Revisiting lipid – general anesthetic interactions (I): Thinned domain formation in supported planar bilayers induced by halothane and ethanol

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Abstract: A long-standing question in anesthesia is that of the molecular mechanism. Do anesthetics target proteins or change membrane properties or both? We used temperature-dependent magnetic A/C mode atomic force microscopy (AFM) to study interaction of the volatile anesthetics halothane and ethanol with model membranes made from supported planar bilayers (SPBs) of 1,2-dioleoyl-sn-3-glycerol-3-phosphocholine (DOPC), dioleoyltrimethylammonium propane (DOTAP), or 1,2-dipalmitoyl-sn-3-glycerol-3-phosphocholine (DPPC). We found that the incorporation of halothane or ethanol induces structural changes in the bilayer. These compounds cause thickness reduction in L\text{α} bilayers (either globally or in domains) and the formation of domains with reduced thickness in L\text{β} phase bilayers. We propose that an anesthetic-induced increased area per lipid drives local chain disorder, thus promoting local phase change. The characteristics of SPBs with halothane or ethanol incorporated were compared with characteristics of the L\text{α} and L\text{β} phases of anesthetic-free SPBs.

Key words: atomic force microscopy, anesthesia, lipid bilayer domains, phase transition

Introduction

The phenomenon of domain formation in model membranes is of great interest, as membrane structural changes define changes in membrane functional properties. This may also be true for anesthesia. Indeed, there have been paradigm shifts in the molecular theory of anesthetic action over the past century. The correlation found by Meyer and Overton (1, 2) between anesthetic potency and lipid solubility suggested that alteration of the lipid membrane structure was key to anesthetic efficiency. As membrane protein studies became possible at the molecular level, attention shifted to the direct action of anesthetics on ligand-gated ion channels (3, 4). More recently, it has been suggested that volatile anesthetics indirectly affect membrane protein by altering the lateral pressure within bilayers (5). Also, a multiple-sites-of-action hypothesis has been forwarded in which general anesthetics may bind to many protein and lipid sites with relatively low affinity (1–10 mmol/L) (6). Despite considerable study, the exact molecular effect of volatile anesthetics on membranes is only beginning to emerge. It has been demonstrated that volatile anesthetics can induce a change in lipid packing in membranes (7–9). For example, several experimental and theoretical studies were devoted to investigation of changes in membrane properties due to the incorporation of alcohols. For example, Holte and Gawrish (10) found that ethanol partitions near the lipid–water interface close to the lipid glycerol backbone and upper segments of lipid hydrocarbon chains and increases the degree of motional disorder in lipid chains. The implication is that a contributing factor in the physiological effect of ethanol results from the mole-
cules dissolving in specific lipid hydrophobic sites, which significantly modifies membrane structure and may lead to bilayer thinning. Thinned bilayers could contain both interdigitated lipids (interpenetration of hydrocarbon chains of the upper leaflet to the lower leaflet of the bilayer, resulting in disappearance of the bilayer midplane) and lipids with vastly increased tilt angles. It has been reported that bilayer thinning occurs when the local concentration of the species in the bilayer is higher than a threshold value (11). Bilayer thickness changes may have a strong effect on the function of membrane-associated proteins because of a mismatch of the protein–lipid hydrophobic interaction region. Changes in lipid packing in the presence of ethanol have been examined by X-ray diffraction, and they have been suggested in fluorescence spectroscopic studies (12, 13). Interdigitated domains caused by ethanol were proposed by Mou et al. (14) to explain the thinned dipalmitylophosphatidylcholine (DPPC) bilayer they observed using atomic force microscopy (AFM). Recently, AFM studies by McClain and Breen (15) suggest that 2-propanol-induced DPPC bilayer thinning is strictly due to interdigitation.

Halothane is a clinical anesthetic that has received increasing attention as a model molecule for fundamental studies on anesthetic action. Molecular dynamics simulations (16) of halothane in a DPPC bilayer revealed that halothane self-associates and also is distributed nonuniformly across the membrane interior. This resulted in significant modifications of the lipid bilayer, including thinning. A decrease in lipid wobble and an increase in tail defects of the lipid system were also observed. A temperature-dependent AFM study may provide valuable information about the effect of anesthetics on the thinned-bilayer formation by monitoring the effect of anesthetics on the chain-melting phase transition. Very recently, three temperature-dependent AFM studies involving bilayers have been reported (17–19). In these papers, increases in temperature were used to induce Lβ−Lα phase changes in the supported planar bilayers (SPBs). In our studies of DNA–cationic bilayer interactions as a function of temperature, we have shown that topology can be accurately measured using magnetic A/C (MAC) mode AFM to temperatures as high as 70 °C in a liquid sample cell (19). Temperature-dependent AFM allows a novel examination of halothane-induced changes in lipid bilayer properties and permits powerful reexamination of some previous results (14) for anesthetic–DPPC bilayer interactions, because the characteristics of both the gel- and the liquid-phase SPB can be measured on the same sample in the presence and absence of anesthetic.

In the present work, we have concentrated our efforts on studying the incorporation of ethanol and halothane into SPBs. We employed MAC mode AFM to study anesthetic-induced changes in supported phospholipid bilayers of dioleoylphosphatidylcholine (DOPC), DPPC, or dioleoyltrimethylammonium propane (DOTAP). The goal of this study was to extend the knowledge that exists in the literature for ethanol and to examine the incorporation of halothane into a bilayer. We were able to measure the bilayer properties as a function of temperature and to induce the Lα−Lβ phase transition in DPPC, and we used a temperature increase to irreversibly drive ethanol out of the SPBs. In the following companion paper (20), the results of our AFM studies for halothane are compared with those obtained using spectroscopic techniques.

**Materials and methods**

Dioleoylphosphatidylcholine (DOPC, lyophilized or in chloroform solution), dioleoyltrimethylammonium propane (DOTAP, chloroform solution), and dipalmitoylphosphatidylcholine (DPPC) were obtained from Avanti Polar Lipids Inc. (Alabaster, Ala.) and were used without further purification. Tris–EDTA (TE) buffer, pH 7.6, and distilled, ultrapure water were used in the generation of all vesicles. Freshly cleaved ASTM-V2 quality, scratch-free ruby mica (Asheville-Schoonmaker Mica Co., Newport News, Va.) was used throughout this study as a substrate. In some cases, mica was modified by addition of a dilute solution of 3-aminopropyltriethoxysilane (APTES) using standard procedures (21).

All vesicles were prepared using the “dry” method as previously reported (22). An appropriate aliquot of phospholipid chloroform solution was measured into a small vessel, and the chloroform was removed using a stream of dry nitrogen. The dry phospholipid was then resuspended in buffer to its final concentration and stirred for 30 min. The solution was sonicated (Branson 1200; Branson Ultrasonics, Danbury, Conn.) at room temperature for 10-min periods. Between each 10-min period, there was a 15-min “rest” interval where the solution was placed in an ice bath and then stirred at room temperature. For this method, the solutions were cycled an average of 10 times or until they were observed to clear.

Supported planar bilayers were prepared for AFM imaging by the method of vesicle fusion (22, 23). Aliquots of liposome solution were deposited on modified or unmodified freshly cleaved mica. After a controlled period of time, the mica was gently rinsed with ultrapure water, and the surface was imaged under water in the liquid cell, at room temperature and at various higher temperatures.

Addition of ethanol and halothane was performed in two ways: (i) by addition of pure anesthetic into the vesicle solution with an incubation time of 1.5 h and (ii) by addition of an ethanol/water mixture (50% by volume) or pure liquid halothane into the AFM liquid cell with the established supported bilayer and incubation for the same period of time. A time of 1.5 h was shown by fluorescence experiments to be optimal for halothane distribution between buffer and membrane (20).

We employed MAC mode AFM, where a magnetically coated probe oscillates near its resonant frequency driven by an alternating magnetic field. This technique has proven to be advantageous for measuring supported planar bilayers in liquid media (22, 24, 25). All images were taken using a Pico SPM microscope with AFM-165 scanner (Molecular Imaging Inc., Phoenix, Ariz.). Magnetically coated type I Macelevs® (Molecular Imaging Inc.) were used for MAC mode imaging. Their specifications include a length of 85 µm, a force constant of 0.06–0.1 N/m, a resonant frequency of 20–40 kHz in water, and a tip radius of curvature quoted as less than 10 nm. The standard MAC mode fluid cell (Molecular Imaging Inc.) was used throughout. The scanning speed was 1–2 lines per second. The height scale

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was calibrated using colloidal gold spheres of 5 nm and 14 nm in diameter (26). For elevated-temperature experiments, the AFM Temperature Controller and Hot Mac Mode Stage from Molecular Imaging Inc. were used. Heating and cooling were performed at $\Delta T = 1 \, ^\circ\text{C/min}$, with the possibility to hold at any given temperature. The mean value(s) of the bilayer thickness was calculated using between 50 and 70 measurements over several scanned regions, where appropriate. The standard deviation is quoted at the 99% confidence level.

**Results and discussion**

**DPPC**

The changes in a $L_\beta$ DPPC bilayer structure as a function of incorporation of halothane and ethanol were examined and compared with the effect of temperature. The reason for using ethanol was to compare our data, obtained by MAC mode AFM, with the results of previous studies (14, 15). There are several studies in the literature that have examined incorporation of ethanol and other alcohols into phospholipid bilayers using various methods, including fluorescence (27) and AFM (14, 15). It has been suggested that a high concentration of ethanol in a lipid-containing aqueous solution can induce transformation of a lipid bilayer from ($L_\beta$) gel phase to the fully interdigitated ($L_{\beta I}$) phase of mono-unsaturated phosphatidylcholine (PC) lipids, which alters the $L_\beta$–$L_\alpha$ melting transition temperature of the phospholipid bilayer (28). In our study, ethanol was added to a supported DPPC bilayer on mica. After 1.5 h, the cell was then rinsed and imaged in water. In the absence of ethanol, we measured the thickness of a pure supported DPPC bilayer on mica as 5.5 nm, from natural defects, which is in good agreement with the value of 5.7 nm reported earlier (14). We observed the formation of thin domains in the presence of ethanol (Fig. 1). The thickness of the higher domains was 5.6 nm, and that of the lower domains was 3.6 nm (Table 1). The difference in thickness of the two domains was 2.0 nm, compared with 1.9 nm previously reported (14). The ethanol/SPB system remained stable at room temperature for up to 2 days. Changing the relative concentration of ethanol only changed the extent of thin domains, but not their thickness. The stability of the thin domains suggests that ethanol remains preferentially in the bilayer at room temperature. When the bilayer was heated to 50 °C (i.e., above the melting transition point, $T_m = 41 \, ^\circ\text{C}$) while imaging was performed and then slowly cooled back to room temperature, the lower domains disappeared and the original bilayer thickness was restored. This reversibility of the ethanol effect is in agreement with data of Mou et al. (14), although the latter authors were not able to simultaneously image and scan temperature. These findings suggest that ethanol repartitions into water when DPPC is at elevated temperature and that this repartitioning depends on the phase of the bilayer. Recently, we investigated the temperature dependence of a pure DPPC SPB (data not shown). As the tempera-
ture approached the Lβ–Lα phase transition, thinned domains appeared. The heights of the domains were exactly the same as those induced by ethanol, described above. When holding at a temperature of 50 °C, the lower domains coalesced and grew in extent. There is every reason to believe that these thinner domains represent a DPPC SPB in its Lα state. The value of 3.6 nm for Lα DPPC thickness is in the range previously determined using X-ray scattering (Xhead-to-head = 3.6–4.0 nm) (29). A similar range was also calculated by Feller et al. (30) by molecular dynamics simulation. Interestingly, the simulation illustrated that the DPPC lipid chains in the Lα phase displayed both some interdigitation and significant tilting. However, there was no indication of the completely interdigitated LβI phase, where methyl end groups would be exposed to water.

We have also examined the incorporation of halothane into a DPPC bilayer to compare the effects of ethanol and halothane. The halothane was added into the vesicle solution or into the liquid cell with a preformed supported bilayer. In Fig. 2 we illustrate the effects of incorporating halothane into vesicles prior to SPB formation. In the presence of halothane, we observed similar domain formation in a DPPC bilayer to that induced by ethanol. The thickness of the higher and lower domains was measured as 5.5 and 3.5 nm, respectively, giving the same difference of 2.0 nm as we observed in the case of ethanol (Table 1). The surface coverage of the thin domains was 2–3% at lower halothane concentrations (Fig. 2b) and comparable to that naturally formed in pure DPPC SPBs (1–2%). At higher halothane content, we observed a considerable amount of lower domain surface coverage (25–50%, Fig. 2c). We were able to image the surface during slow heating of the bilayer above the melting transition point (Tm = 41 °C). Heating the system to 42 °C did not induce considerable changes in the domain structure. After 1 h of scanning at 42 °C, lower domains in the bilayer were still present. A further increase in temperature to 50 °C

| Table 1. Dependence of thickness (nm) of supported planar bilayers (SPBs) on the incorporation of halothane and ethanol. |
|---------------------------------|---------------------------------|---------------------------------|
| SPB | Thickness of pure lipid bilayer | Thickness after addition of ethanol | Thickness after addition of halothane |
| DOTAP | 3.7 ± 0.2 | 3.6 ± 0.2 | 3.5 ± 0.2 |
| DOPC | 5.4 ± 0.1 | 5.3 ± 0.3 | 5.0 ± 0.5 |
| DPPC | 5.5 ± 0.1 | 5.6 ± 0.2 | 5.5 ± 0.1 |

Note: MR is the ratio of the number of moles of halothane in the membrane to the number of moles of lipids.

*DOTAP, dioleoyltrimethylammonium propane; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

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demonstrated that the remaining thicker domains melted at a higher temperature than the pure DPPC free-standing bilayer and the phase transition was broad. This is consistent with the melting of a pure DPPC SPB (data not shown).

In the alternate preparation, halothane was added into the liquid cell containing the supported DPPC bilayer covered with 1 mL of water. After the addition of halothane, no rinsing was performed. In Fig. 3, we follow the SPB containing
halothane as a function of temperature and time. In this case we observed a very interesting transition of the bilayer containing halothane at room temperature during the first 30 min of scanning. Domains (thickness of higher domains was 4.5 ± 0.2 nm) initially present (Fig. 3a) disappeared and the bilayer became generally thin (3.3 ± 0.3 nm, Fig. 3b). Because the bilayer was supported on mica, we consider that halothane first causes structural changes in the upper leaflet, and scanning at room temperature might induce equilibration of halothane between the two leaflets. After this thinner bilayer is formed, the system is stable during scanning at room temperature and becomes thinner yet at a temperature higher than that of the Lβ–Lα transition for a pure DPPC bilayer. Further thinning was observed to start at 50 °C and was complete at 62 °C (Figs. 3c–e). After the system was cooled back to room temperature (Figs. 3f–h), bilayer thick-
ness was 3.9 ± 0.2 nm, which is lower than that of pure Lα DPPC bilayer. The initial thickness of pure DPPC without halothane was restored during a similar cooling process (data not shown). The molar ratio of the amount of halothane in the membrane to that of lipid was estimated as $n_{\text{halo}} / n_\text{L} = 3.4$ at room temperature and has been shown to increase by a factor of 4 above the chain-melting transition temperature (31). The amount of halothane in the bilayer was estimated using the partition coefficient of halothane in DPPC provided by Simon et al. (31). This increase in halothane partitioning during heating is probably responsible for a saturation of the bilayer with halothane and stabilization of thinned domains when the system was cooled back to room temperature.

The persistence of thinner domains and the complete thinning of the bilayer show that interaction of halothane with the lipids is very strong and increases at elevated temperature. These results are consistent with those reported in the companion paper (20) as well as with those obtained by Simon et al. (31) and by Hill (32).

When compared with ethanol, halothane shows different behavior during heating. Whereas ethanol leaves the bilayer, thus restoring its initial thickness, halothane partitioning increases with temperature, leading to more extensive domain formation. The reason for this difference in behavior likely originates from the higher hydrophobicity of halothane and its lower solubility in water (17 mmol/L) (20) compared with that of ethanol. Ethanol can escape during heating when the mobility of phospholipids and ethanol is high, whereas for solubility reasons halothane remains in the bilayer.

The transitions observed upon heating the bilayer with and without halothane were compared. For a pure DPPC bilayer, we observed a broad main transition between 42 °C and 52 °C and further change between 52 °C and 60 °C; upon cooling back to room temperature, a transition between 35 °C and 36 °C occurred. When the bilayer with halothane incorporated was heated, the lower temperature transition was not observed, and only one transition at 52–60 °C occurred. The presence of halothane likely changes structural properties of the bilayer in such a way that the other transitions observed for a pure bilayer are not possible. The transition below $T_m$ likely involves reorganization of water molecules at the interface. In the presence of halothane, water molecules are exchanged for halothane molecules, which eliminates the low-temperature transition.

The inhomogeneous domain formation observed with both ethanol and halothane incorporation is presumably due to a cooperative mechanism of anesthetic penetration and partitioning into the bilayer. This mechanism may include localized dehydration of headgroups by disruption of the coordinated hydration shell. The existence of such a hydration shell has been suggested by molecular dynamics simulations (33). Additionally, the initial rupture of the hydration shell may promote inhomogeneous anesthetic incorporation into the bilayer at the exposed location. After water shell rupture, further incorporation of anesthetic into the bilayer increases the headgroup area and motional disorder in lipid chains and leads to formation of the thinned state. The possible role of water in the mechanism of anesthesia was emphasized in previous studies (33, 34). Ueda and co-workers (34, 35) have obtained evidence, through proton NMR measurements and FTIR spectroscopy, for the release of bound interfacial water by inhalation anesthetics in a water-in-oil emulsion. This suggested that some anesthetics compete with water for binding sites. Both ethanol and halothane are able to form hydrogen bonds. Halothane contains a so-called acidic hydrogen, which is considered to be involved in a “hydrogen bond breaking effect”. It has been shown that anesthetics with “acidic hydrogen” and therefore with high “hydrogen bond breaking” ability have higher anesthetic potency than halogenated molecules containing no acidic hydrogen (36). An NMR study (10) has shown that ethanol incorporates into the lipid–water interface near the lipid glycerol backbone and upper segments of lipid hydrocarbon chains and increases the degree of motional disorder in the lipid chains, which promotes formation of a thinned bilayer.

Molecular dynamics simulations (16) indicate that halothane induces lateral expansion and contraction of the Lα DPPC bilayer. Koubi et al. (16) reported a thickness reduction of 8 Å (from 42 to 34 Å; 1 Å = 0.1 nm) and an increase in area per lipid of 16% (from 63.6 to 72.0 Å²). We observed a thickness reduction from 5.5 to 3.5 nm (2.0 nm) in the Lα DPPC bilayer due to incorporation of halothane. We also have observed a thinning of a pure DPPC bilayer to 3.6 nm upon heating above 42 °C. Thus, incorporation of halothane produces a similar thinning effect as heating the bilayer above the chain melting transition temperature. The difference in an Lα DPPC SPB with and without halothane is small (0.1 nm) compared with that predicted (0.8 nm (16)). This might reflect the differences between supported planar bilayers and free-standing bilayers.

The fact that very similar DPPC bilayer changes are observed for both ethanol and halothane incorporation suggests a common mechanism. Moreover, because the formation of thin bilayer domains surrounded by thick domains is analogous to what we observe for a DPPC SPB near the Lα–Lβ phase transition temperature, it is likely that ethanol and halothane induce something similar to a local phase change in the SPB. The mechanism may include initially smaller Lα-like domains coalescing into larger thin domains. The stability of these domains for periods of days would be due to a cage effect by the surrounding thicker and less mobile Lβ regions. Fluorescence results from our companion paper (20) also suggest that increased disorder of the lipid packing and penetration of water near the headgroup region of DPPC small unilamellar vesicles is induced by the incorporation of halothane.

**DOPC**

To gain insight into how the lipid phase might affect the interaction of ethanol and halothane with SPBs, we used DOPC. DOPC exists in a fluid phase (Lα) at room temperature and serves as a fluid-phase analog of the DPPC bilayer. A pure DOPC SPB (Fig. 4a) has a thickness of 5.4 nm, as we reported earlier (22). In the presence of ethanol (Fig. 4b) or halothane (Fig. 4c) at a molar ratio of 7.2:1, the DOPC SPB transforms to a uniform thickness of 3.5 and 3.6 nm, respectively (Table 1). We assume that the higher lipid disorder of a DOPC bilayer results in much less cooperativity in
Fig. 4. Topography images and cross sections showing the effects of incorporation of ethanol or halothane into a dioleoylphosphatidylcholine (DOPC) bilayer. (a) Pure DOPC bilayer; (b) DOPC bilayer with ethanol, $C_{\text{EtOH in water}} = 1.4$ mol/L; (c) DOPC bilayer with halothane, at an estimated molar ratio of halothane in the membrane to lipid of 7.5:1. Ethanol and halothane were added into the vesicle solution prior to SPB formation. The scale bars in all panels are 500 nm long. The arrows in all cross sections represent 4.0 nm in height.

Fig. 5. Topography images and cross sections of DOPC bilayer with halothane, at an estimated molar ratio of halothane in the membrane to lipid of 3.7:1. (a) Initial scan; (b) after 40 min of scanning. Halothane was added into the vesicle solution prior to SPB formation. The scale bars in all panels are 500 nm long. The arrows in all cross sections represent 4.0 nm in height.
the incorporation of anesthetic at these higher molar ratios, and thus no domains are formed.

Interestingly, at a lower concentration of halothane (molar ratio = 3.6:1), domain formation was observed (Fig. 5a). These domains were 5.0 and 3.6 nm thick (Table 1), and the domain shape and size changed during 40 min of scanning (Fig. 5b). The fact that DOPC domains were not well resolved and more dynamic compared to DPPC bilayer may be explained by higher mobility of anesthetic in DOPC bilayer than in DPPC Lβ bilayer. Thinned DOPC domains were observed in the presence of ethanol as well (Fig. 6a).

Although the ethanol-induced domains were more stable at room temperature than those induced by halothane, they started to disappear at 35 °C as the sample was heated and simultaneously imaged. We found that heating the DOPC bilayer with ethanol incorporated and then cooling it back to room temperature revealed changes in SPB surface coverage (Figs. 6a–c) and bilayer thickness. Initially, we observed a bilayer with domains of 5.5 and 3.5 nm. Upon heating and slow cooling, the bilayer was restored to 5.3 nm. This implies that the release of ethanol from DPPC is largely due to elevated temperature rather than the lipid phase, since the ethanol–DOPC system is stable at room temperature but releases ethanol at elevated temperature.

For an SPB with anesthetic incorporated, changes in surface coverage as a function of temperature can be used to provide insight into the amount of lateral expansion the SPB undergoes during incorporation. For the ethanol-containing bilayer, surface coverage increased by 42.3% (compared to the surface coverage at room temperature) upon heating to 43 °C (observed after 50 min of scanning at 43 °C). After
the bilayer was slowly cooled back to room temperature, the surface coverage was decreased by 6.2% compared to the initial value before heating. The increase in surface area at 43 °C (with a concomitant decrease in thickness to 3.6 nm) indicates an increase in lipid disorder. Restoring of the bilayer full thickness and a decrease of surface area indicate ethanol escape and formation of the L\(_{\alpha}\) DOPC bilayer. For our conditions, we estimate that incorporation of ethanol produces a 10% increase in DOPC area per lipid compared to that of a pure DOPC bilayer at room temperature. This estimation has been made on the basis of surface changes we observed and known DOPC area per lipid in pure DOPC bilayer without ethanol (\(A_{\text{DOPC}} = 64 \text{ Å}^2\)) (37). The increase in DOPC area per lipid can be compared to that of an L\(_{\alpha}\) DPPC bilayer (16%) in the presence of halothane at high concentrations (16) and to that of DPPC in the presence of 2-propanol (15) (57.8% — from 52 Å\(^2\) to 90 Å\(^2\)). The smaller increase in the case of DOPC can be explained by taking into account the liquid phase of DOPC bilayer, which is more disordered compared to gel-phase DPPC bilayer, and also to differences in the volume of incorporated molecules.

At a higher concentration of ethanol (3 mol/L) in the vesicle incubation solution, we observed formation of small disk-shaped aggregates and clusters of these aggregates upon exposure of the solution to a mica surface (Fig. 7). No bilayers were observed for this preparation. The height of the circular aggregates was in the range 10–15 nm. We assume that these aggregates could be flattened vesicles, which were stabilized by the partitioning of more ethanol molecules in the upper leaflet. This seems to make vesicles more susceptible to a pre-fusion type of aggregation but resistant to rupture upon adsorption to the mica surface. Mou et al. (14) mentioned previously the existence of such small globular structures in the presence of high concentrations of ethanol.

**DOTAP**

To help elucidate the influence of the headgroup on the anesthetic-induced changes in SPB structure, we performed analogous experiments using DOTAP. DOTAP is L\(_{\alpha}\) at room temperature, and its headgroup carries a positive charge at neutral pH. A pure DOTAP supported bilayer was described in our earlier publication (18). Halothane was incorporated into the DOTAP bilayer via incubation in the vesicle solution at an estimated molar ratio of halothane in the membrane to lipid of 7.3:1. An aqueous solution of halothane (41 mmol/L) was added into the vesicle solution prior to SPB formation. The scale bars in all panels are 500 nm long. The arrows in all cross sections represent 4.0 nm in height.

**Conclusions**

We have demonstrated by AFM that the thinned domains in DOPC, DPPC, and DOTAP supported planar bilayers in-
duced by halothane and ethanol are similar, regardless of whether the headgroup is the zwitterionic PC or the cationic TAP. This is consistent with the molecular dynamics simulations of Koubi et al. (16) for halothane in the Lα phase of DPPC, where halothane was observed to partition preferentially on the lipid side of the headgroup region. A room temperature DPPC bilayer showed stable domains when either ethanol or halothane was present in the bilayer. Upon heating above the phase transition temperature, ethanol was released whereas halothane was retained. This indicates that the effects we observe are solely due to anesthetic within the bilayer and not an effect of anesthetic content in aqueous solution. In a fluid-phase DOPC bilayer, inhomogeneous, dynamic domains were formed at a ratio of incorporated anesthetic to lipid of less than 7:1. DOPC SPBs completely thinned when this ratio was 7.3:1. The fact that domain formation was almost universally observed implies that anesthetic-induced bilayer thinning is a cooperative process. For gel-phase bilayers, it seems that volatile anesthetics induce a localized phase transformation to Lα, whereas for lipids already in Lα, even thinner domains are generated. Heating of the DPPC bilayer with halothane showed that halothane affects the transition in the bilayer. For the anesthetics studied here, the mechanism likely involves partitioning of the anesthetic preferentially near the headgroups. This is in agreement with the findings of the following companion paper and with previous theoretical and experimental studies. The fact that thinning seems to be universal means that this effect cannot be ignored when considering the physiological mechanism of anesthesia, since membrane thinning may affect the function of some membrane-associated proteins.

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