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Differential effects of oleuropein, a biophenol from *Olea europaea*, on anionic and zwiterionic phospholipid model membranes

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Abstract

Oleuropein (Ole) is the major phenolic constituent of the olive leaf (*Olea europaea*) and it is also present in olive oil and fruit. In the last years several compounds from olive tree, oleuropein among them, have shown a variety of biological activities such as antimicrobial or antioxidant. A phospholipid model membrane system was used to study whether the Ole biological effects could be membrane related. Ole showed a significant partition level in phospholipid membranes, i.e. 80%, at lipid-saturating conditions. Moreover, fluorescence quenching experiments indicated a shallow location for Ole in membranes. Ole promoted weak effects on zwiterionic phospholipids such as phosphatidylcholine or phosphatidylethanolamine. In contrast, differential scanning microcalorimetry, light scattering and fluorescence anisotropy pH titration studies revealed strong effects on anionic phospholipids such as phosphatidylglycerol at physiological pH and salt conditions. These effects consisted on perturbations at the phospholipid membrane surface, which might involve specific molecular interactions between Ole and the negatively charged phosphate group and therefore modify the phospholipid/water interface properties. It is proposed that Ole induces lipid structures similar to the gel–fluid intermediate phase (IP) described for PG membranes, in a similar way than low ionic strength does. These effects on phosphatidylglycerol may account for the antimicrobial activity of Ole.

Keywords: Oleuropein; Phospholipid membranes; Phosphatidylglycerol; DSC; Fluorescence; Gel–fluid intermediate phase; Antimicrobial

Abbreviations: $\langle r \rangle$, steady-state fluorescence anisotropy; 16-NS, 16-doxyl-stearic acid; 5-NS, 5-doxyl-stearic acid; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphate; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine]; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DSC, differential scanning calorimetry; H_{II}, inverted hexagonal-H_{II} phase; HPLC, high performance liquid chromatography; IP, gel–fluid intermediate phase; K_P, phospholipid/water partition coefficient; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; Ole, oleuropein; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; T_c, onset temperature of the gel to liquid-crystalline phase transition; T_m, gel to liquid-crystalline phase transition temperature; T_m^{off}, offset temperature of the IP region; T_m^{on}, onset temperature of the IP region; VHSV, viral haemorrhagic septicaemia virus

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1. Introduction

Leaves and drupes from olive tree, *Olea europaea*, are rich in olive biophenols, such as oleuropein, verbascoside, ligstroside, tyrosol and hydroxytyrosol, which have exhibited antioxidant and antimicrobial properties. Oleuropein (Ole) is the major biophenol in olive leaf and fruit (Benavente-García et al., 2000) (Fig. 1). This compound is a phenolic secoiridoid glycoside with hydroxyaromatic functionality deriving from the shikimate and phenylpropanoid metabolism. Hydroxytyrosol and oleuropein, have been shown to be potent radical scavengers (Visioli et al., 1998; Saija et al., 1998; Benavente-García et al., 2000; Gordon et al., 2001; Briante et al., 2001; Saija and Uccella, 2001; Paiva-Martins et al., 2003). In addition, the presence of some biophenols in olive oil and drupes, among other factors, has been related to the prevention of coronary artery disease and atherosclerosis because of their capability to inhibit platelet aggregation (Carluccio et al., 2003), arachidonic acid metabolism modulation (Kohyama et al., 1997) and to inhibit LDL peroxidation (Visioli et al., 1995, 2002; Visioli and Galli, 2002).

Furthermore, some of these compounds have demonstrated to have antimicrobial activity by inhibiting the growth of a wide variety of bacteria (Aziz et al., 1998; Bisignano et al., 1999), fungi (Tassou et al., 1991; Aziz et al., 1998) and viruses (Renis, 1969; Hirschman, 1972; Fredrickson, 2000; Ma et al., 2001). In particular, oleuropein has exhibited antibacterial activity against a variety of Gram-positive and Gram-negative human pathogenetic bacterial strains (Bisignano et al., 1999). Besides, oleuropein has shown antiviral activity mostly against enveloped virus (Bisignano et al., 1999; Ma et al., 2001; Fredrickson, 2000; Micol et al., 2005). It is also proposed that the lower antimicrobial capacity of oleuropein in comparison to hydroxytyrosol might be due to its lower capability to penetrate the cell membrane due to its glycosidic structure (Saija and Uccella, 2001).

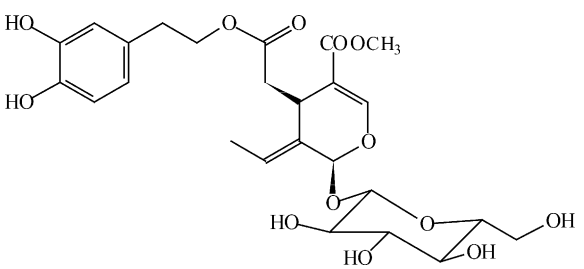

Fig. 1. Chemical structure of the secoiridoid oleuropein.

The putative molecular mechanism of the wide biological activity of Ole has been pointed out only in a few cases. This compound, when digested by β-glucosidases present in separate plant leaf compartments or deriving from intestinal bacteria, yields a glutaraldehyde-like structure which exhibits protein-denaturing, protein-crosslinking and lysine-alkylating properties (Konno et al., 1999).

Some Ole effects may be also related to its capacity to interact with biological membranes. It is presumed that this compound, being a water and lipid soluble molecule, can get through cell membranes in some extent reaching intracellular targets in a similar way than hydroxytyrosol does (Kohyama et al., 1997). In fact, it has been shown that a significant amount of Ole reaches intracellular targets in isolated erythrocytes (Saija and Uccella, 2001). Ole partition coefficient has been previously determined in octanol/water and oil/water systems through UV absorbance or HPLC measurements (Saija et al., 1995, 1998; Gordon et al., 2001; Paiva-Martins et al., 2003) but it has not yet been determined in a phospholipid model membrane system. In addition, the association of Ole with biological membranes may promote changes in the membrane physical properties and therefore modulate membrane-dependent biological processes. To this respect, we have recently shown that anthraquinones, some of them bearing a glucoside moiety, promote important changes on the surface of membranes containing negatively charged phospholipids which could account for their antimicrobial activity (Alves et al., 2004).

Some authors have previously studied the interaction of Ole with model membranes containing phosphatidylcholine and linoleic acid postulating that Ole may act as an internal radical scavenger against lipid peroxidation within biomembranes (Saija et al., 1998). In contrast, other authors have recently proposed a superficial location for Ole in phospolipid bilayers where this compound would play a role as an effective antioxidant (Paiva-Martins et al., 2003).

In this work, phospholipid model membranes were used first to determine the phospholipid/water partition

coefficient of Ole in a membrane system. Second, Ole's effect on the thermotropic properties of model membranes composed of zwiterionic or anionic phospholipids was studied by differential scanning calorimetry (DSC). Fluorescence spectroscopy studies were used to study the potential location of Ole in the membrane. Finally a combination of DSC, fluorescence anisotropy pH titration and light scattering studies was used to determine the effect of Ole on the phospholipid/water interface of membranes composed of anionic phospholipids such as phosphatidylglycerol (PG). The results obtained support a superficial location of oleuropein in membranes and show significant changes on the surface of PG membranes to account for part of its biological effects.

2. Experimental procedures

2.1. Materials

1,2-Dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DMPG), 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DPPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (DMPS), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), 1,2-dimyristoyl-*sn*-glycero-3-phosphate (monosodium salt) (DMPA), and 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) were obtained from Avanti Polar Lipids (Birmingham, AL, USA). Stock solutions of lipids were prepared in chloroform/methanol (1:1) and stored at -20 °C. The spin labels 5-doxyl-stearic acid (5-NS) and 16-doxyl-stearic acid (16-NS), and the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Molecular Probes, Inc. (Eugene, OR). Acrylamide was obtained from Sigma (St. Louis, MO, USA). All other compounds were of analytical or spectroscopic reagent grade. Double-distilled and deionized water was used throughout this work. Oleuropein (Ole) was obtained from Extrasynthese (Genay, France) and further purified by reverse phase preparative high performance liquid chromatography. Ole stocks were prepared in ethanol and its concentration was determined by using a molar absorptivity value of $2985 \pm 25\ M^{-1}\ cm^{-1}$ at 280 nm.

2.2. Determination of oleuropein partition coefficient in model membranes

Fluorescence measurements of Ole were recorded with an SLM-8000C spectrofluorimeter fitted with Glan–Thompson polarisers. The partition coefficient, K_P, of Ole was determined from its intrinsic fluorescence intensity increase upon the incorporation to large unilamellar vesicles (LUVs) composed of DMPC at 30 °C, as compared to that in the aqueous phase, from experiments where Ole concentration was kept constant and phospholipid concentration was varied (Mateo et al., 2000). The phospholipid/water partition coefficient, K_P, was defined as

$$K_P = \frac{n_L / V_L}{n_W / V_W}, \tag{1}$$

where n_i stands for moles of compound in phase i and V_i for volume of phase i; the phase was either aqueous ($i = W$) or lipidic ($i = L$). The quantitation of K_P was done according to:

$$\Delta I = \frac{\Delta I_{max}[L]}{1/(K_P \gamma) + [L]}, \tag{2}$$

where ΔI ($\Delta I = I - I_0$) stands for the difference between the fluorescence intensity of Ole measured in the presence (I) and in the absence of the phospholipid vesicles (I_0); $\Delta I_{max} = I_\infty - I_0$ is the maximum value of this difference once the limiting value is reached (I_∞) upon increasing the phospholipid concentration [L], and γ is the molar volume of the phospholipid ($0.737\ M^{-1}$ for DMPC in the fluid phase) (Marsh, 1990).

Multilamellar vesicles (MLVs) were formed by resuspending the dried phospholipid in the same buffer as the one used for DSC experiments (described later in this section). The vesicle suspension was then heated at a temperature above the phase transition of the phospholipid and vortexed several times. LUVs with a mean diameter of 90 nm were prepared from these MLVs by 10× pressure extrusion through 0.1 μm polycarbonate filters (Nucleopore, Cambridge, MA, USA) and phospholipid content was determined (Böttcher et al., 1961). Samples were excited at 280 nm and fluorescence emission was recorded at 315 nm.

2.3. Quenching of oleuropein fluorescence in model membranes

Differential quenching data using 5-NS and 16-NS spin probes were analyzed by the Stern–Volmer plot of I_0/I versus $[Q]_L$, where I_0 and I stand for the fluorescence intensity of Ole in the absence and in the presence of the quencher, respectively, and $[Q]_L$ is the quencher concentration in the phospholipid phase given by:

$$[Q]_L = \frac{K_{PQ} V_T}{V_W + V_L K_{PQ}} [Q]_T, \quad (3)$$

where $K_{PQ} = [Q]_L/[Q]_W$ is the partition coefficient of the quencher between the phospholipid phase and the aqueous phase, $[Q]_T$ the concentration of the quencher in the total volume V_T ($V_T = V_L + V_W$) and V_L and V_W are, respectively, the volume of the lipid and aqueous phases. For 5-NS and 16-NS, K_{PQ} in the fluid phase are 89,000 and 9730 respectively (Wardlaw et al., 1987). Experiments were carried out in LUVs of DMPC containing Ole at 30 °C, at lipid-saturating conditions, by adding aliquots from a 1 mM ethanolic solution containing either the 16-NS or 5-NS spin probes to the vesicles suspension. Measurements were taken immediately after preparation.

The hydrophilic quencher acrylamide was used in order to study the presence of Ole molecules accessible to the aqueous phase and to confirm the possible location of this compound in phospholipid model membranes. LUVs composed of DMPC were incubated with the appropriate amounts of Ole for 30 min at 30 °C before the quenching experiments. Then quenching of Ole was performed in the absence and in the presence of DMPC vesicles, at lipid-saturating conditions, by adding acrylamide from a 4 M acrylamide/water solution to the Ole/vesicles or Ole/buffer suspensions. Acrylamide quenching data were analyzed by the modified Stern–Volmer plot that accounts for both collisional and static quenching modes (Eftink and Ghiron, 1976).

2.4. Differential scanning calorimetry measurements

Chloroform/methanol solutions containing 2.4 μmol of total phospholipid (DMPC, DMPG, DMPS, DMPA, DMPE, DPPG, or DEPE) and the appropriate amount of Ole were dried under a stream of oxygen-free N_2 in order to obtain a thin film at the bottom of small thick-walled glass tubes. Last traces of solvent were removed by keeping the samples under high vacuum for >3 h. MLVs were formed by incubating the dried lipid on 1.6 ml of buffer (10 mM HEPES, 0.1 mM EDTA) with the desired pH and NaCl concentration for about 15 min at a temperature above the gel to liquid-crystalline phase transition, but below the temperature of the lamellar liquid-crystalline to hexagonal-H_{II} phase transition for DEPE containing vesicles and at 50 °C for DMPG vesicles (above the broad transition observed under low salt conditions) (Riske et al., 2002) with occasional vigorous vortexing and three freeze-thaw cycles. For all samples, the medium pH was checked to be stable at the desired value. Thermograms were recorded on a high-resolution Microcal MC-2 differential scanning microcalorimeter, equipped with a DA-2 digital interface and data acquisition utility for automatic data collection as previously described (Micol et al., 2001; Villalain et al., 2001; Caturla et al., 2003). After the thermal measurements, the phospholipid content of the sample was determined as above-mentioned.

2.5. Steady-state fluorescence anisotropy

DMPG or DMPC model membranes (MLVs) containing different Ole concentrations were prepared as described for DSC measurements with 10 mM HEPES, 0.1 mM EDTA and 100 mM NaCl at different pH values. Sample preparation was essentially performed as previously described (Riske et al., 2002). The sample pH was adjusted by addition of HCl below pH 7.4 and was controlled before and after the measurements. The phospholipid suspensions were adjusted to a final phospholipid concentration of 1 mM in order to eliminate the effect of lipid dilution in the thermal behavior of DMPG. Aliquots of DPH in *N*,*N*′-dimethylformamide (3.1×10^{-4} M) were directly added into the lipid dispersion to obtain a probe/lipid molar ratio of 1/200. Samples were incubated for 1 h well above the gel to liquid-crystalline phase transition temperature and 2 min more at 50 °C for DMPG samples, and measurements were taken immediately thereafter. The steady-state fluorescence anisotropy, $\langle r \rangle$, of DPH was measured in a Varian Clary Eclipse spectrofluorimeter as previously described (Caturla et al., 2003). The temperature was controlled with a Varian temperature

controller and the measures were done at a constant rate of 0.25 °C/min over the range from 10 to 55 °C. The samples were stabilized at the desired temperature and measurements were done with continuous mixing. The steady-state anisotropy $\langle r \rangle$, defined by:

$$\langle r \rangle = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2G I_{VH}}, \quad (4)$$

where the G-factor, accounting for differential polarization sensitivity, was determined by measuring the polarized components of fluorescence of the probe with horizontally polarized excitation ($G = I_{HV}/I_{HH}$).

2.6. 90° light scattering measurements of DMPG/oleuropein dispersions

An SLM-8000C spectrofluorimeter (Spectronics Instruments, Urbana, IL, USA) set at a wavelength of 305 nm was used. The temperature was controlled within the range 10–40 °C using a Haake external bath and measured utilizing a thermoprobe placed inside the cuvette. MLVs composed of DMPG and containing Ole at different molar percentages were stabilized for 5 min at the desired temperature before measurements were done. Final lipid concentration in the cuvette was 3 mM.

3. Results

3.1. Determination of the phospholipid/water partition coefficient of oleuropein

Previous studies have pointed out a significant partition of Ole in hydrophobic phases using oil/water or octanol/water systems (Saija et al., 1995, 1998; Gordon et al., 2001; Paiva-Martins et al., 2003). Therefore, we used a membrane model system composed of LUVs of DMPC to determine the phospholipid/water partition coefficient, K_P (Eq. (1)). The membrane model system possesses a higher physiological relevance as an approximation to biological membranes compared to the abovementioned systems. For that purpose the fluorescent properties of Ole were utilized. Fluorescence spectra of Ole in dilute solutions (5×10^{-5} M) were recorded in different aqueous solutions (pH 7.4 and 5.5), cyclohexane and ethanol (data not shown). Fluorescence in water and saline buffers was much less intense than in solvents such as cyclohexane, methanol or ethanol. The maxima for the fluorescence excitation and emission spectra were 280 and 315 nm respectively.

The increase in the fluorescence intensity observed for Ole in the presence of phospholipid vesicles as compared to that in the aqueous phase was used to quantify its K_P (Eq. (2)) (Fig. 2) (Mateo et al., 2000; Caturla et al., 2003). A two parameters (ΔI_{max} and K_P) fitting procedure was performed, as described in Section 2, on the experimental data obtained from the enhancement of Ole fluorescence upon addition of LUVs composed of DMPC at 30 °C. Then, a K_P value of $(0.920 \pm 0.080) \times 10^3$ was obtained at lipid-saturating conditions (5 mM DMPC versus 50 μM Ole), which are closer to physiological conditions. This value was similar to that one obtained for other antioxidant phenolic compounds such as non-galloylated catechins (Caturla et al., 2003).

3.2. Localization of oleuropein in membranes by fluorescence quenching

The relative quenching of Ole fluorescence by the lipophilic spin probes 5-NS and 16-NS, and incorporated into the fluid phase of DMPC vesicles, was

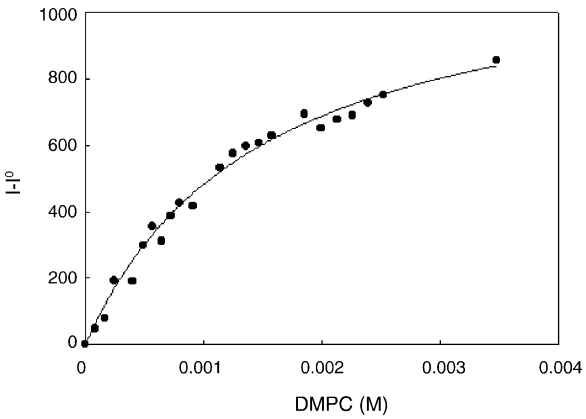

Fig. 2. Determination of the partition coefficient, K_P, of oleuropein from the fluorescence intensity increase upon incorporation to phospholipid LUVs of DMPC at 30 °C. ΔI stands for the difference between the fluorescence intensity of the oleuropein measured in the presence (I) and in the absence of lipid (I_0). The solid line is the best fit of Eq. (2) to the experimental data.

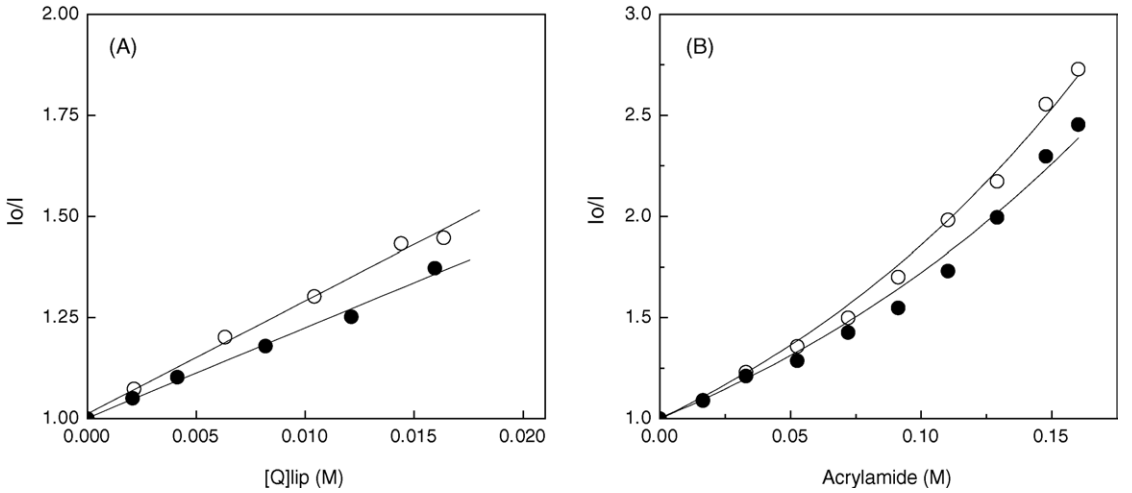

Fig. 3. Stern–Volmer plots for the quenching of oleuropein fluorescence by spin probes or acrylamide. (A) Stern–Volmer plots for the quenching of Ole fluorescence at a concentration of 50 μM by 5-NS (◯) and 16-NS (●) and incorporated into LUVs of DMPC at 30 °C at lipid-saturating conditions. (B) Quenching of Ole fluorescence by acrylamide. Ole was either dissolved in buffer (◯) at a concentration 50 μM, or incorporated into LUVs of DMPC (●) at lipid-saturating conditions.

utilized to determine the transverse location of Ole into the lipid bilayer. The addition of any of the two nitroxide quenchers resulted in a decrease of the Ole fluorescence. The Stern–Volmer plots of the fluorescence intensity changes for Ole were linear within the utilized concentration range of the quencher in the lipid phase (Fig. 3A). Ole cromophore group seemed to be quenched by both spin probes with a similar efficacy (Fig. 3A), although a little higher quenching was observed for 5-NS, a probe that covers the membrane interface region (Ellena et al., 1988). This result suggests a location of the cromophore group of the molecule (diphenylethanol moiety, Fig. 1) closer to 5-NS probe in phospholipid membranes composed of PC. A similar behavior and quenching by using these probes have been previously observed for (−)-epigallocatechin gallate, a galloylated catechin (Caturla et al., 2003).

Accessibility of Ole to membranes was further studied and confirmed by using acrylamide, a hydrophilic quencher which quenches essentially fluorophores present in the aqueous phase or in the lipid/water interface (Chalpin and Kleinfeld, 1983). Fig. 3B shows the quenching of Ole fluorescence by acrylamide in buffer or in the presence of DMPC vesicles. The upward curvature of the quenching plots, previously described (Eftink and Ghiron, 1976; Caturla et al., 2003), was due to the presence of two quenching effects: collision (dynamic quenching) and complex formation (static quenching) between the acrylamide and the fluorophore. As it is shown in Fig. 3B, Ole was quenched with a similar efficacy by acrylamide both in aqueous buffer and in the presence of DMPC vesicles, which corroborates a shallow location of this molecule at the membrane.

3.3. Effect of oleuropein on the thermotropic behavior of neutral or negatively charged phospholipid model membranes

The effect of Ole in the thermotropic behavior of several phospholipids was studied by using model membranes. Although a screening of Ole concentrations was performed, only DSC profiles corresponding to dispersions of pure phospholipid and phospholipid containing the highest concentration of Ole utilized are shown in some cases in Fig. 4 for the sake of briefness. Firstly, thermograms for pure DMPC, a zwiterionic phospholipid, and DMPC containing 30 mol% of Ole are shown in Fig. 4A, concentration at which no drastic changes in the thermotropic behavior of this phospholipid were observed. The onset temperature,

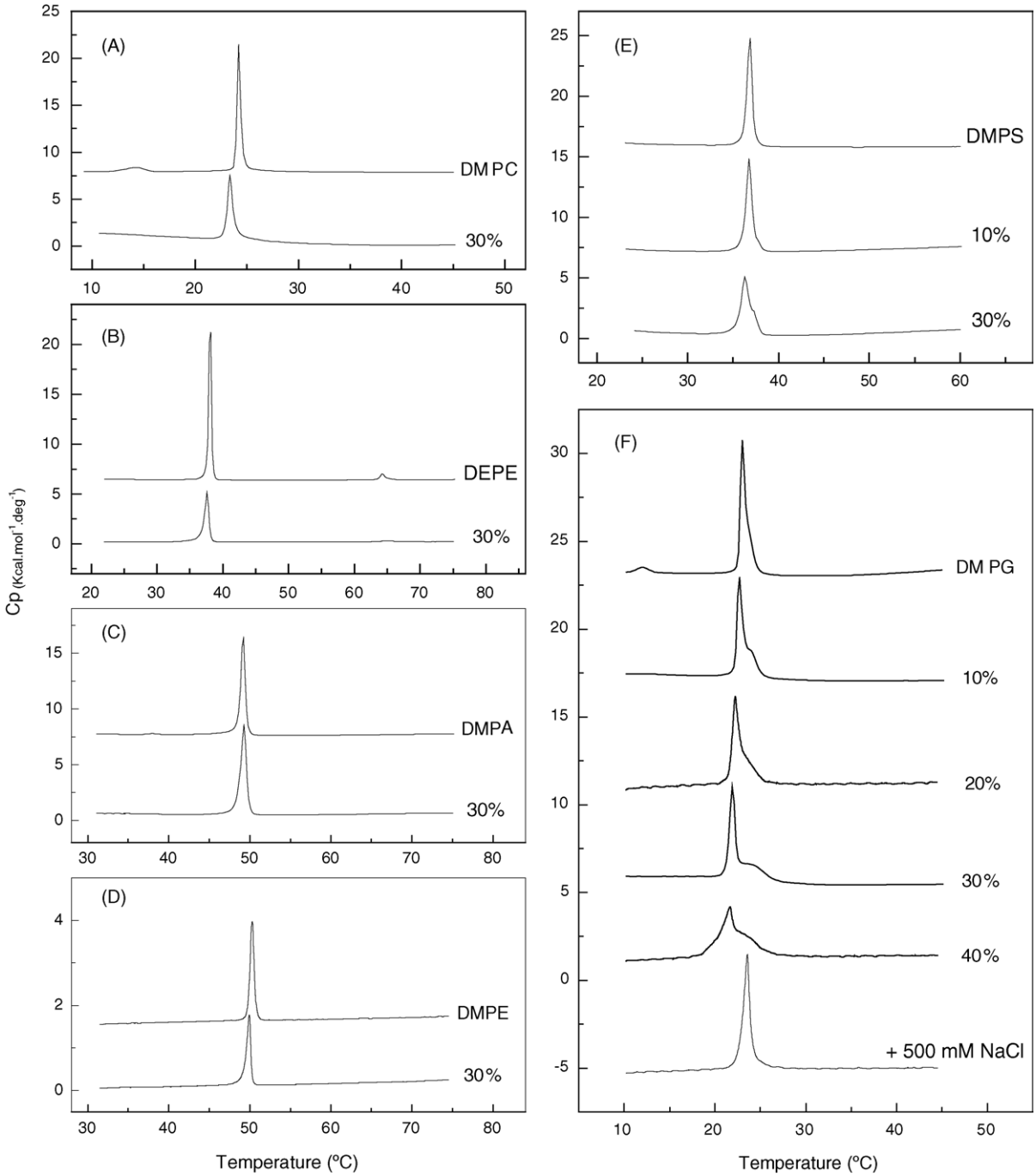

Fig. 4. Differential scanning calorimetry heating thermograms for phospholipid dispersions composed of DMPC (A), DEPE (B), DMPA (C), DMPE (D), DMPS (E) or DMPG (F), and these phospholipids containing Ole at different molar percentages in 10 mM HEPES, 0.1 mM EDTA, 100 mM NaCl, pH 7.4 buffer, unless otherwise stated. The concentration of Ole in the mixtures (molar % of total) is indicated in the curves.

T_c, of the gel to liquid-crystalline ($L_\beta \rightarrow L_\alpha$) phase transition was slightly shifted to lower temperatures, its enthalpy faintly decreased, but neither additional lipid phase transitions nor significant broadening were observed at 30 mol% of Ole (i.e. 0.3 mol fraction).

Secondly, the effect of Ole on model membranes composed of DEPE, an unsaturated zwiterionic phospholipid bearing a *trans* configuration of the C=C bond, was also studied through DSC in order to determine the effect of Ole on non-lamellar phases such as the inverted hexagonal-H_{II} phase. Aqueous dispersions of pure DEPE usually undergo two phase transitions: a gel to liquid-crystalline ($L_\beta \rightarrow L_\alpha$) phase transition in the lamellar phase (∼37 °C), and a lamellar liquid-crystalline to hexagonal-H_{II} ($L_\alpha \rightarrow H_{II}$) transition (∼65 °C) (Gallay and De Kruijff, 1984) (Fig. 4B). The incorporation of Ole into DEPE decreased the temperature of the lamellar liquid-crystalline to hexagonal-H_{II} phase ($L_\alpha \rightarrow H_{II}$) transition and induced a gradual broadening of this transition, which was completely abolished for DEPE dispersions containing 30 mol% Ole. In contrast, the gel to liquid-crystalline phase transition of DEPE maintained its onset temperature almost invariable upon incorporation of amounts of Ole as high as 30 mol%, although the transition enthalpy was decreased (Fig. 4B).

Since the fluorescence spectroscopy results obtained in this work suggested a superficial location for Ole in membranes in agreement with a recent study (Paiva-Martins et al., 2003), the possibility of interactions between oleuropein and charged phospholipids at the phospholipid/water interface was explored. For that purpose, the effect of Ole on model membranes having different phospholipid head groups and bearing the same acyl chain composition was studied. The effect of Ole on several negatively charged phospholipids such as DMPA, DMPS or DMPG, and also on DMPE, a saturated phosphatidylethanolamine, was studied at physiological pH. Multilamellar vesicles composed of pure DMPA, DMPE, DMPS or DMPG and these phospholipids containing several percentages of Ole are shown in Fig. 4C–F respectively. No significant effect was observed when a concentration of Ole as high as 30 mol% was incorporated into multilamellar vesicles composed either of DMPA (Fig. 4C) or DMPE (Fig. 4D). A weak effect, consisting in the appearance of a shoulder corresponding to a new transition peak at temperatures above the gel to liquid-crystalline phase transition, was observed when the same amount of Ole, i.e. 30 mol%, was incorporated into DMPS vesicles (Fig. 4E).

In contrast, when Ole was incorporated into DMPG vesicles, more drastic changes were observed (Fig. 4F). At 10 mol% Ole, a broad shoulder appeared at temperatures above the gel to liquid-crystalline ($L_\beta \rightarrow L_\alpha$) phase transition, which became broader as Ole content increased. The sharp transition corresponding to pure phospholipid, 23 °C, was concomitant to the broad transition at all Ole concentrations studied (Fig. 4F). The gel to liquid-crystalline ($L_\beta \rightarrow L_\alpha$) phase transition of DMPG was also shifted to lower temperatures as the concentration of Ole was raised. This complex behavior of DMPG is identical to that one described for this phospholipid when very low ionic strength conditions are used (Epand and Hui, 1986; Heimburg and Biltonen, 1994; Riske et al., 1997; Lamy-Freund and Riske, 2003). In these conditions, DMPG undergoes two phase transitions: one corresponding to the main gel–fluid transition, and a second broad one, called post-transition. Between these two temperatures, it has been proposed the presence of reversible structures postulated as extended bilayer networks (Schneider et al., 1999) or as gel–fluid intermediate phase (IP) (Heimburg and Biltonen, 1994; Riske et al., 2002). These structures are characterized by low turbidity, high viscosity and high curvature. It has been recently reported that these features might be due to the presence of perfored unilamellar vesicles (Riske et al., 2004). Our results indicate that Ole, at 100 mM NaCl, is able to produce an identical effect on DMPG to that one appearing for pure DMPG at much lower salt conditions. The behavior showed by DMPG/Ole dispersions was reversed by very high NaCl concentrations, such as 500 mM NaCl (Fig. 4F).

3.4. Effect of oleuropein on the light scattering of DMPG dispersions at physiological salt and pH conditions

DMPG gel–fluid intermediate phase have also been previously monitored by light scattering measurements (Heimburg and Biltonen, 1994; Riske et al., 1997; Lamy-Freund and Riske, 2003). At low ionic strength, DMPG dispersions undergo a sharp decrease of the light scattered at $\theta = 90^\circ$ at the temperature corresponding to the gel to liquid-crystalline phase transition.

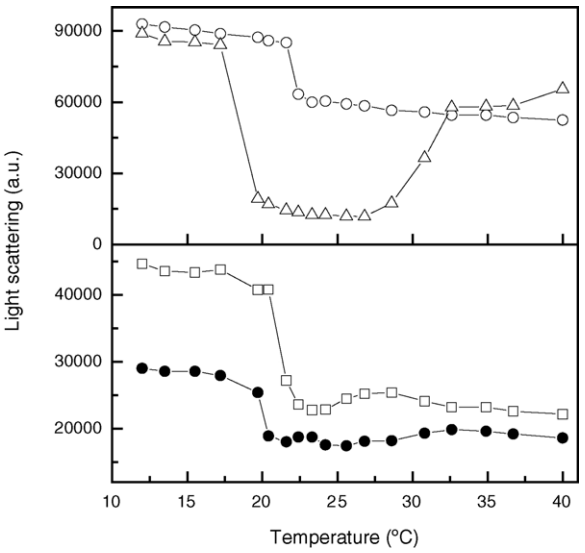

Fig. 5. Temperature dependence of 90° light scattering (λ = 305 nm) of 3 mM DMPG dispersions in 10 mM HEPES, 0.1 mM EDTA, pH 7.4 buffer, no salt added (△), and DMPG dispersions containing different amounts of Ole in 100 mM NaCl-HEPES buffer: (◯) pure DMPG, (□) 20 mol% Ole and (●) 40 mol% Ole. Curves are the average of three independent measurements.

Furthermore, an increase in the scattering is observed at higher temperatures, approximately 30–32 °C, which corresponds to the post-transition (Fig. 5, upper panel). In contrast, pure DMPG undergoes only a sharp transition at 22 °C in the presence of 100 mM NaCl (Fig. 5, upper panel). The incorporation of Ole in DMPG vesicles, at an ionic strength of 100 mM NaCl, promoted a light scattering profile much similar to that one shown by the pure phospholipid at low ionic strength (Fig. 5, lower panel). Nevertheless a less dramatic change of the light scattered was observed compared to that one shown by pure DMPG at low salt condition. The low scattering region, which could correspond to the gel–fluid intermediate region, became broader as the Ole content increased, in agreement with the DSC data (Fig. 4F).

3.5. Effect of oleuropein on the structural order of DMPG model membranes as a function of the pH

The fluorescent probe DPH has been demonstrated to monitor the structural order of acyl chains in deep regions of the phospholipid palisade (Kaiser and London, 1998) and is equally distributed in the gel and fluid phases (Lentz et al., 1976). To test the role of the head group protonation of DMPG on Ole effect, the pH titration of the fluorescence anisotropy of the probe DPH incorporated into DMPG dispersions was studied in the presence and in the absence of Ole at 100 mM NaCl (Fig. 6). For pure DMPG at pH 7.4, $\langle r \rangle$ decreased slightly as temperature increased, with a sharp drop occurring at approximately 23 °C (Fig. 6A, open symbols), which corresponds to the phospholipid gel to liquid-crystalline phase transition temperature (T_m) observed by DSC (Fig. 4F; Fig. 6B, normal traces). This behavior was independent of the medium pH at pH values above 5.5–6 (Fig. 6A, open symbols), which is consistent with DMPG having fully deprotonated phosphate groups. In contrast, at pH values of 4.5 an below (Fig. 6A, open symbols), the anisotropy decreased more steadily and the T_m increased gradually up to 40 °C (Fig. 6A), which correlated with the increase of the transition temperature of the wide transition peaks observed through DSC at these pH values (Fig. 6B, normal traces). This behavior has been assigned to the protonation of the PG phosphate group (Riske et al., 2002).

On the contrary, the DPH fluorescence anisotropy behavior of DMPG dispersions containing Ole was quite different. At pH values of 7.4 and 5.5 (Fig. 6A, closed symbols), DMPG/Ole dispersions yielded a wide phospholipid transition in which two transitions were clearly observed. The T_m values of these transitions corresponded exactly to those of the transitions observed by DSC, i.e. a first sharp transition and a second wider transition located at higher temperatures (Fig. 6B, bold traces). This is the first time in which the two-steps transition has been clearly observed by the use of fluorescence anisotropy. At pH 4.5, the two-steps transition occurring in the presence of Ole did not take place (Fig. 6A, closed symbols) and the anisotropy profiles were more similar to those of pure DMPG, although the decrease of the anisotropy was more gradual in the presence of Ole than in pure phospholipid. At pH 4.0 and below, the anisotropy behavior was very similar in both cases, in the absence and in the presence of Ole (Fig. 6A, open and closed symbols respectively). This effect was corroborated by using DSC (Fig. 6B, normal and bold traces respectively).

Fluorescence anisotropy values of the probe DPH incorporated in lipid bilayers depends on the viscos-

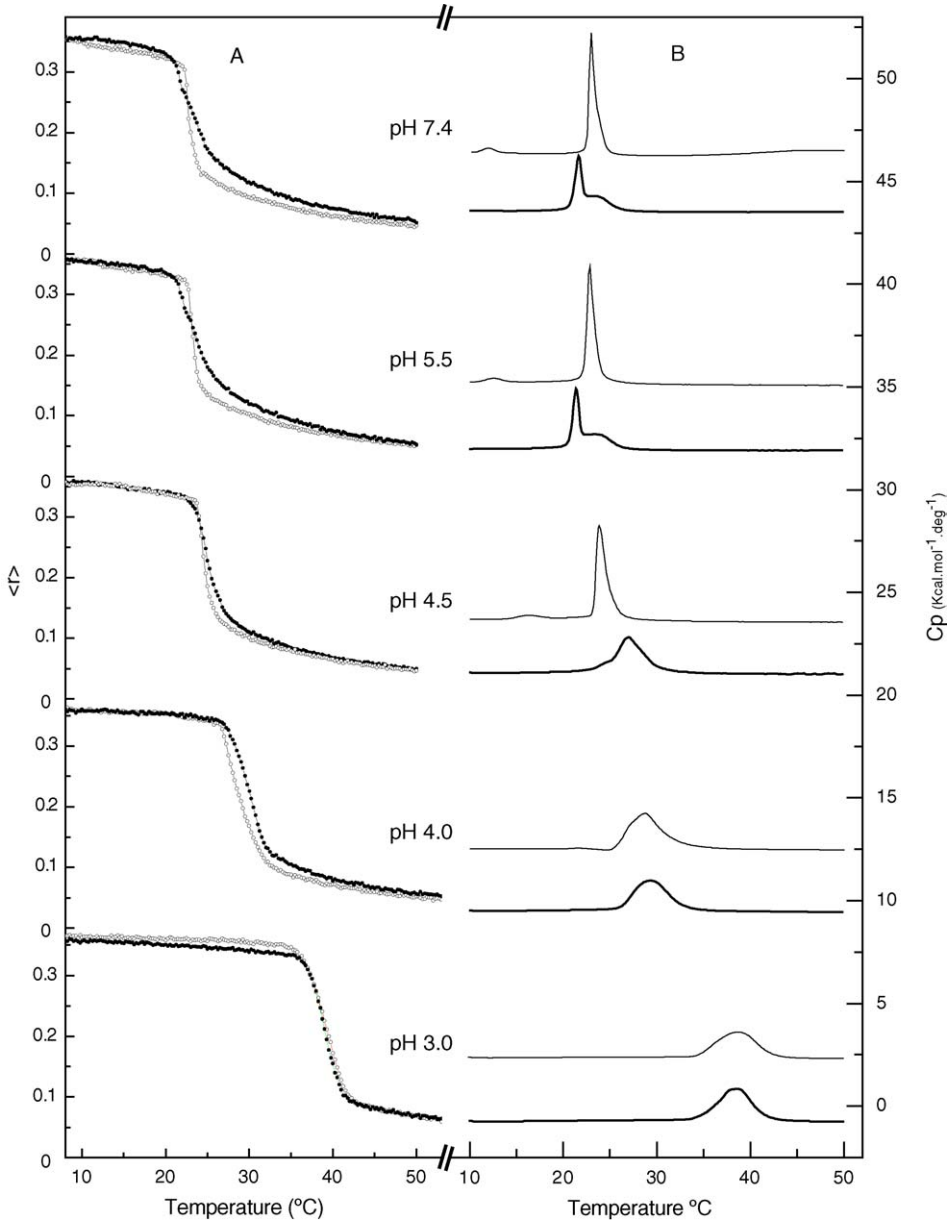

Fig. 6. (A) Steady-state anisotropy, ⟨*r*⟩, of DPH incorporated into DMPG vesicles containing Ole as a function of temperature for pure DMPG (◯) and DMPG containing 40 mol% Ole (●) at different pH values. Curves are the average of three independent measurements. (B) DSC heating thermograms for pure DMPG (normal trace) and DMPG containing 40 mol% Ole (bold trace) at different pH values.

ity of the local molecular environment (Harris et al., 2002). DMPG dispersions containing Ole showed an increase of the anisotropy values compared to pure phospholipid (Fig. 6A, bold and open symbols respectively), meaning a decrease in membrane fluidity. This effect was especially strong above the gel to liquid-crystalline phase transition and within the pH range 7.4–5.5 which coincides with the conditions at which IP has been observed by DSC (Fig. 6B). Moreover, this region exhibiting high DPH

anisotropy values in the DMPG/Ole mixtures coincides with the low scattering region observed in Fig. 5 (lower panel). Furthermore, no changes were observed on DPH anisotropy for DMPC vesicles, a zwiterionic phospholipid, in the presence of oleuropein (data not shown for briefness) neither below nor above the temperature of the gel to liquid-crystalline transition.

3.6. Effect of oleuropein on the ionic strength dependence of DPMG dispersions

The presence of the IP has shown to be strongly dependent of the ionic strength (Riske et al., 2002). At very low NaCl concentrations, i.e. 10 mM NaCl (Fig. 7A), DMPG exhibits a wide and complex DSC phase transition delimited by a sharp transition (T_m^{on}),

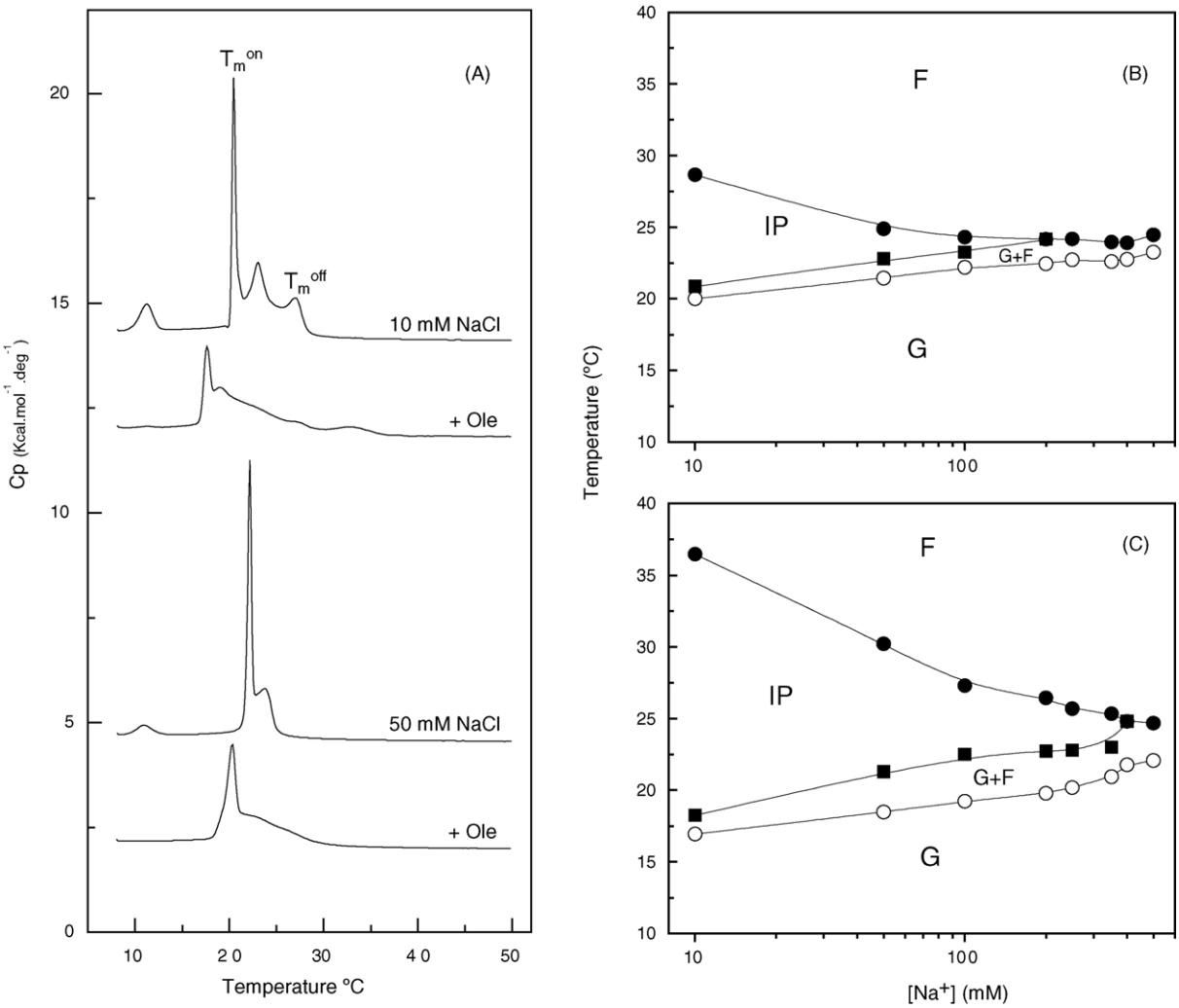

Fig. 7. (A) DSC heating thermograms for DMPG dispersions in 10 mM HEPES, 0.1 mM EDTA, pH 7.4 buffer, containing either 10 mM or 50 mM NaCl in the absence and in the presence of 40 mol% Ole. T_m^{on} and T_m^{off} are the onset and offset temperatures of the transitions delimiting the broad transition region of the phospholipid.Partial phase diagrams as a function of sodium concentration obtained from DSC experiments for aqueous dispersions of DMPG (B) and DMPG containing 40 mol% Ole (C). The open and closed circles were obtained from the temperature of the onset and completion temperatures respectively of the heating scans. Closed squares represent the completion temperature of the regular gel to liquid-crystalline phase transition. Pretransition details have been omitted for clarity. G, gel phase; F, fluid phase; IP, intermediate phase.

probably corresponding to a lipid domain undergoing a regular gel to liquid-crystalline phase transition, and a broader transition (T_m^{off}). A complete fluid phase only exists above T_m^{off} as seen by electron spin resonance (Riske et al., 2001). This broad chain-melting process has been attributed to the gel–fluid intermediate structures promoted by the strong repulsions between negatively charged head groups (Riske et al., 2002). When 40 mol% of Ole were added to DMPG dispersions at 10 mM NaCl, T_m^{on} was shifted to lower temperatures and the transition became broader (Fig. 7A), showing a DSC profile similar to that one exhibited by DMPG at 2 mM NaCl (Riske et al., 2002). A similar behavior was observed at 50 mM NaCl (Fig. 7A), the presence of Ole in DMPG dispersions shifted the T_m^{on} to lower temperatures and broadened the whole transition, yielding a DSC profile similar to that one obtained for DMPG at a lower NaCl concentration in the absence of Ole.

4. Discussion

It has been previously reported that oleuropein may interact with biological membranes, therefore it is reasonable to think that this feature might be responsible for part of its biological activity. To get insight this fact, we have studied the association, localization and effect of Ole in biological membranes through the use of phospholipid model membranes composed of zwiterionic or anionic phospholipids. The results obtained are discussed in order to establish a membrane-related mechanism for Ole.

Previously determined partition coefficients in organic/water systems yielded around 50–60% of Ole within the organic phase (Saija et al., 1998; Edgecombe et al., 2000; Paiva-Martins et al., 2003). In this work, the oleuropein phospholipid/water partition coefficient has been calculated in a membrane/water system considering lipid-saturating conditions, as expected to occur under physiological conditions. Under these conditions and after applying Eq. (1), around 80% of Ole molecules were found to be associated to phospholipid membranes. Although oleuropein exhibits a significant affinity for phospholipid membranes, is also rather soluble in water, which may allow it to interact with its molecular targets both in water-soluble and lipophilic environments. This feature opens a wide spectrum of possible molecular mechanisms to account for its biological activity. In fact, Ole has been recently shown to modulate the activity of several extra- and intracellular enzymes (Polzonetti et al., 2004). Considering that no pharmacokinetic data in humans are available so far, it has to be pointed out that membrane affinity and localization studies performed in this work may have a physiological significance, since Ole concentration used was much lower than those found in plasma rabbits fed with virgin oil and oleuropein (Coni et al., 2000).

Previous studies on the localization of oleuropein and its possible effects on membranes have yielded controversial results. Prior DSC studies shown by other authors have postulated that Ole physically immerses into phospholipid membranes composed of phosphatidylcholine and could be responsible for a membrane fluidifying effect (Saija et al., 1998). On the contrary, a recent study on the antioxidant capability and location of Ole in liposomes has proposed a shallow localization of Ole at the surface of phospholipid bilayers composed of soybean PC based on fluorescence quenching studies with probes located at different depths in the phospholipid palisade in which no changes in lipid order were observed (Paiva-Martins et al., 2003). To this respect, the DSC results obtained in this work using high sensitivity microcalorimetry and deriving from dispersions composed of Ole and different neutral or charged phospholipids (DMPC, DMPE, DEPE or DMPA) (Fig. 4) did not show significant changes of the $L_\beta \rightarrow L_\alpha$ phase transition, even at concentrations of Ole as high as 30 mol%. No significant transition broadening or lipid separation phenomenon was noticed from DSC thermograms either, which also supports the absence of strong molecular interactions due to Ole at the level of phospholipid acyl chains in these phospholipids. Nevertheless, it has to be considered that PE forms more ordered and packed bilayers than PC due to the larger size of PC, so any perturbation on the phospholipid acyl chains promoted by Ole would be more easily observed in PC than in PE membranes, in spite of the unsaturated character of DEPE (Boggs, 1980). In contrast, the liquid-crystalline to hexagonal-H_{II} transition of DEPE was affected at relatively low concentrations of Ole ($\geq$2 mol%) and a shift to lower temperatures was observed. This effect can be explained as a tendency of Ole to promote inverted hexagonal (H_{II}) phase at lower temperatures than those of the pure phospholipid.

These results suggest that hydrophobic interactions between acyl chains of these phospholipids are barely perturbed by Ole. Then, membrane interactions with Ole at the level of phospholipid acyl chains should be discarded. In addition, the fluorescence quenching studies performed in this work using spin probes indicated a shallow location for Ole, which agrees with a recent work (Paiva-Martins et al., 2003) (Fig. 3). This fact was confirmed by using the hydrophilic quencher acrylamide.

The DSC (Fig. 4F) and light scattering (Fig. 5) results obtained in this work strongly support a remarkable effect of Ole on the anionic phospholipid DMPG. The DSC profile of DMPG dispersions containing Ole in the presence of 100 mM NaCl was almost identical to that one observed for the pure phospholipid at much lower ionic strength. This profile consisted on a complex broad transition, which is similar to that one previously assigned to the gel–fluid intermediate region (Heimburg and Biltonen, 1994; Riske et al., 2002; Lamy-Freund and Riske, 2003). In addition, the presence of a high salt concentration in DMPG/Ole dispersions abolished this effect in DSC experiments (Fig. 4F), which has been previously demonstrated for the IP (Heimburg and Biltonen, 1994; Riske et al., 2002), corroborating the presence of this phase. Moreover, the evidence of the formation of this phase was confirmed by the presence of two transitions through light scattering measurements, which delimitate the gel–fluid region (Fig. 5).

It has also been demonstrated in this work that the presence of these structures in DMPG/Ole mixtures is highly dependent on the medium pH. The presence of a broad transition observed by DSC (Fig. 6B) and a more gradual anisotropy decrease at the phase transition induced by Ole in DMPG dispersions (Fig. 6A), both being signs of the IP (Riske et al., 2002; Lamy-Freund and Riske, 2003), were only observed at 5.5–7.4 pH values, at which the phosphate group of DMPG is negatively charged. In contrast, this behavior was eliminated at pH values below 4.5 what corresponds with the protonation of this group. An apparent pK of 2.9 has been previously estimated for the phosphate group of DMPG in 100 mM salt (Watts et al., 1978). In contrast, at low salt conditions, i.e. 6 mM NaCl, its pK has been estimated to be near 4.7 by using pH-anisotropy titration (Riske et al., 2002), which is closer to the value derived from our pH-anisotropy titration of DMPG/Ole dispersions.

Besides, the higher anisotropy values observed in the fluid phase of DMPG dispersions containing Ole at pH values 7.4–5.5 compared to those observed for pure phospholipid (Fig. 6A) support that fluid DMPG membranes exhibit a higher viscosity or a lower mobility in the presence of Ole. This may be consistent with the presence of high viscosity structures as those previously observed in gel–fluid or IP phases (Schneider et al., 1999; Heimburg and Biltonen, 1994). All these results point out that Ole may exert a similar effect to that one observed in DMPG membranes at low salt concentration, although 100 mM NaCl is present. An analogous effect has been recently observed for other glycosylated phenolic compound such as the anthraquinone barbaloin (Alves et al., 2004).

The behavior of the IP in DMPG dispersions as a function of sodium ion concentration was comprehensively studied in the absence and in the presence of Ole. Fig. 7B and C show the partial phase diagrams of DMPG dispersions in the absence and in the presence of 40 mol% Ole respectively, constructed from the DSC and fluorescence anisotropy results. The phase diagram of pure DMPG (Fig. 7B) shows a narrow boundary region, which may correspond to the coexistence of the gel and the fluid phases (G + F). Besides, a broad region corresponding to the gel–fluid intermediate phase (IP), in which the coexistence of different lipid phases may take place (Riske et al., 2001), is present at low ionic strength. The broad IP region becomes sharper as NaCl concentration increases (Riske et al., 2002). These two regions of phase coexistence are delimited by the onset (starting) and completion (ending) temperatures of the whole transition observed by DSC, which defined the solidus and fluidus lines of the diagrams. Pure DMPG dispersions show an onset temperature, which increases gradually as NaCl concentration raise up. At the same time completion temperature decreases as a function of the NaCl concentration, becoming horizontal approximately at 100–200 mM NaCl, salt concentration range at which IP vanishes and only a sharp gel to liquid-crystalline phase transition is observed (Riske et al., 2002). In contrast, the region corresponding to the IP was much broader in the presence of Ole (Fig. 7C), showing a very wide transition covering a temperature range of 17–37 °C at 10 mM NaCl, compared to a narrower IP region in pure DMPG (20–28 °C). In addi-

tion, the salt concentration at which IP was abolished in DMPG/Ole mixtures was shifted to almost 400 mM NaCl.

Among those phospholipids, which are able to stabilize their polar head groups through hydrogen bonds at physiological pH (PE, PS, PA and PG) (Boggs, 1980), PG exhibits the weakest interaction between head groups due to the strong repulsions between them. These interactions probably take place through hydrogen bonds between the glycerol of one polar head and the negatively charged phosphate of the neighbor (Zubiri et al., 1999). In fact, DMPG displays the most pronounced alterations in its phase transition by salt removal compared to DMPS or DMPA (Epand and Hui, 1986), as it happens in this work when Ole is incorporated in model membranes composed of these phospholipids. Monovalent cations, such as Na^+ or K^+, easily brake these bridges, allowing phospholipids to move independently and diminishing head group repulsions (Leckband et al., 1993). Ole is more likely to be uncharged at physiological pH, since its partition coefficient is quite similar at pH values of 5.5 and 7.4 (Gordon et al., 2001). Then, we postulate that Ole may establish interactions, probably through hydrogen bonds, with glycerol polar head or phosphate groups, in which the presence of the ionized polar group of PG seems to be essential as derived from our pH titration results on fluorescence anisotropy. Thus, Ole–PG interaction would be stronger than Na^+–PG interaction, therefore shifting Na^+ ions from the polar head groups and promoting an effect similar to that one obtained at lower ionic strength. The glucopyranosil group of Ole has a markedly hydrophilic character and a high number of hydroxyl groups able to donate or accept hydrogen bonds. In addition, our fluorescence results support a superficial location of the diphenylethanol moiety of Ole. Considering these facts, both groups are candidates for establishing this interaction with PG. In addition, no effect was observed by DSC when 40 mol% Ole was incorporated in DPPG membranes at pH 7.4 and 100 mM NaCl (data not shown). This fact reinforces the hypothesis of an interaction of Ole at the level of PG phosphate group since DPPG is not completely deprotonated even at pH 8.5 (Riske et al., 2002).

The transition temperature of ionized DMPG is also known to increase when the ionic strength increases as a consequence of the augmentation of the gel state stability (Träuble et al., 1976; Riske et al., 1997). The DSC results shown in this work reveal that T_c temperature of the gel to liquid-crystalline transition of DMPG was decreased upon Ole incorporation, which strongly supports the conclusion that Ole effect on DMPG membranes is contrary to high ionic strength effect and may be related to changes in membrane surface potential.

Therefore, we can conclude that the presence of Ole in PG containing membranes, at physiological pH and salt conditions, promotes the formation of structures similar to the gel–fluid intermediate phase, which may lead to drastic changes in the membrane physical properties and probably destabilize bilayers in a similar way to low salt conditions (Epand and Hui, 1986; Riske et al., 1997). These changes may involve molecular interactions between Ole and phosphatidylglycerol polar head groups, probably through hydrogen bonds, which might vary the surface potential and membrane curvature of anionic phospholipid domains. This fact may interfere with those processes highly dependent on negatively charged phospholipids, which may account for its wide antibacterial and/or antiviral activities. The biological relevance of the gel–fluid intermediate phase of DMPG has been recently pointed out since it could promote the formation of aqueous pores and/or disruption effects in biological processes such as disruption of prokaryotic cell membranes, changes in intracellular membrane topology or targeted drug delivery (Riske et al., 2004).

Regarding Ole antimicrobial activity, it has to be stated that bacterial membranes contain significant amounts of negatively charged phospholipids such as phosphatidylglycerol and cardiolipin (up to 40 and 5% respectively) (Lill et al., 1990), therefore the effect of Ole on these phospholipids could also account for its wide antibacterial effect. Furthermore, Ole has shown to be an effective antiviral agent against enveloped virus (Bisignano et al., 1999; Ma et al., 2001; Fredrickson, 2000). A recent study of our group (Micol et al., 2003, 2005) has demonstrated that Ole is a virucidal agent against the viral haemorrhagic septicaemia rhabdovirus (VHSV), an enveloped (−)-RNA rhabdovirus, and inhibits viral induced fusion. Ole may promote drastic changes on the membrane surface to interfere the binding of proteins to phospholipid domains enriched in negatively charged phospholipids such as phosphatidylglycerol or phosphatidylserine. Anionic phospholipids have been shown to play a cru-

cial role on the viral entry process. For instance, it has previously been published that the interaction of glycoprotein G of rhabdoviruses with anionic phospholipids of cellular membranes seems to be a necessary step for a successful viral fusion (Gaudin et al., 1992; Lenard, 1993; Estepa and Coll, 1996). Furthermore, we have also reported that a synthetic peptide (p2) deriving from the PS-binding domain of glycoprotein G of VHSV is able to mediate aggregation, lipid mixing and leakage with negatively charged phospholipid vesicles (Nunez et al., 1998).

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