Induced structural changes of a supported planar bilayer after exposure to halothane — A real-time atomic force microscopy study

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Abstract: Atomic force microscopy (AFM) was used to study the effect of the general anesthetic halothane on a supported DOPC phospholipid bilayer, under conditions of high anaesthetic loading. The presence of the anaesthetic in the lipid-induced restructuring of the supported bilayer, erosion of the bilayer from the mica surface, and the formation of lipid aggregates. Addition of halothane to lipid vesicles prior to adsorption on the mica surface inhibited the lysis of adsorbed vesicles and thus no planar bilayer formation was observed.

Key words: lipid bilayer restructuring, atomic force microscopy, halothane, anesthésie.

Résumé : On a fait appel à la microscopie des forces atomiques (MFA) pour étudier l’effet de l’anesthésique général, halothane sur une bicouche supportée de phospholipide DOPC, dans des conditions de charge élevée d’anesthésique. La présence de l’anesthésique dans le lipide induit une restructuration de la bicouche supportée, une érosion de la bi-couche de la surface du mica et la formation d’agrégats de lipides. L’addition d’halothane aux vésicules de lipide avant l’adsorption sur la surface du mica inhibe la lyse des vésicules adsorbées et on ne peut alors observer de formation de bicouche planaire.

Mots clés : restructuration d’une bicouche de lipide, microscope de forces atomiques, halothane, anesthésie.

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Introduction

Although, general anaesthetics are known to have a direct interaction with biological cell membranes (1), the exact sequence of events leading to anesthetic action remains unknown at the molecular level. Presently, there is significant evidence that the targets of volatile anaesthetics are ligand-gated ion channels embedded in cell membranes (2). However, it has also been hypothesized that the interaction between anaesthetics and these ion channels occurs indirectly, via a change in the microenvironment of the phospholipid bilayer surrounding the ion channels (3). Regardless, since anaesthetic potency increases with lipophilicity, an understanding of the changes that occur within model biological membranes in the presence of anaesthetics will provide insight regarding anaesthetic mechanisms.

A supported 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) planar bilayer is often chosen as a model biological cell membrane, because its behavior is well-characterized and membrane mimetic (4). In addition, DOPC is stable in the form of a supported planar bilayer for a period of days (5, 6), allowing for long-term observations of interactions with anaesthetics.

The anaesthetic used in this study, halothane, is an amphiphilic molecule. Fluorescent studies have indicated that halothane resides predominantly in the headgroup region of a model DOPC bilayer (5). Moreover, using atomic force microscopy (AFM) we have recently shown that halothane likely has a heterogeneous lateral distribution in gel-phase 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) SPBs (6). Finally, molecular dynamic simulations revealed halothane is distributed unevenly across the membrane interior (7). It is of interest how this uneven distribution affects the stability of a supported planar bilayer. One would expect uneven distribution of halothane within a supported phospholipid bilayer to decrease line tension, resulting in destabilization, and possibly restructuring of the bilayer.

Supported planar bilayer restructuring after the addition of chitosan was previously observed by Fang and Chan (8). The presence of the protein chitosan within the bilayer induced growth clusters to form within the bilayer, and aggre-
gation of lipids was observed. A decrease in bilayer line tension was cited as the cause of the changes. In addition, Fang and Chan reported an erosion of fragments of the lipid membrane from the mica surface.

In the present work, magnetic A/C mode (MAC) AFM was used to examine the effect of the presence of the general anaesthetic halothane on a mica-supported planar DOPC bilayer. The halothane-induced changes in a DOPC SPB were monitored over a 22 h period.

Methods

Materials

DOPC in chloroform solution was used without further purification (Avanti Polar-lipids Inc., Alabaster, Maine). Filtered (0.1 µm pore size) ultrapure water was used for the preparation of all solutions. Freshly cleaved ASTMV-2 quality, scratch-free ruby mica (Asheville-Schoonmaker Mica Co., Newport News, Virginia) was used as the SPB substrate for imaging.

Supported DOPC bilayer in water

A 0.7 mg/mL DOPC solution was prepared in ultrapure water using the method previously described (9). Briefly, an aliquot of DOPC suspended in chloroform was transferred into a dry vessel. The solution was then dried under a stream of nitrogen to remove the chloroform. The phospholipid was then resuspended in ultrapure water and stirred for 30 min. The solution was then sonicated and stirred for successive 10 min cycles at room temperature until it became clear (Branson 1200, Dansbury, Connecticut). The solution was then filtered (0.2 µm pore size) and sonicated for 10 min prior to deposition onto the mica substrate.

Supported planar bilayers were prepared via vesicle fusion (9). DOPC solution (75 µL, 0.7 mg/mL) was deposited on unmodified, freshly cleaved mica and incubated for 45 min. The surface was then gently rinsed with ultrapure water. All bilayers were imaged in ultrapure water at room temperature.

Supported DOPC bilayer containing halothane

Halothane (60 µL) was added to the AFM cell containing a previously imaged supported DOPC bilayer and incubated at room temperature for 30 min (creating a 10% v/v solution in ultrapure water). A series of exposure times were tested. Thirty minutes was the minimum time needed to observe change, and allows imaging of further changes in bilayer structure. The liquid above the bilayer was then gently removed from the cell with a pipette, and fresh ultrapure water was immediately added to prevent drying of the sample. Under these conditions the solution would be saturated with halothane (17.5 mmol/L).

AFM imaging conditions

All imaging was done using MAC mode AFM in which a magnetically coated type I Maclevers® (Molecular Imaging Inc.) were used. The cantilever used had a force constant of 0.6 N/m and the tip radius of curvature was quoted as being within the range of 5–10 nm. A standard MAC mode fluid cell (Molecular Imaging Inc.) was used in all experiments.

Results and discussion

To study the effect of halothane’s presence on the supported phospholipid bilayer, it was first necessary to obtain a single planar supported bilayer via vesicle fusion on the mica surface. In the absence of halothane, a uniform supported planar DOPC bilayer was observed (Fig. 1A). Viscoelasticity analysis (9) was used to confirm the presence of a single supported bilayer (data not shown).

Next, halothane was added to a preformed SPB as described in the Methods section. The addition of halothane resulted in a restructuring of the supported DOPC bilayer. Images obtained 6 h and 45 min after halothane addition to the supported planar bilayer displayed large structures, which are presumably lipid aggregates (Fig. 1B). The largest aggregate observed had a height of 100 nm and was imaged at 7 h after halothane addition. It is uncertain whether this is the maximum size of aggregate, or whether they continue to increase in size indefinitely. Control experiments where halothane was added directly to the mica surface did not produce images comparable to those with a bilayer. Further control experiments were performed where the SPB was treated in the same way as for halothane addition, with the exception that an equivalent volume of water was injected. No changes in the SPB were observed under these conditions.

Although there is no evidence that these aggregates result from uncontrolled foreign substances acting upon the bilayer, viscoelasticity analysis should provide additional insight into the nature of the aggregates, possibly confirming that they are lipid-based. Viscoelasticity (i.e., phase) images provide information about a sample’s ability to dissipate energy transferred from interactions with the AFM tip. Differences in viscoelasticity result from differences in phase, and thus viscoelasticity analysis can be used to distinguish samples that are different in lipid phase. For instance, the solid mica support can be distinguished from liquid- or gel-phase SPBs. Fig. 2A displays an aggregate of interest. Topography images indicate the presence of larger entities on the mica substrate (Fig. 1A). In the corresponding viscoelasticity (i.e., phase) image, areas of only two different viscoelasticities are evident. These two represent the bilayer and the mica support. There is no phase contrast between the large aggregates (identified by a circle in Fig. 1C) and the domain that surrounds them, suggesting that the aggregates are lipid based and possibly multilamellar. The formation of these lipid aggregates could result from erosion of the lipid from the mica support and restructuring of the lipids owing to a destabilizing effect of halothane on the supported bilayer. This destabilization would result from a decrease in line tension caused by uneven distribution of halothane within the bilayer.

Significantly, we were able to monitor the spontaneous formation of a lipid aggregate at times ranging from 4 h and 25 min to 5 h after initial halothane addition (Fig. 2).
lipid aggregation observed is concluded to be solely because of the presence of halothane in the bilayer, as there is minimal perturbation of the system by the AFM probe during imaging. Moreover, a supported planar DOPC bilayer is stable in the absence of halothane over a period of up to days (6). The aggregate formation, observed after 4 h and 25 min, indicates halothane (at a high concentration regime) has a prolonged destabilizing effect on a supported phospholipid bilayer.

The results above suggest that in the presence of halothane a DOPC bilayer has a lower energy when curved rather than when planar. We postulate this is related to a ten-
Nonuniformly sized curved structures were observed ad- 

onto the mica surface. This yielded no bilayer formation. 

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resides in a liquid-phase bilayer compared to a gel-phase 

supported by the observation that halothane preferentially 

density of halothane to reside in a region of the bilayer in 

which the space between head groups is increased. This is 
supported by the observation that halothane preferentially 

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thane, halothane-containing DOPC vesicles were deposited 

onto the mica surface. This yielded no bilayer formation. 
Nonuniformly sized curved structures were observed ad-
sorbed to mica. These are presumably nonlysed vesicles 

(Fig. 3).

Fig. 3. Topography image of DOPC vesicles deposited on a mica surface. Halothane was added to the vesicle solution prior to sur-

face deposition. Nonuniform curved structures are evident.

Taken together, these observations are consistent with 

halothane’s uneven bilayer distribution, which destabilizes 

the planar structure leading to an energetically favored 
curved configuration. The destabilization of the bilayer in 

the presence of the drug, attributed to a decrease in line ten-
sion, could be the consequence of many factors. First, 

the presence of halothane within the bilayer likely has a deter-
genent effect, as its shape (Fig. 4) is much different from that 
of the cylindrical DOPC headgroup/tail unit. Thus, insertion 
of halothane may disrupt the packing of individual cylindri-
cal headgroup/tail units within the bilayer. The molecular 
dynamics study of halothane in uneven distribution of 

halothane within the bilayer could further disrupt the stabili-
y of packed lipid units within a bilayer, therefore contri-
buting to decreased line tension and inducing further restruc-
turing. Since this behavior was not observed in previous 
studies at low halothane loading conditions (approxi-
mately 0.5 mmol/L halothane solution) (5), we postulate that 
the destabilization is concentration dependent. Furthermore, 
the anaesthetic interaction with the SPB may be co-

operative. Initial aggregation of the SPB appears to enhance 

with slight changes in lateral pressure that result in large conformational changes of embedded proteins (3). The lipid aggregation we observed in the present work could be due to pooling of halothane near the headgroup region. This, coupled with our fluorescence-based halothane distribution results (5), suggests that halothane may induce changes in lateral pressure gradients in phospholipid bilayers. Thus, in addition to direct anesthetic protein interactions, asymmetric anesthetic distribution may contribute to the mechanism of anesthesia. The question, then, is whether these two possible mechanisms compete or are synergistic.

**Conclusion**

DOPC vesicles were deposited onto a mica surface, both 
in the absence of the drug and after the addition of 

halothane. Height and viscoelasticity analysis indicate the 
lipid is stable in the form of a single planar bilayer in the ab-

sence of halothane. However, in the presence of the drug, a 
curved bilayer structure is favored. The addition of 

halothane to vesicles in solution followed by vesicle deposi-
tion did not yield the formation of a supported bilayer. How-
ever, adhesion of curved vesicles on the mica surface was observed. Together, these results indicate the destabilizing effect of halothane on a planar lipid bilayer at high anaesthetic loading regimes. To our knowledge, this is the first study to date of supported phospholipid bilayer restructuring induced by a general anaesthetic.

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