to higher temperatures (Fig. 2B). The greater sensitivity of the H_{II} transition to perturbation by losartan is undoubtedly attributable to the much lower enthalpy and entropy of this fluid–fluid transition, relative to that for chain-melting [38]. The direction of the shift indicates that the fluid lamellar phase is stabilized relative to the H_{II} phase by losartan. From the data given in Table 2 and Eq. 1, a value of $K=1.2$ is estimated for the preferential partitioning of losartan into the lamellar fluid phase, relative to the H_{II} phase, of DEPE dispersions. This is consistent with a superficial or interfacial association of losartan with phosphatidylethanolamine bilayers rather than a penetration into the hydrophobic core. Such a location of the antagonist would increase the lipid headgroup area and consequently disfavor formation of the inverted H_{II} phase. Correspondingly, the accompanying perturbation of the tight lipid packing in the gel phase, will shift the gel to liquid-crystalline phase transition to lower temperature.

ESR spectroscopy was performed to study the effect of losartan on the chain motion and packing in bilayer membranes by using different positional isomers of phosphatidylethanolamine (*n*-PESL) spin probes incorporated in DMPE, DPPE or DEPE membranes. The ESR spectra obtained showed that losartan shifted the chain-melting phase transition to lower temperatures and broadened the region of phase coexistence, in agreement with DSC data (Figs. 1 and 2). Although mirroring the effects on the calorimetric transition, changes in chain dynamics precede the heat capacity maxima. This is a characteristic feature of phosphatidylethanolamine lamellae, as registered for instance by deuterium NMR spectroscopy [40]. A shift to lower temperatures and widening of the chain-melting region was also induced by losartan in phosphatidylcholine bilayers [15].

The incorporation of the antagonist in DMPE, DPPE or DEPE membranes at mole fraction $x=0.20$ does not significantly affect the chain mobility of the 5-PESL spin label (Figs. 3–5). This is consistent with a more superficial interaction of losartan with phosphatidylethanolamines under the conditions used. Perturbation of the chain mobility is almost negligible in the gel and fluid phases, outside the chain-melting transition region. These results are in contrast to the situation with phosphatidylcholines [15]. Even at a mole fraction $x=0.10$, losartan induced a decrease in chain mobility of phosphatidylcholine bilayers in both the gel and fluid phases. Additionally, this change was registered more sensitively by the spin label on the 5-position than by spin labels positioned further down the chain. Because of this observation, it was concluded that the antagonist is located closer to the interfacial region than to the

hydrophobic core of the phosphatidylcholine bilayer. It was proposed that one possible mode of insertion is location of losartan's polar hydroxyl group near the carbonyl groups of the phospholipids, with which it could form hydrogen bonds. This difference in the location of losartan in phosphatidylethanolamine and phosphatidylcholine membranes could be a consequence of the different degrees of hydration of the surfaces of the membranes composed of these two classes of lipids. Hydrogen bonding between the lipid headgroups in phosphatidylethanolamine membranes results in a more tightly packed and less hydrated structure that could resist the penetration of losartan beyond the superficial membrane interface.

Interestingly, the spin-spin broadening in the spectra of the 12-PESL spin probe incorporated in the gel phase of DMPE or DPPE is increased in the presence of losartan rather than being decreased as is the corresponding situation with phosphatidylcholine bilayers [15]. In the gel phase of phosphatidylcholine bilayers, the antagonist was found to improve the solubility of the lipid probes, and possibly of other components. This again suggests a different mode of association with the less strongly hydrated phosphatidylethanolamine bilayer membranes, such that close packing of the lipid chains is increased in the gel phase by the presence of losartan.

At high mole fractions ($x = 0.60$), losartan was found to insert deeper in the phosphatidylcholine bilayers, affecting more strongly the mobility of 14-PCSL spin probes for DMPC and DPPC membranes while the mobility of 5-PCSL was less affected [15]. In the case of DMPE membranes, when losartan was incorporated at high concentration ($x = 0.60$), the formation of a second component in the outer wings of the 14-PESL spin label spectra in the fluid phase was observed (data not shown). This indicates that at high concentrations, the antagonist is located deeper also within DMPE bilayers. Indeed, the effect is greater in DMPE bilayers than in phosphatidylcholines (specifically in DMPC), because a second more motionally restricted spectral component is not observed with the 14-position labels in the latter case. In our previous study [15], we proposed that the deeper penetration may be caused by self-association of losartan with intermolecular bonding of its polar hydroxyl groups that results in a more hydrophobic behavior of the antagonist. Self-association of losartan may be enhanced in phosphatidylethanolamine bilayers by intermolecular hydrogen bonding between the latter lipids. This, possibly together with hydrophobic mismatch, may account for the presence of the second more motionally restricted lipid component in DMPE bilayers, where the latter would correspond to lipids at the interface with the losartan complexes.

^{31}P-NMR spectroscopy was performed to further investigate the effect of losartan on the structural transition from the lamellar liquid-crystalline phase to the hexagonal H_{II} phase of DEPE membranes. Our ^{31}P-NMR data confirmed the results obtained by DSC, showing that the presence of the antagonist stabilizes the fluid lamellar phase relative to the H_{II} phase. Formation of hexagonal-phase intermediates has been associated with membrane fusion [41]. A substance which is located near the surface of a bilayer can inhibit the formation of a hexagonal phase, as well as inhibiting membrane fusion phenomena and other mechanisms of lipid exchange [42]. The present results with DEPE indicate that losartan can function in this manner.

In conclusion, our results show that losartan affects the thermotropic behavior of phosphatidylethanolamine membranes, while the molecular mobility of the membranes is not significantly affected, in contrast to the situation with phosphatidylcholine bilayers. Furthermore, the interactions of the antagonist with phosphatidylethanolamine membranes, at least at low drug concentrations, are more superficial than with phosphatidylcholine bilayers.

Acknowledgements

This work was carried out at the Max-Planck-Institut für biophysikalische Chemie, Göttingen, Germany, as part of a European Community training Project for Training and Mobility of Researchers financed by the European Commission. E.T. was the recipient of a TMR Marie Curie Research Training Grant (ERBFMBICT972467). We are grateful to MSD Sharp and Dohme GmbH company for providing losartan. We also acknowledge both Merck and Du Pont companies. We would like to thank Frau B. Angerstein for the synthesis of spin-labeled phospholipids and for excellent technical assistance

and Frau I. Dreger for her help in the preparation of the manuscript.

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