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Perturbation of Peptide-Induced Lateral Phase Separations in Phosphatidic Acid Bilayers by the Inhalation Anesthetic Methoxyflurane

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SUMMARY

GALLA, H. J., AND J. R. TRUDELL. Perturbation of peptide-induced lateral phase separations in phosphatidic acid bilayers by the inhalation anesthetic methoxyflurane. *Mol. Pharmacol.* 19:432–437 (1981).

The effect of methoxyflurane in a clinical concentration range on a phospholipid-peptide interaction in model membranes was studied by electron paramagnetic resonance. Thermotropic phase transition curves of negatively charged phosphatidic acid bilayers in the presence of the peptide antibiotic polymyxin-B were obtained using the spin probe 2,2,6,6-tetramethylpiperidine-1-oxyl. Polymyxin interacts strongly with phosphatidic acid membranes, causing lateral phase separations. Domains of peptide-bound phospholipids are formed in a matrix of a bilayer of unbound phosphatidic acid. The phase transition of the polymyxin-phosphatidic acid domain is decreased by about 13° as compared with a pure phosphatidic acid bilayer. The phosphatidic acid matrix surrounding these domains exhibits a somewhat broadened and lowered phase transition due to the presence of some monomeric polymyxin. Addition of the inhalation anesthetic methoxyflurane progressively disrupts the polymyxin-induced lateral phase separation, leading to a homogeneously dispersed phase at concentrations above 100 mmoles of anesthetic per mole of lipid. Application of high pressure antagonizes the effect of the anesthetic on the phase transition temperature of the free phosphatidic acid matrix and antagonizes the disruption of the phosphatidic acid-polymyxin domain. In a phosphatidic acid bilayer containing polymyxin, 4 moles %, a pressure of 100 atmos absolute (ATA) antagonizes the effect of 30 mmoles of methoxyflurane per mole of lipid. The latter concentration is equal to that produced in nerve membranes during clinical anesthesia, while a pressure of 100 ATA is sufficient to reverse anesthesia in mice and tadpoles. A much larger antagonism of the pressure and inhalation anesthetic effects is observed in the phase transition temperature of the surrounding phosphatidic acid phase than in the polymyxin-phosphatidic acid domains. However, at methoxyflurane concentrations greater than 100 mmoles/mole of lipid, when the polymyxin-phosphatidic acid domains are completely dispersed and form a homogeneous bilayer structure, the effect of pressure is only 20% of that observed in a pure phosphatidic acid vesicle containing the same concentration of methoxyflurane.

INTRODUCTION

The nerve membrane may be considered to be a lipid bilayer membrane containing proteins which control nerve cell function such as the transport of sodium ions through the membrane. The question of whether the site of anesthetic action is in the membrane lipid, the membrane protein, or the lipid-protein interface is still unanswered. The membrane-expansion theory of anesthesia (1) states that anesthetics are dissolved in the hydrophobic lipid region, leading to a membrane expansion which may block nerve function. Conversely, the protein perturbation hypothesis considers the protein conformational state as the primary site of anesthetic action (2).

A pressure of approximately 100 atmos absolute has been shown to antagonize the anesthetic effect in tadpoles and mice (3, 4). Trudell *et al.* (5, 6) reported antagonistic anesthetic-pressure effects in phospholipid bilayers that favor the lipid-expansion hypothesis of anesthesia. Even if it is accepted that anesthetics affect both protein and lipid, the question of what is the interaction between the two components remains open. In an attempt to provide a molecular mechanism for anesthetic action, Trudell (7) has suggested that anesthetics perturb lateral phase separations that exist near controlling proteins in nerve cell membranes. However, it has not been

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possible to study lateral phase separations in natural nerve cell membranes. Therefore such theories lack experimental verification.

This paper deals with the effect of an inhalation anesthetic on peptide-induced lateral phase separations as a model for the mechanism of anesthesia. We investigated the effects of methoxyflurane on the interaction between negatively charged phosphatidic acid and polymyxin, a peptide antibiotic. Polymyxin has been shown to interact strongly with negatively charged lipids (8, 9). Domains of polymyxin molecules binding three to five phosphatidic acid molecules are formed in a surrounding phosphatidic acid bilayer matrix. Pressure causes significant changes in the proportion of the total phosphatidic acid that is in the lipid-peptide clusters (10). Moreover, phosphatidic acid membranes have been shown to be strongly perturbed by inhalation anesthetics (11). A concentration of 100 mmoles of methoxyflurane per mole of lipid broadens the phosphatidic acid phase transition and lowers it by about 10°. This is a much greater effect than in phosphatidylcholines, where a decrease in lipid phase transition temperatures of only 2° was reported (12). Therefore, this polymyxin-phosphatidic acid system is a suitable model for the investigation of anesthetic and pressure effects on lipid-protein interactions.

The present paper demonstrates that low concentrations of methoxyflurane disturb the cooperative interactions in the polymyxin-phosphatidic acid domain which leads to the dispersal of the domain. At methoxyflurane concentrations greater than 100 mmoles/mole of lipid, there is only a single homogeneous polymyxin-phosphatidic acid phase. Pressure partially antagonizes the anesthetic effect in the phosphatidic acid-polymyxin domains, but the lowering of the transition temperature in the homogeneous phosphatidic acid-polymyxin phase at an anesthetic concentration higher than 100 mmoles/mole of lipid is not antagonized by pressure.

MATERIALS AND METHODS

Dipalmitoylphosphatidic acid (disodium salt) and polymyxin-B sulfate were obtained from Sigma Chemical Company, St. Louis, Mo. The dipalymitoylphosphatidic acid did not exhibit contaminants when assayed by thin-layer chromatography on silica gel with $CHCl_3$:MeOH:H_2O in a 65:35:4 ratio and was free of calcium ion. It was used without further purification. Methoxyflurane was a gift from Abbott Laboratories, North Chicago, Ill. All measurements were performed in a pH 9.0 sodium borate buffer containing 81.6 ml of 0.05 M sodium borate and 18.4 ml of 0.2 M boric acid.

Lipid dispersions were prepared by sonication of 20 mg of dipalmitoylphosphatidic acid in 8 ml of the above buffer for 2 min at 55°. Polymyxin was added from a stock solution of 5 mg/ml in the same buffer, and the dispersion was resonicated for 2 min at 55°. Liquid methoxyflurane was added in the given amount to the lipid dispersion in a close 10-ml vessel. The amount of methoxyflurane applied in the experiments is given as millimoles with respect to the molar lipid content based on the lipid/water partition coefficient of 20 measured in our laboratory by gas chromatography (5, 11). The samples were stirred magnetically at 55° for 2 hr. After cooling to room temperature, they were centrifuged at 5000 × *g* for 1 hr and kept at room temperature overnight. The pellets (0.4 ml) were suspended with a Vortex mixer after addition of 40 μl of a 3×10^{-3} M solution of TEMPO.[2] Samples (10 μl) were transferred to a 1-mm inner diameter quartz EPR sample tube pressurizable to 100 atmos absolute helium. The temperature was regulated to ±0.1° by a thermostated nitrogen stream and measured with a thermocouple. EPR spectra of the TEMPO label were measured on a Varian E-104A spectrometer with on-line digitized recording. A typical spectrum is shown in Fig. 1. The high-field hyperfine extrema in a matrix (H) has a smaller hyperfine splitting than in the polar water phase (P) (13). The ratio $f = H/(H + P)$ of the EPR signal intensities is a partition coefficient for the spin probe and measures the fraction of the membrane in a fluid state. Curve fitting for the evaluation of the fluidity parameter, f, as function of temperature to derive phase transition curves was performed using a DEC PDP 11/03 computer. The standard deviation of f was less than 1%.

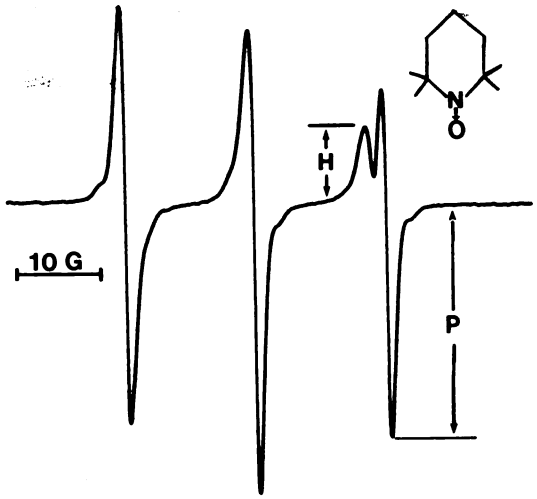

FIG. 1. *EPR spectrum of TEMPO in a phosphatidic acid bilayer*
The ratio $H/(H + P)$ is a partition coefficient for TEMPO and is a measure of the fraction of a membrane in the fluid state.

RESULTS

Phase transition curves of dipalmitoylphosphatidic acid in the presence of polymyxin-B. Phase transition curves of dipalmitoylphosphatidic acid in the presence and absence of polymyxin are shown in Fig. 2. The fluidity parameter (f) is plotted as function of temperature. Pure phosphatidic acid membranes exhibit a phase transition of T_m = 55° at pH 9.0 and a sodium ion concentration of 0.05 M. Addition of polymyxin leads to the appearance of a second, lower phase transition between 32° and 48° with a midpoint at 42°. This lower phase transition was first determined by fluoresence polarization (8), and equivalent results have been obtained from EPR experiments (10). It is attributed to a domain in which three phosphatidic acid molecules were bound

[2] The abbreviation used is: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

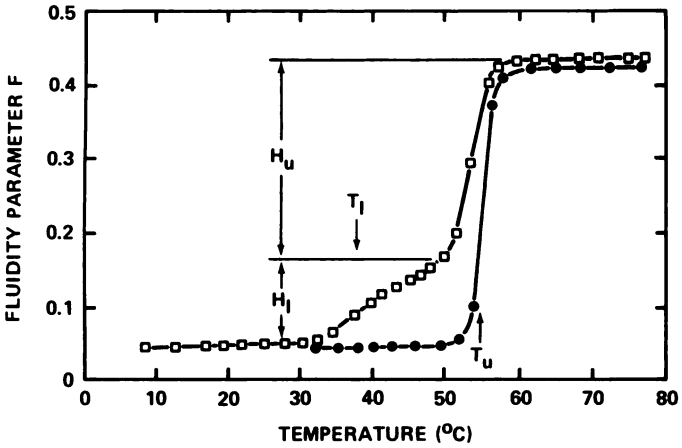

FIG. 2. *Phase transition curves of dipalmitoylphosphatidic acid bilayers at pH 9.0 and a sodium concentration of 0.05 M without* (●——●) *and after incorporation of polymyxin, 4 moles %* (□——□)

A second phase transition step with a lowered phase transition temperature, T_l, as compared with that of mixed phosphatidic acid bilayers, appears at T_u. The step height of each of these phase transitions is designated by H_l or H_u, respectively.

to polymyxin to form cooperative clusters. The heights of the steps in the resulting phase transition curve H_l and H_u in Fig. 2 are considered to represent the amount of lipid in the polymyxin-bound domain or in the surrounding phosphatidic acid phase.

Effect of methoxyflurane. Increasing amounts of the anesthetic methoxyflurane were added to dipalmitoylphosphatidic acid bilayers containing polymyxin. Figure 3A shows the phase transition curve for a membrane preparation containing polymyxin, 6 moles %. *Arrow 1* gives the height, H_l, of the low melting phase transition step in the absence of methoxyflurane. Addition of methoxyflurane, 75 mmoles/mole of lipid, reduces this step from a relative height given by the ratio $H_l/(H_l + H_u) = 0.45$ to a value 0.2. Both the phase transition temperature of the free phosphatidic acid and of the polymyxin/phosphatidic acid domains are lowered and the width of the free phosphatidic acid phase transition is broadened by methoxyflurane. The effect of a higher methoxyflurane concentration is demonstrated in Fig. 3B for phosphatidic acid bilayers containing polymyxin, 4 moles %. Again, the lower phase transition step attributed to the polymyxin-phosphatidic acid domain diminishes with increasing amounts of methoxyflurane and disappears at 100 mmoles/mole of lipid.

The upper phase transition temperature (T_u in Fig. 2) is also lowered by about 7°. Further addition of methoxyflurane up to 200 mmoles/mole of lipid broadens and lowers this phase transition by an additional 7° as well as increases the fluidity of the membrane below the lipid phase transition temperature. At 15°, for example, the fluidity parameter increases from $f = 0.05$ to $f = 0.095$ in the presence of 200 mmoles of methoxyflurane per mole of lipid. Corresponding results are obtained at polymyxin concentrations of 6 moles %; however, in these bilayers more methoxyflurane is necessary to obtain a complete loss of the lower phase transition step of the phosphatidic acid-polymyxin domain.

These results are shown quantitatively in Fig. 4. The ratio $H_l/(H_l + H_u)$, obtained from the phase transition curves, is plotted as a function of methoxyflurane concentration for membrane preparations containing polymyxin, 4 or 6 moles %. Under atmospheric pressure we observe a decrease in the ratio $H_l(H_l + H_u)$ with increasing anesthetic concentration. The extrapolated straight line leads to values of 70 or 120 mmoles of methoxyflurane per mole of lipid where the lower transition step of the phosphatidic acid-polymyxin domain disappears, if the membrane contains polymyxin, 4 or 6 moles %, respectively.

Antagonistic effect of pressure. Helium pressure of 100 atmos was applied to phosphatidic acid bilayers containing polymyxin, 4 moles %, in the presence of methoxyflu-

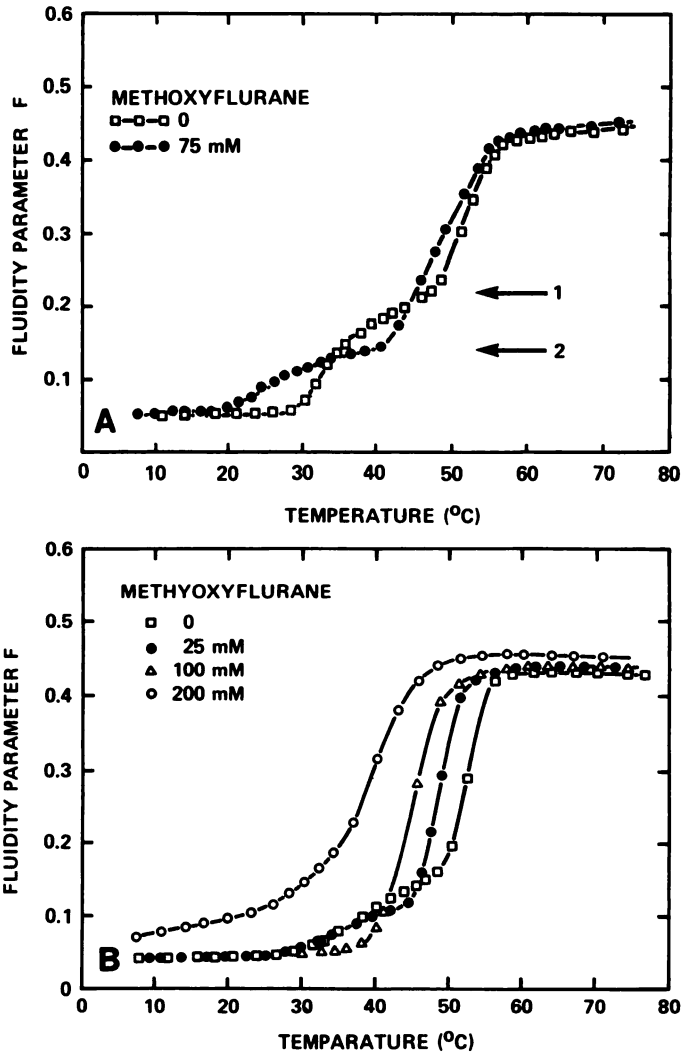

FIG. 3. *Phase transition curve of dipalmitoylphosphatidic acid bilayers*

A. Polymyxin, 6 moles %, in the absence (□——□) and presence (●——●) of 75 mmoles of methoxyflurane per mole of lipid. *Arrows 1* and *2* point out the step height of the lower phase transition step designated as H_l in Fig. 2.

B. Equivalent bilayer preparations as in A but with polymyxin, 4 moles %. The phase transition curves are given for different amounts of anesthetic between 25 and 200 mmoles/mole of lipid. The lower phase transition disappears at concentrations of methoxyflurane greater than 100 mmoles/mole of lipid.

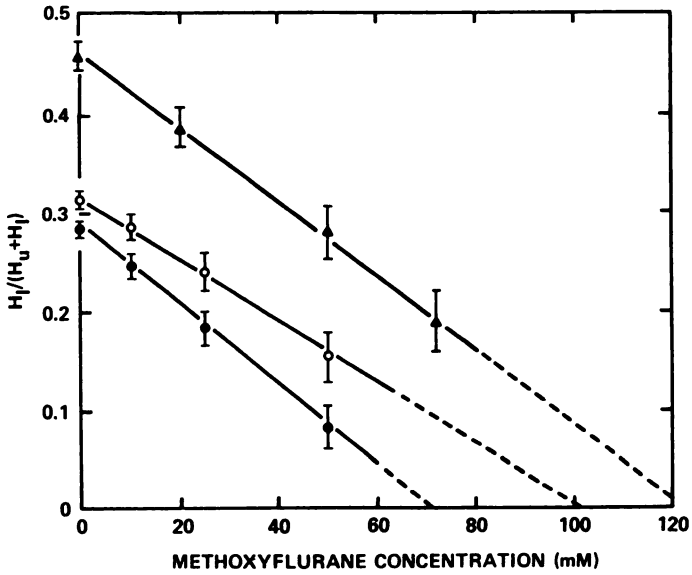

FIG. 4. *The ratio $H_l/(H_l + H_u)$ obtained from measurements as shown in Figs. 2 and 3 is plotted against methoxyflurane concentration for dipalmitoylphosphatidic acid bilayers containing polymyxin, 6 moles % (▲——▲) or 4 moles % (●——●)*

Measurements with the latter sample were also performed at 100 atmos helium pressure (○——○).

rane. At methoxyflurane concentrations less than 100 mmoles/mole of lipid we observed an antagonistic pressure effect. The step height, H_l, increased with pressure at a given anesthetic concentration. Figure 5 shows an example at 50 mmoles of methoxyflurane per mole of lipid. Pressure raises the ratio $H_l/(H_l + H_u)$ from 0.9 to 1.7. This pressure effect is lower at lower methoxyflurane concentrations, as shown in Fig. 4. In the absence of anesthetic, pressure increases the ratio $H_l/(H_l + H_u)$

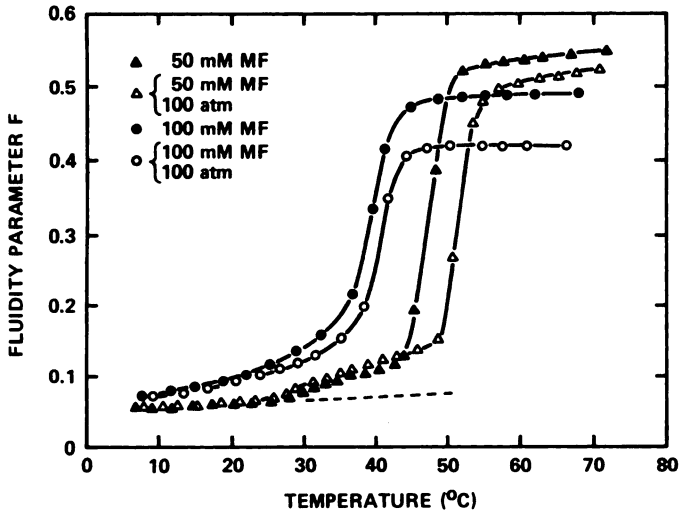

FIG. 5. *Pressure dependence of the phase transition curves of dipalmitoylphosphatidic acid bilayers containing polymyxin, 4 moles %, and additional treatment*

Methoxyflurane, 50 mmoles/mole of lipid at atmospheric pressure (▲——▲); methoxyflurane, 50 mmoles/mole of lipid and application of 100 atmos helium (△——△); methoxyflurane, 100 mmoles/mole of lipid at atmospheric pressure (●——●); and methoxyflurane, 100 mmoles/mole of lipid at 100 atmos helium (○——○).

only from 0.28 to 0.31. In an earlier paper (10) we interpreted this pressure effect as a pressure-induced reorganization of the phosphatidic acid-polymyxin complex resulting from the redistribution of polymyxin dissolved in the surrounding phosphatidic acid phase. Extrapolation of the straight line in Fig. 4 at 100 atmos helium pressure for phosphatidic acid bilayers containing polymyxin, 4 moles %, leads to a concentration of 100 mmoles of methoxyflurane per mole of lipid necessary for the complete disappearance of the low melting phosphatidic acid-polymyxin domain. Therefore, an additional 30 mmoles of anesthetic per mole of lipid are required at 100 atmos as compared with normal pressure to disperse the complex.

The phase transition temperature T_m of the free phosphatidic acid phase is lowered from 53° in the absence of methoxyflurane to 48° at 50 mmoles of anesthetic per mole of lipid if polymyxin, 4 moles %, is present (Fig. 5). However, a pressure of 100 atmos helium increases the phase transition temperature to 52°, which means that pressure antagonizes the anesthetic effects in this concentration range. This antagonism is much less at concentrations of methoxyflurane greater than 100 mmoles/mole of lipid. In a manner corresponding to that represented in Fig. 5, the lipid phase transition temperature is broadened and lowered to 42°. A sigmoidal curve is obtained which shows no evidence for the existence of phosphatidic acid-polymyxin domains. Pressure of 100 atmos helium shifts the phase transition curve by only 2°. This result is in striking contrast to our earlier results in pure phosphatidic acid membranes, where a 10° shift in phase transition temperature could be reversed by 100 atmos helium pressure.

DISCUSSION

We have investigated the effect of methoxyflurane on lateral phase separations caused by the peptide antibiotic polymyxin-B in phosphatidic acid bilayers. In earlier studies (8, 9) it was demonstrated that polymyxin strongly binds to negatively charged phosphatidic acid. Very recently, a strong interaction of polymyxin with negatively charged phosphatidylglycerol was demonstrated in monolayers (14) as well as in bilayer vesicles.[3] In the latter, polymyxin causes the same type of domain formation as in phosphatidic acid bilayers. Therefore our present study using phosphatidic acid bilayers can be considered as a model for a variety of negatively charged lipids that are present in large amounts in nerve membranes (15).

The most significant result is that inhalation anesthetics perturb protein-induced lateral phase separations. We have demonstrated the disruption of phosphatidic acid-polymyxin domains which were formed within a surrounding phosphatidic acid bilayer matrix. A model of these domains is shown in Fig. 6. The polymyxin-phosphatidic acid domain (Fig. 6) contains numerous subunits consisting of one polymyxin molecule binding at least three phosphatidic acid molecules. The incorporation of this highly asymmetrical peptide causes an elastic distortion of the bilayer surface and the assembly of

[3] F. Sixl and H. J. Galla, unpublished observations.

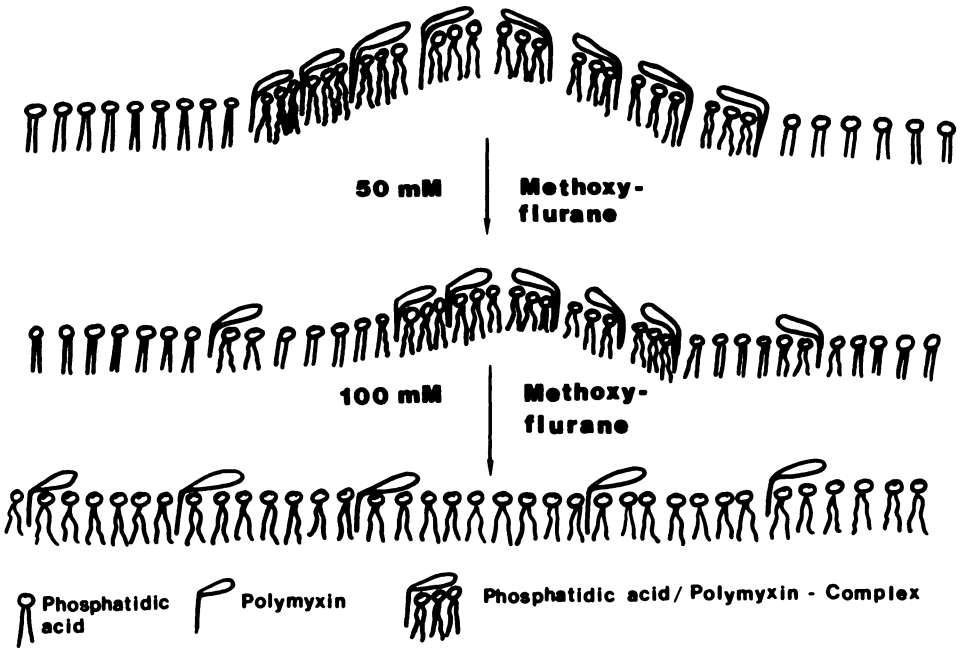

FIG. 6. *Model for the polymyxin/phosphatidic acid domain (from ref. 8)*
Addition of a small amount of methoxyflurane disperses some of the subunits and therefore decreases the amount of phosphatidic acid in the polymyxin-phosphatidic acid domains. At a higher concentration of methoxyflurane (e.g., Fig. 4) the domains are completely dispersed.

many of these subunits into cooperative domains. The domains exhibit a curvature with respect to the bilayer surface and form cusps in order to minimize elastic distortion energy (16).

The dissolution of an inhalation anesthetic in these phosphatidic acid-polymyxin domains lowers their cooperativity, reduces the requirement to relieve elastic distortion energy, and results in dispersion of the complexes into a single homogenous mixed phase. A concentration of 100 mmoles of methoxyflurane per mole of phosphatidic acid in a bilayer containing polymyxin, 4 moles %, causes the phosphatidic acid-polymyxin domain to be dispersed completely. Only one broad phase transition is observed which higher concentrations of methoxyflurane further broaden and lower. Pressure antagonizes the anesthetic effect on the polymyxin-phosphatidic acid domain at concentrations of methoxyflurane up to 100 mmoles/mole of lipid. The low temperature phase transition step increases upon application of 100 atmos helium pressure as is shown in Figs. 4 and 5. Pressure antagonism of the anesthetic effect is also observed in the phase transition of the surrounding free phosphatidic acid matrix. However, at anesthetic concentrations higher than 100 mmoles/mole of lipid, the broadened phase transition between 30° and 45° (Fig. 5) is elevated only 2° by 100 atmos pressure. This is in contrast to our earlier result obtained in pure dipalmitoylphosphatidic acid bilayers (11), where we observed an almost complete pressure antagonism: the phosphatidic acid phase transition temperature was lowered by about 10° after incubation with 100 mmoles of methoxyflurane per mole of lipid, but this effect was completely antagonized by 100 atmos pressure. In contrast, the 2° increase with 100 atmos helium pressure in phase transition temperature in phosphatidic acid-polymyxin bilayers containing methoxyflurane is comparable to the small increase previously observed in phosphatidylcholine bilayers (12).

Monolayer experiments (17) have shown that phosphatidic acid forms a much more condensed lipid film than does phosphatidylcholine. Polymyxin added to phosphatidic acid bilayers is mainly incorporated into phosphatidic acid-polymyxin domains but is also dissolved as monomers within the free phosphatidic acid bilayer surrounding the domain (10). Therefore, the upper phase transition at T_u (Fig. 2) is also lowered to some extent after polymyxin incorporation as a result of the disturbance of the high packing density of the pure phosphatidic acid matrix. Addition of methoxyflurane to phosphatidic acid bilayers containing polymyxin leads to the progressive dispersion of the phosphatidic acid-polymyxin domains into monomeric subunits. We suggest that a new lipid phase with a much lower packing density and higher fluidity than pure phosphatidic acid is formed. The dispersed polymyxin-phosphatidic acid subunits create a lipid matrix in which anesthetic molecules are readily soluble. The compressibility (dV/dP) of this new phase may be completely different from that of pure phosphatidic acid bilayers in the presence of methoxyflurane. In the latter case, methoxyflurane is extruded from the bilayer by pressure (11). If polymyxin is present, this is not the case, as seen in Fig. 5. Extrusion of the anesthetic by pressure is not observed in phosphatidylcholine bilayers even though the anesthetic-induced increase in chain mobility is antagonized by pressure. We suggest that polymyxin causes phosphatidic acid bilayers to respond to inhalation anesthetic more like pure phosphatidylcholine bilayers by lowering the packing density and increasing the surface area per phospholipid.

It is noteworthy that in this model system the effect of 30 mmoles of methoxyflurane per mole of lipid is antagonized by 100 atmos pressure (Fig. 4). The early Meyer-Overton theory of anesthesia predicted that clinical anesthesia will be produced whenever the concentration of any anesthetic in a nerve membrane reaches 20–50

mmoles/mole of lipid. A concentration of 30 mmoles of halothane per mole of phospholipid-cholesterol was recently measured in an aqueous vesicle suspension equilibrated with a clinical concentration of 1.3% halothane in air (18). Helium pressures of approximately 100 atmos have been shown to reverse the effects of a clinical concentration of anesthetics in a variety of species, including luminous bacteria, tadpoles, and mice (3, 4).

These results offer support for theories that propose that anesthetic-induced disruption of lateral phase separations near membrane-solvated proteins may be a mechanism of anesthesia. They add to the evidence that proteins are capable of forming lateral phase separations in the phospholipid matrix that surrounds them (2, 8, 14). Furthermore, these protein-induced phase separations are strongly affected by inhalation anesthetics, leading to disruption of long-range protein-protein cooperativity. Polymyxin is largely an extrinsic membrane protein that binds ionically to the surface of negatively charged bilayers and hydrophobically with a hydrocarbon tail. As such, it is only the beginning of a nerve membrane model for studying molecular theories of anesthesia. However, the evidence presented here for disruption of protein-induced lateral phase separations by an anesthetic will facilitate interpretations of less dramatic results that may be obtained with neural proteins.

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