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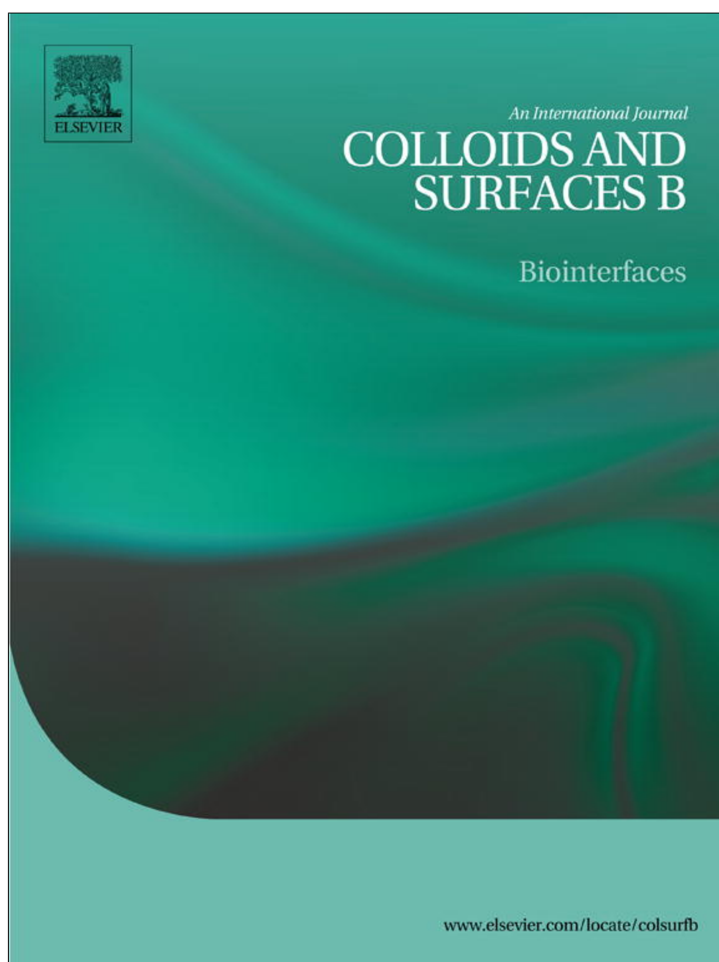

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Effects of propofol and other GABAergic phenols on membrane molecular organization

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A B S T R A C T

$GABA_A$ receptor is the main inhibitory receptor of the central nervous system. The phenols propofol and thymol have been shown to act on this receptor. $GABA_A$ is an intrinsic protein, the activity of which may be affected by physical changes in the membrane. Taking into account the lipophilicity of phenols, their interaction with the membrane and a consequent non-specific receptor modulation cannot be discarded. By using Langmuir films, we analyze the comparative effects on the molecular properties of the membrane exerted by propofol, thymol and other related compounds, the activities of which on the $GABA_A$ are under investigation in our laboratory. All the compounds were able to expand phospholipid films, by their incorporation into the monolayer being favored by less-packed structures. Nonetheless, they were able to be incorporated at lateral pressures above the equilibrium pressure estimated for a natural membrane. Epifluorescence images revealed their presence between phospholipid molecules, probably at the head-group region. Hence, all results indicated that the phenols studied were clearly able to interact with membranes, suggesting that their anesthetic activity could be the combined result of their interaction with specific receptor proteins and with their surrounding lipid molecules modulating the supramolecular organization of the receptor environment.

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1. Introduction

The $GABA_A$ receptor (GABA-R), the main inhibitory receptor of the central nervous system, is a ligand-gated ion channel that mediates fast synaptic inhibition in brain and spinal cord. GABA-R is unique among neurotransmitter receptors in the number of allosteric ligands that modulate its function [1,2]. The GABA-R ligands include drugs other than the GABA neurotransmitter, such as benzodiazepines, barbiturates, anesthetics, neurosteroids, ethanol and the convulsant picrotoxinin, which behave as allosteric modulators or channel blockers. This wide spectrum of drugs modifies GABA-R function by directly interacting either with these binding sites or with other as yet not well-described sites, present in the receptor complex [3,4]. The phenols propofol and thymol have been shown to act on this receptor as positive allosteric modulators or as direct agonists, according to the concentration assayed. These activities are mediated by their interaction with a specific site in the GABA-R [5,6].

GABA-R is a membrane intrinsic protein whose activity may be affected by surface-active compounds and by physical changes in the membrane [7–11]. Thus, given the lipophilicity of thymol and propofol, their interaction with the membrane region surrounding the receptor and a consequent non-specific receptor modulation cannot be discarded.

Recently we determined several lipophilic parameters for two phenol derivatives (PDs) with known GABAergic activity (thymol and propofol), and another three (carvacrol, eugenol and chlorothymol) that are structurally related with the former. The results obtained, based on the octanol–water partition coefficient ($\log P_{o/w}$), retention data in high performance liquid chromatography (HPLC) using C18 and immobilized artificial membrane (IAM) columns at different temperatures, and partition coefficients determined in phospholipid liposomes, demonstrated the high capacity of all the compounds assayed to interact with membrane phases. In addition, this supported the possible participation of some kind of alteration of the GABA-R lipid environment as part of the receptor modulation exerted by phenolic compounds [12].

The interaction between surface active compounds and phospholipids has been extensively studied in several model membrane systems, including liposomes and Langmuir monolayers [13,14]. In the present study, using Langmuir dpPC films, we analyze the comparative effects of these five PDs on the molecular properties of the membrane. Langmuir films constitute an informative and convenient membrane model because they permit subtle control

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of the membrane molecular packing. Three types of experimental approaches were used in the work: (i) surface pressure–molecular area isotherms, (ii) compound penetration capacity at different lateral surface pressures, and (iii) topographic film analysis through epifluorescence microscopy imaging.

2. Materials and methods

2.1. Materials

Propofol (2,6-bis(isopropyl)-phenol), thymol (5-methyl-2-isopropyl-phenol), carvacrol (2-methyl-5-isopropyl-phenol), eugenol (2-methoxy-4-prop-2-enyl-phenol) and chlorothymol (5-methyl-4-chloro-2-isopropyl-phenol) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and used without further purification. 1,2-Dipalmitoyl-phosphatidylcholine (dpPC) was from Avanti Polar Lipids (Alabaster, USA) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($DiIC_{18}$) was from Molecular Probes (Invitrogen, Argentina). Water was bidistilled in an all-glass apparatus (pH 6.5 ± 0.3). Other drugs and solvents used were of analytical grade. The compounds were dissolved in DMSO at 400× concentration, so that the final concentration of DMSO in the testing solution was 0.25% (v/v). Controls contained the same amount of DMSO, when so required.

2.2. Surface pressure–molecular area isotherms

Monomolecular layers were prepared and monitored essentially according to Garcia and Perillo [15]. The equipment used was a Minitrough II (KSV, Finland). A constant volume (15 μl) of chloroformic solution of dpPC (1 mg/ml) was spread over an aqueous surface; about 5 min were allowed for the evaporation of chloroform. Lateral surface pressure (π) was measured by the Wilhelmy plate method. Reproducibility was within ±0.01 nm^2 and ±0.001 mN/m for molecular area (A) and π, respectively.

π values were measured at different molecular areas of the phospholipid, in the absence or presence of each assayed phenol, at different concentrations in the subphase. For these experiments, we used a rectangular trough fitted with two barriers that move synchronously by electronic switching. The signal corresponding to the surface area (automatically determined by the Minitrough according to the relative position of the two compression barriers) and the output from the surface pressure transducer (measured automatically by the Minitrough with a platinized Pt foil 5 mm wide × 20 mm long × 0.025 mm thick) were fed into a personal computer through a serial interface using a specific software. Before each experiment, the trough was rinsed and wiped with 70% ethanol and several times with bidistilled water. The absence of surface-active compounds in the pure solvents and in the subphase solution (bidistilled water) was checked before each run by reducing the available surface area to less than 10% of its original value after enough time was allowed for the adsorption of possible impurities that might be present in trace amounts. The monolayer was compressed at a constant low rate of 20 mm^2/s at 28 ± 0.5 °C.

The onset of phase transition points was identified from a minimum and π_c from a maximum in the variation of the compressibility modulus (K) vs. molecular area plot. For this, K values were calculated from π–A isotherm data by applying Eq. (1):

$$K = -(A_\pi)\left(\frac{\partial \pi}{\partial A}\right)_\pi \tag{1}$$

where A_π is the molecular area at the indicated surface pressure.

From the π–A isotherms, the interfacial concentration of phenol expressed as mass per unit area (Γ) was calculated according to the following equation derived from the Gibbs surface tension equation [16]:

$$\Gamma = \frac{c}{RT}\cdot\frac{\partial \gamma_{\text{lip}}}{\partial c} \tag{2}$$

where c is the PD concentration in the subphase; γ_{lip} is the surface tension of dpPC monolayer (calculated from: $\gamma_{\text{lip}} = \gamma_{\text{w}} - \pi$, with γ_{w} being the surface tension of water at 25 °C) at a particular molecular area. An ideal behavior of drug solution was assumed, so phenol activity coefficient was equal to one. PD concentration in the subphase varied from 0 to 500 μM.

2.3. Penetration rates of phenol derivatives in lipid monomolecular layers at the air–water interface

In penetration experiments we used a circular Teflon trough (4.5 cm diameter and 0.5 cm depth). The subphase (8.5 ml, 15.9 cm^2 of surface area) was under continuous stirring with a miniature Teflon-coated rod spinning at 150–250 rpm. These experiments were performed at constant surface area but at different initial π (π_i), in order to measure the increment in π induced by PD penetration into the dpPC monolayer as a function of time ($\Delta\pi_t$), after the injection of 100 μM of each compound in the subphase. Accordingly, the compound penetration rate at each π_i was determined as the maximal value of the first derivative calculated from these π–time curves. In all penetration experiments, the injection of each PD in the subphase was made after the stabilization of the π_i (between 5 and 10 min approximately).

2.4. Epifluorescence microscopy of monolayers

DPPC was dissolved in chloroform (1 mg/ml), into which a small volume of a concentrated solution of $diIC_{18}$ in methanol was added to a final concentration of 1 mol%. The lipid mixture was dispersed onto an aqueous subphase and compressed in the same conditions detailed in point 2.2, and observed with an inverted epifluorescence microscope. Briefly, a KSV Minisystems surface barostat was mounted on the stage of a Nikon Eclipse TE2000-U (Tokyo, Japan) microscope, which was supplied with 20× long-working distance optics and with a fluorescence filter (excitation range: 520–553 nm, and emission range: 578–633 nm). The Teflon trough used had a 35 mm diameter quartz window at its base, which allowed the observation of the monolayer through the trough. Each PD was injected in the subphase at 100 μM final concentration, and images were taken at different π with a color Nikon DS-5 M video camera with a supported resolution up to 2560 × 1920 pix (capture). The images were then analyzed using the public domain Java image-processing program, ImageJ (National Institutes of Health, Bethesda, USA).

3. Results and discussion

3.1. Surface pressure–area isotherms

It is important to note that none of the PDs studied was able to form stable monomolecular layers by itself (results not shown); consequently the changes in the dpPC monolayers induced by each of these compounds were interpreted directly as its interaction with the phospholipid monolayer.

Fig. 1A–E shows π–A compression isotherms of dpPC in the absence (Control) or in the presence of each assayed PD present at different concentrations (20, 100 and 500 μM) in the aqueous subphase. Control isotherms obtained in the presence of DMSO 0.25% (v/v) were not different from those performed at 0% DMSO.

All compounds expanded the dpPC π–A isotherm in a concentration dependent manner. Although the well-known dpPC

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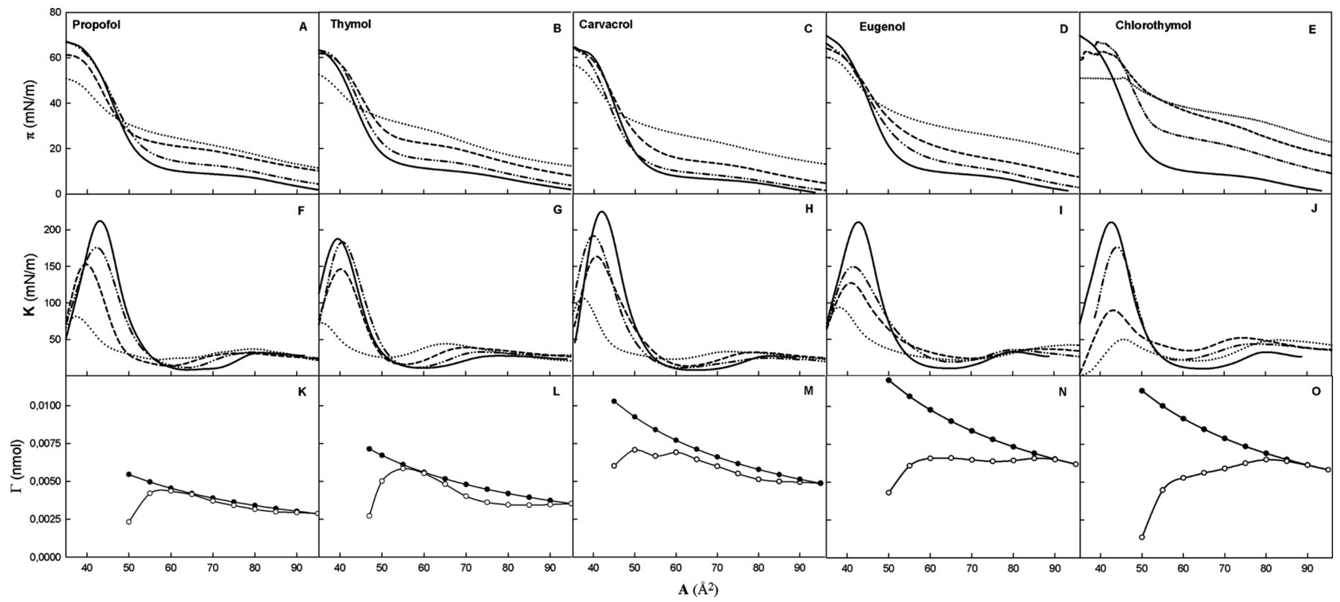

Fig. 1. Effect of compression and PD bulk concentration on the interfacial properties of dpPC Langmuir films and PD surface concentration. Curves were obtained in the absence (Control, solid line) or in the presence of PDs at different final concentrations (20 μM, long dash; 100 μM, short dash; and 500 μM, dotted line). Upper panels (A–E), π–A compression isotherms. Middle panels (F–J), K values calculated from curves in upper panels according to Eq. (1). Lower panels (K–O), ○, Γ values calculated from experimental data (π–A isotherms) using Eq. (2); ●, theoretical amount of PD per unit area, assuming both an ideal PD–dpPC mixture and a constant amount of PD in the monolayer along the whole compression process (Ideal values).

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bi-dimensional phase transition from liquid-expanded (LE) to liquid-condensed (LC) phase states was evident in all the isotherms obtained at different compound concentrations, all PDs studied induced a significant increase in π within the phase transition region with respect to the Control.

Minimum molecular areas (A_{min}) reached in the presence of phenols were similar to those corresponding to the Control isotherm. Collapse pressures (π_c) were almost unaffected up to 100 μM of PD. At 500 μM, the monolayer was destabilized by all PDs considering the decrease of π_c, which was more significant for the more lipophilic compounds (propofol and chlorothymol).

Fig. 1F–J depicts *K*-area plots calculated from π–*A* isotherms in panels A–E. This parameter reflects the physical state of the monolayer and helps to localize bi-dimensional phase transition. The higher the *K* values, the lower the interfacial elasticity. While in gaseous monolayers the *K* value is of the same magnitude as the surface pressure, in liquid condensed phases it varies between 12.5 and 250 mN/m [15,17]. A maximum in *K* vs. molecular area curve indicates the onset of phase transition. In the presence of propofol, chlorothymol, carvacrol and thymol, bi-dimensional phase transition occurred at molecular areas lower than in Control while, in the presence of eugenol, changes took place at bigger areas. The appearance of dpPC LC phase in Control monolayers at molecular areas below 50 Å^2 was evidenced by the marked increase in *K* values (full lines in Fig. 1F–J).

During the compression, not only the onset of the LE–LC phase transition but also its completeness arose later, at smaller molecular areas in the presence of most of the assayed PDs, than in the Control. This indicated that these compounds enhanced the elasticity of the LC phase in dpPC monolayers [18]. The latter behavior was not exhibited by eugenol. At this point, it is necessary to highlight that all compounds, at the highest concentration assayed (500 μM), did not permit the development of condensed phases (*K* values remained at levels comparable with the respective surface pressures). A similar behavior was evidenced by 100 μM chlorothymol. That observation reinforced the concept that the presence of a high concentration of phenols in the subphase induced a more elastic dpPC monolayer.

Γ values for the different phenol derivatives, determined from π–*A* isotherms according to Eq. (2), were plotted as a function of molecular areas (Fig. 1K–O, empty circles). Filled circles represent the amount of phenolic molecules per unit area, at different molecular areas, calculated by assuming that the total amount of each compound per unit area at the beginning of compression (maximum area) remained in the monolayer all through the compression process, without releasing or incorporating phenol molecules from the subphase (Ideal values). Equal results were obtained at all the concentrations assayed. Thus, for the sake of simplicity, only data corresponding to the 500 μM PD concentration are shown. All compounds reached similar maximal surface concentrations in the monolayer ($\Gamma_{max} \cong$ 5–8 pmol/mm^2). However, from the comparison of the compressional dependence of experimental and theoretical Γ values, two different types of behaviors could be identified: (i) propofol, thymol and carvacrol surface concentrations remained quite similar to Ideal values at the highest molecular areas (at the beginning of compression) but underwent a significant decrease at low molecular areas, within the LC phase; (ii) eugenol and chlorothymol concentrations in the monolayer followed the same trend as Ideal values, but only at very high molecular areas, at the beginning of the compression, and then deviated from the Ideal values with a decreasing Γ that reached a quite constant regime. This behavior suggested the expulsion of molecules from the monolayer upon compression (while the area diminished), which became more noticeable starting at molecular areas corresponding to the LE–LC transition region, approximately. Thus, while the former compounds were expelled from the dpPC monolayer only at π corresponding to the LC phase, the latter were released at both phases (LE and LC) during the compression. It is interesting to note that, in spite of the type of behavior shown, the beginning of the strongest decrement in Γ values in each graph (Fig. 1K–O) coincides with the zone of minimum values of the compressibility modulus (Fig. 1F–J) and with the two-phase (LE–LC) coexisting region of the monolayer (Fig. 1A–E). The fact that A_{min} (panels A–E) were not significantly affected strongly suggests that it is PD and not dpPC molecules that are expelled from the film upon compression. Γ values did not exhibit an increasing trend at any stage of the compression process, indicating that none of the compounds studied was able to increase its concentration in the monolayer by incorporating new molecules from the subphase. So, at all the molecular packings of dpPC, the PDs studied reached saturating interfacial concentrations which were in equilibrium with their bulk concentrations.

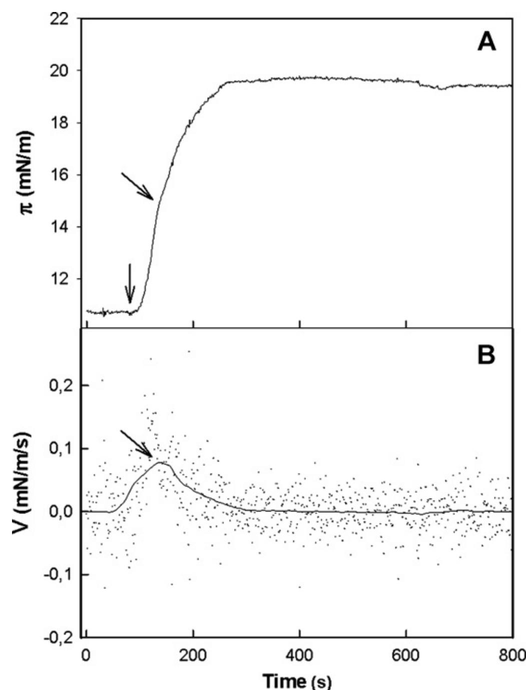

Fig. 2. Penetration rate analysis. Typical curves of variation of lateral pressure (π) as a function of time (A) and its first derivative (B), after the injection of 100 μM carvacrol in the subphase. Before the compound injection, the monolayer was stabilized at a constant π (π_i: initial pressure) (vertical arrow in panel A). The maximal penetration rate (V_{MAX}) is indicated by oblique arrows in both panels.

3.2. *Penetration rates of phenols in lipid monomolecular layers at the air–water interface*

The penetration of all phenolic compounds in the monomolecular layers of dpPC can be evidenced through the time-dependent variation of π at constant area and at known molecular packing (initial surface pressure, π_i). A typical curve is depicted in Fig. 2A, which shows the variation of π as a function of time at a π_i = 10.7 mN/m, after the injection of 100 μM PD (carvacrol) in the subphase. π values underwent an abrupt increase due the compound's penetration in the monolayer from the subphase, and then π evolved toward a plateau. The time-dependent penetration rate (*V*) was calculated at different π_i and for all PDs from the first derivative of typical π–time plots and, from its maximum, V_{MAX} was determined as shown in Fig. 2B. V_{MAX}–π_i plots (Fig. 3) showed a decreasing trend, which indicated that the entrance rate of these compounds into the monolayer was favored by the less-packed film structures. Note

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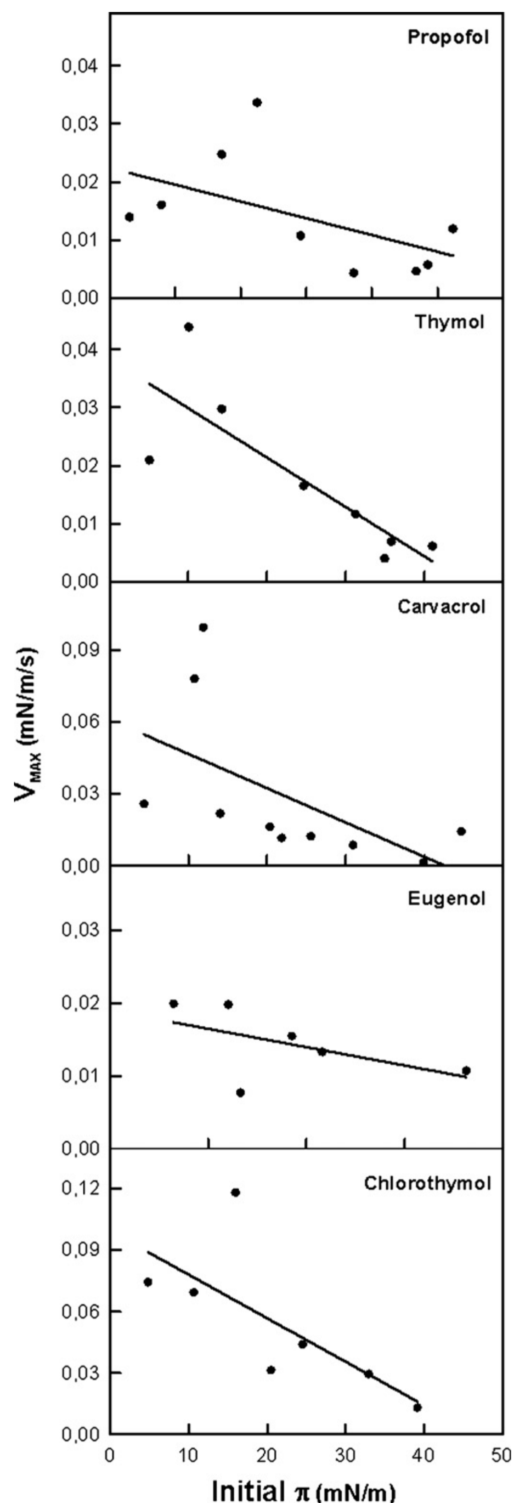

Fig. 3. Maximal penetration rates of PDs (V_{MAX}). V_{MAX} were determined as shown in Fig. 2 in the presence of 100 μM of each of the assayed compounds and at different π_i. Straight lines were fitted to data to note the trend followed by V_{MAX} values as a function of π_i.

that V_{MAX} can also be interpreted as a maximal monolayer deformation rate. This might be associated with a decreasing viscosity of the monolayer upon PD incorporation, which would facilitate further incorporation. This can be interpreted as a positive feedback loop, operating mainly at the beginning of drug incorporation, which would be counterbalanced by a decreasing trend of cohesive forces impairing the retention of PD at the film. The combination of both opposite phenomena at a certain point would become optimal, leading to V_{MAX}.

Changes in π ($\Delta\pi$) induced by phenol penetration into the monolayer diminished as the membrane presented a more tightly packed state. Since data could not be fitted to any simple function, the maximal π allowing drug penetration ($\pi_{cut\text{-}off}$) [15] could not be determined. However, all PDs were able to be incorporated in the monolayer at pressures above 35 mN/m (the bilayer's internal pressure estimated for a natural membrane bilayer) (results not shown).

3.3. *Epifluorescence microscopy of monolayers*

Epifluorescence microscopy enables the phase behavior of a monomolecular layer to be visualized during compression, allowing the observation of possible effects of lipophilic drugs on domains that are formed upon increasing the quantity of membrane components taking part in the most packed phases. The lipophilic fluorescent probe $DiIC_{18}$ shows preferential partition in the LE phase of the lipid monolayer [19]. In consequence, LE and LC lipid phases are represented by bright ($DiIC_{18}$ enriched) and dark ($DiIC_{18}$-depleted) domains.

Fig. 4 shows the epifluorescence images corresponding to two successive stages of the LE–LC coexistence region for dpPC monolayer in the absence (Control) or the presence of phenols in the subphase. PDs were added to the subphase at 100 μM. The fluorescence microphotographs of Control samples (Fig. 4A and B) show the formation of LC domains at the onset of the phase transition during monolayer compression, which exhibit the characteristic triskelion structure, curving in a counterclockwise direction as expected for pure L-dpPC [20]. At the concentration assayed, chlorothymol inhibited the development of a definite condensed phase, as indicated by the low *K* values. In agreement, typical LC dark domains were not visualized in the presence of this PD, perhaps due to the uniform partition of the fluorescent probe within the whole monolayer. In the presence of the rest of the PDs studied, the LC domains observed within that phase coexistence region were usually smaller and more ramified than those observed in the Control monolayer, and triskelion structures seemed to be absent (Fig. 4C and D).

Fig. 5 shows the results of quantitative analysis of images taken at equivalent stages within the phase coexistence region for all samples. All microphotographs analyzed were captured at molecular areas around 70% of the area corresponding to the onset of the phase transition.

It was widely described that the size and shape of domains in phospholipid monolayers are controlled by the competition of molecular interactions between the domains (LC phase) and the surrounding fluid (LE) phase line tension, and the electrostatic repulsion between excess dipole moments within the condensed phase [20]. However, since such lipids are chiral, the mesoscopic structure will be also influenced by the optical isomerism of the constituent molecules [21,22].

The chirality of the enantiomeric compounds within compact domains can be evident in clockwise or counterclockwise curvatures of those domains. Epifluorescence microscopic studies of triskelion of dpPC established that the handedness of the highly ordered domains is directly related to the absolute configuration of the enantiomer. Thus, mesoscopic chirality is triggered by the microscopic chirality present at the molecular level [23]. For phospholipids, the impact of chirality on the microscopic and mesoscopic level is determined by the headgroup size in relation to the cross section of the aliphatic chains. Phosphatidic acids (PAs) and phosphatidylethanolamines (PEs), which are characterized by small headgroup sizes, form circular domains [20,24], while dpPC,

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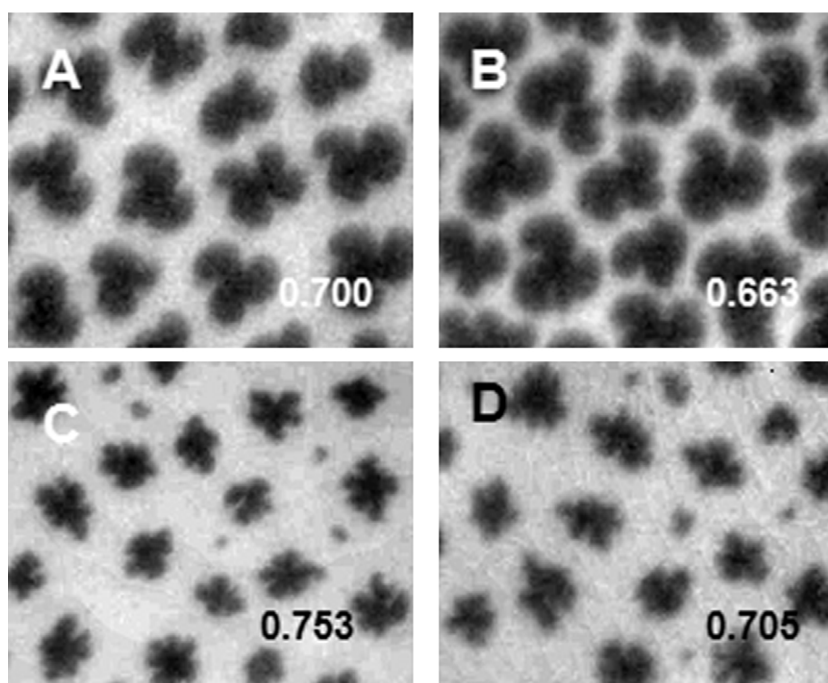

Fig. 4. Epifluorescence microphotographs of dpPC monomolecular layers. Microphotographs represent typical image fields taken at two successive compressing stages of the LE–LC coexistence region of the monolayer, in the absence (Control) (A and B) or in presence of 100 μM of thymol (C and D). Numbers in the picture refer to relative molecular areas (molecular area corresponding to each stage divided by the molecular area at the onset of the LC–LE coexistence region) and indicate that monolayers are being compared at similar compressing stages.

with its larger headgroups, shows a chiral structure in the mesoscopic domain shape. The elimination of chiral shapes in the LC domains, induced by the presence of every assayed compound, would indicate its location between phospholipid molecules, probably at the head group region, changing the molecular orientation within the domain.

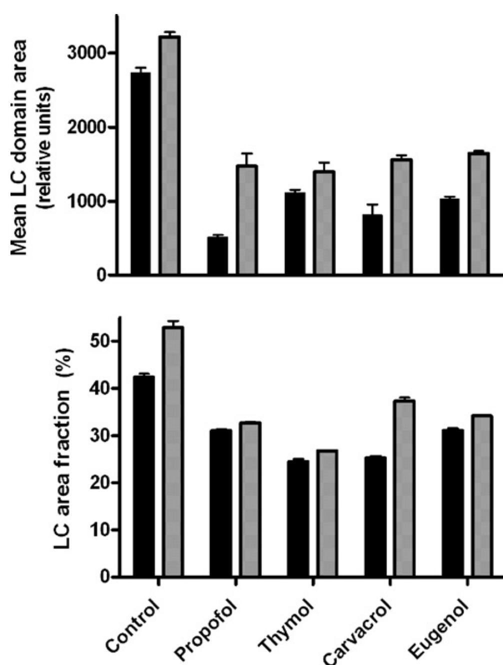

Fig. 5. Quantitative analysis of LC domains from epifluorescence microphotographs. Values correspond to image analysis of microphotograph in the absence (Control) or presence of the PDs (see Section 2). (A) Relative mean LC domain area and (B) fraction of LC area at successive compressing stages within the LE–LC coexistence region. Each value corresponds to the mean ± sem of 3–4 different fields taken at similar relative molecular areas (between 0.630 and 0.830) corresponding to the first (black bars) and the second (gray bars) compression stage.

Considering the analysis of microphotographs, all compounds induced LC domains smaller in size than those in the Control, with propofol being the PD exhibiting the strongest effect. In spite of the number of domains increasing in the presence of phenols, the LC fraction area diminished as a consequence of the smaller size of domains. This behavior is in agreement with the fact that, during the compression, in the presence of PDs, the condensed phase not only appears at smaller molecular areas but also the film presents lower compressibility values, indicating a more elastic LC phase for dpPC monolayers in the presence of phenols, as explained above.

The role of electrostatic interactions, mainly dipolar interactions, is a subject of intense study. It is known that small changes in the charge distribution within the head group of phospholipids can drive important variations in domain shape. This kind of study confirms the decisive contribution of in-plane (parallel to the interface) and out-of-plane (perpendicular to the interface) dipole moments on the domain shape [25,26]. The in-plane dipole moment in dpPC is relatively high with respect to other phospholipids with smaller polar heads (i.e. dpPE), mainly due to its larger molecular tilt in the LC domains [26]. This larger dipolar repulsion is the main force that enables dpPC domains to develop elongated arms (triskelions). However, the presence of phenols between dpPC molecules would reduce this dipolar repulsion in the tilt direction, allowing domain growth in various directions, where the dipolar repulsion cannot prevail over the line tension. Thus, it would be expected that the interaction of all assayed phenols with dpPC monolayers can be explained as an intercalation of phenols between dpPC molecules diminishing the molecular repulsion among phospholipid headgroups. This hypothesis is reinforced by the results described by Kane and Floyd [27] in which the incorporation of local anesthetic within the dpPC monolayer decreased the average in-plane electric dipole moment, due to the inclusion of electrically neutral particles between dpPC molecules.

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Finally, considering that epifluorescence microscopy allows domain changes to be observed, the degree of uniformity in the distribution of any compound capable of interacting with the phospholipid monolayer can be directly visualized [27]. Thus, it is interesting to note that the loss of typical domain shape of the Controls induced by the presence of PD, even at low π, would suggest that the PD molecules are localized with a uniform distribution over the entire monolayer instead of being accumulated in specific regions.

4. Conclusions

In the present study, we analyzed the effects of five phenolic GABAergic compounds on the molecular properties of a phospholipidic Langmuir film used as a model membrane, and obtained the following main results:

- All compounds were able to expand the dpPC π–A isotherm in a concentration-dependent manner, destabilized the monolayer at high concentrations and induced a more elastic LC phase in dpPC monolayers. The destabilizing effect of PD was more significant for the more lipophilic compounds.
- The incorporation of PDs into the monolayer was favored by less-packed film structures. However, all PDs were able to be incorporated in the monolayer at pressures above the estimated equilibrium pressure for a natural membrane bilayer.
- The epifluorescence imaging analysis revealed the presence of PDs in the membrane between phospholipid molecules, probably at the head group region, changing the molecular orientation and diminishing the repulsion among phospholipid headgroups.

These results indicate that the PDs studied are clearly able to interact with membranes. Considering that many drugs have intracellular targets, which requires their transport across phospholipid bilayers, drug–lipid interactions are unavoidable. Furthermore, although specific pharmacological regulation of membrane protein functions, such as $GABA_A$ receptor, can be analyzed using well-described theories of ligand–receptor interactions, it should be considered that many compounds that regulate the receptor protein function are noticeably lipophilic, which may change the physical properties of the lipid bilayer. Thus, it is possible that any anesthetic activity of lipophilic phenols could be the combined result of the interaction of the phenol molecules with specific receptor proteins ($GABA_A$ receptor) and with the surrounding lipid molecules modulating the supramolecular organization of the receptor environment.

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