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Membrane Disordering Effects of Anesthetics Are Enhanced by Gangliosides

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The effects of anesthetic drugs on lipid order were evaluated by the fluorescence polarization of the probe molecule, 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated into vesicles of dimyristoylphosphatidylcholine (DMPC) and DMPC with 10 mol% ganglioside (G_{D1a}). Anesthetics (enflurane, chloroform, diethylether, pentobarbital, ethanol, butanol, hexanol) decreased the fluorescence polarization of DPH in vesicles of DMPC, but relatively large concentrations were required. Addition of gangliosides to DMPC enhanced the lipid disordering effects of anesthetics by several fold. The potencies of these anesthetics in decreasing fluorescence polarization of DPH in DMPC-ganglioside was well correlated with their potencies as anesthetics, and significant decreases in fluorescence polarization occurred at pharmacologically relevant concentrations. These results indicte that gangliosides can enhance the sensitivity of membrane lipids to the disordering effects of anesthetics and suggest that the large ganglioside content of the outer leaflet of the lipid bilayer of neuronal membranes may render this membrane region unusually sensitive to anesthetic agents. (Key words: Alcohols: butanol; hexanol. Anesthetics, volatile: chloroform; diethylether; enflurane. Membranes: fluidity; lipid bilayers. Theories of anesthesia: lipid solubility.)

ANESTHETIC DRUGS frequently have been postulated to alter neuronal functioning by dissolving into membrane lipids, altering lipid physical properties, and thereby altering membrane function.[1] The functions of some membrane proteins, including neurotransmitter receptors,[2] ion channels,[3,4] and enzymes,[5] are altered by changes in membrane order, suggesting that perturbation of neuronal lipids by general anesthetics may alter the excitability of brain tissue. The "disordered lipid" hypothesis has been criticized,[6] however, because most effects of anesthetics on membrane order have been obtained only with drug concentrations much larger than are required for general anesthesia *in vivo*. This lack of response to anesthetics may reflect the insensitivity of probes and lipids (mainly phosphatidylcholine [PC]) used in these studies and does not rule out an effect of anesthetics under other conditions. Pang *et al.*[4] noted that a fluorescent probe of the membrane core, 1,6-diphenyl-1,3,5-hexatriene (DPH), was more sensitive than an ESR probe to the disordering effects of pentobarbital and halothane on PC-phosphatidic acid bilayers. We also used DPH to compare brain synaptic membranes to phospholipid vesicles and found the native membranes to be more sensitive than purified lipids to the disordering effects of ethanol and pentobarbital.[7,8] These data indicated that studies of phospholipid may not provide a definitive test of the disordered lipid hypothesis and suggested importance of other membrane lipids.

The phospholipids of the plasma membrane of nerve are not distributed randomly but are ordered in an asymmetric fashion, with the inner half of the bilayer containing most of the phosphatidylethanolamine and phosphatidylserine and the outer half containing PC.[9] Nerve membranes differ from most other cellular membranes in that the predominant sphingolipids are gangliosides, rather than sphingomyelin.[10] Gangliosides are localized in the outer half of the membrane and constitute 10–20% of the lipid in this monolayer.[10,11] The enrichment of gangliosides in nerve membranes prompted studies of their effects in the physical and functional properties of brain membranes.[12,13] The localization of gangliosides suggested to us that vesicles of ganglioside and PC should provide a better model of the outer monolayer of brain membranes than PC alone. Indeed, ganglioside–PC vesicles were found to be more sensitive to the membrane-disordering effects of ethanol and pentobarbital than vesicles of PC alone.[14] This suggested that gangliosides may be an important factor determining the drug sensitivity of biomembranes and raised the possibility that the enhancement of action observed with ethanol and pentobarbital might occur with other anesthetic agents. Our initial study of ganglioside–PC vesicles evaluated the effects of ganglioside composition, ganglioside concentration, PC acyl composition, assay temperature, and probe location.[14] Based on those findings, we chose to compare the effects of ansthetics on vesicles of dimyristolyphosphatidylcholine (DMPC) and DMPC plus 10 mol% disialoganglioside (G_{D1a}) (the major ganglioside of synaptic membranes[15]) using the fluorescence polarization of DPH as a measure of lipid order.

Methods

MATERIALS

Dimyristoylphosphatidylcholine (DMPC) was obtained from Sigma Chemical Co. (St. Louis, Missouri). No

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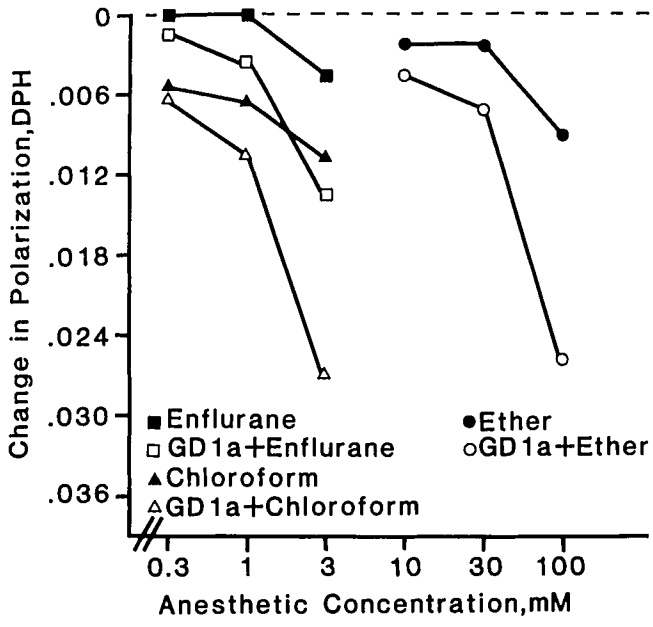

FIG. 1. Effects of anesthetics on the fluorescence polarization of DPH in vesicles of DMPC (solid symbols) or DMPC plus 10 mol% ganglioside (G_{D1a}, open symbols). Squares represent the effects of enflurane, triangles represent chloroform, and circles represent diethylether. Values are the mean from three vesicle preparations; the SEM was 0.001–0.003. For all drugs there was a significant ($P < 0.05$) effect of ganglioside addition. Linear regression analysis of drug concentration *versus* change in polarization gave the following parameters (y-intercept, slope, correlation coefficient-r): enflurane, −.0007, −.0011, .86 (DMPC), −.0001, −.0041, .99 (DMPC + G_{D1a}); chloroform, −.003, −.002, .97 (DMPC), −.002, −.009, .99 (DMPC + G_{D1a}); diethylether, −.0004, −.0001, .98 (DMPC), −.0004, −.0002, .99 (DMPC + G_{D1a}).

impurities were detected by thin layer chromatography, and it was used without purification. Disialoganglioside (G_{D1a}) was obtained from Supelco, Inc. (Bellefonte, Pennsylvania) and 1,6-diphenyl-1,3,5-hexatriene from Molecular Probes, Inc. (Junction City, Oregon). Drugs utilized in these experiments were obtained from the following sources: n-butanol, n-hexanol, anesthetic grade diethyl ether, Fisher Scientific (St. Louis, Missouri); enflurane, Ohio Medical (Arlington, Texas); chloroform (glass distilled), MCB Manufacturing Chemists, Inc. (Cincinnati, Ohio).

Preparation of Lipid Vesicles

DMPC (60 nmol, dissolved in ethanol) was mixed with 10 mol% G_{D1a} dissolved in $CHCl_3OH$ (2:1) and 0.5 nmol of DPH dissolved in tetrahydrofuran. All solutions were stored under argon at −20° C. The mixtures of lipids and probe were dried to a thin film by a stream of nitrogen and stored overnight under vacuum. One milliliter of phosphate-buffered saline (8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 g/l $Na_2HPO_4 \cdot 7\ H_2O$, 0.48 g/l N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, *p*H 7.4) was added to each sample, the tubes were purged with argon and incubated in the dark with occasional vortexing at about 35° C. The samples then were placed in a bath-type sonicator for 1 min. After vigorous vortexing, they were transferred to a 10 × 4 mm quartz cuvette for fluorescence measurements. This method produces multilamellar vesicles.[16]

Fluorescence Measurements

A HH-1 T-format polarization spectrofluorimeter (BHL Associates, Burlingame, California) with fixed excitation and emission polarization filters were used to measure fluorescence intensity parallel ($I_{||}$) and perpendicular ($I_{\perp}$) to the polarization plane of the exciting light.[8] Polarization of fluorescence $[(I_{||} - I_{\perp})/(I_{||} + I_{\perp})]$ and intensity of fluorescence ($I_{||} + 2I_{\perp}$) were calculated by an on-line microprocessor. The excitation wavelength was 362 nm and a 03FCG001 filter (Melles Griot, Irvine, California) was used in the excitation beam and 003FIR045 filters were used for the emitted light. The use of an excitation filter reduced light scattering to negligible levels. Cuvette temperature was maintained by a circulating water bath and monitored continuously by a thermister inserted into the cuvette to a level just above the light beam. Sample temperature was 30° C unless otherwise noted. After determination of the baseline fluorescence polarization, small aliquots (1–5 μl) of anesthetic (diluted with dimethylsulfoxide) were added, the cuvette was capped and the solution was mixed vigorously. Polarization was determined 2–4 min later.[7,8] Each determination was performed with at least three different membrane preparations. Addition of dimethylsulfoxide, the vehicle for the anesthetics, did not alter fluorescence polarization. Gas chromatographic analysis of the solutions containing volatile anesthetics indicated loss of 10–20% of the anesthetic during mixing and determination of fluorescence polarization.

Statistics

Concentration–response curves were compared by analysis of variance for repeated measures. Least-squares linear regression analysis was performed with an Apple II® computer, using Graph Trix® software (Interactive Microware, State College, Pennsylvania).

Results

Enflurane, chloroform, and diethylether decreased the fluorescence polarization of DPH in vesicles of pure DMPC (fig. 1), indicating that the anesthetics increased the fluidity of the lipids. The changes in fluorescence polarization were, however, rather small, and relatively large concentrations of the anesthetics were required.

The effect of these anesthetics was augmented greatly by addition of ganglioside (10 mol% of G_{D1a}) to the DMPC vesicles. The decrease in fluorescence polarization produced by 3 mM enflurane or chloroform or 100 mM diethylether was augmented threefold to fourfold by incorporation of ganglioside into the liposomes (fig. 1). Similarly, two alkanol anesthetics, n-hexanol and n-butanol, produced only small changes in the fluorescence polarization of DPH in DMPC vesicles but markedly decreased polarization in DMPC–ganglioside membranes (fig. 2). Analogous results also have been obtained with ethanol and pentobarbital.[14] The concentration–response curves in Figures 1 and 2 are not linear because concentration is presented on a logarithmic scale. Drug effect was directly proportional to drug concentration, as would be expected for a nonsaturable site of action (see figure legends for regression equations). As indicated previously,[14] ganglioside increased the order of DMPC vesicles. The baseline polarization was 0.142 ± 0.001 (n = 9) for DMPC and 0.174 ± 0.002 (n = 9) for DMPC + G_{D1a}.

In evaluating these results, it was important to compare the potencies of the drugs in fluidizing DMPC and DMPC–ganglioside membranes with their *in vivo* potencies as anesthetics. We estimated the concentration of each anesthetic required to reduce fluorescence polarization by 0.01 by linear regression analysis of change in polarization *versus* drug concentration. These values are plotted *versus* serum concentrations required for anesthesia (fig. 3). The potencies of the drugs in fluidizing DMPC or DMPC–ganglioside membranes were correlated with the anesthetic potencies, but the correlation was somewhat better for DMPC–ganglioside vesicles ($r^2 = 0.96$) than for only DMPC ($r^2 = 0.88$). In addition, the fluidizing concentrations were more comparable to the anesthetic concentrations for DMPC–ganglioside membranes than for DMPC. For example, the concentrations of diethylether, butanol, and ethanol required to fluidize DMPC–ganglioside membranes were similar to those producing anesthesia *in vivo*, but the concentrations needed to fluidize DMPC vesicles were three to five times higher than the anesthetic concentrations (fig. 3). A simple theory linking polarization and anesthetic action would predict lines with unit slope in figure 3. In fact, the slopes are 0.69 (DMPC) and 0.72 (DMPC + G_{D1a}). We have no explanation for this finding.

The experiments presented above were carried out at 30° C, a temperature above the gel–liquid crystalline

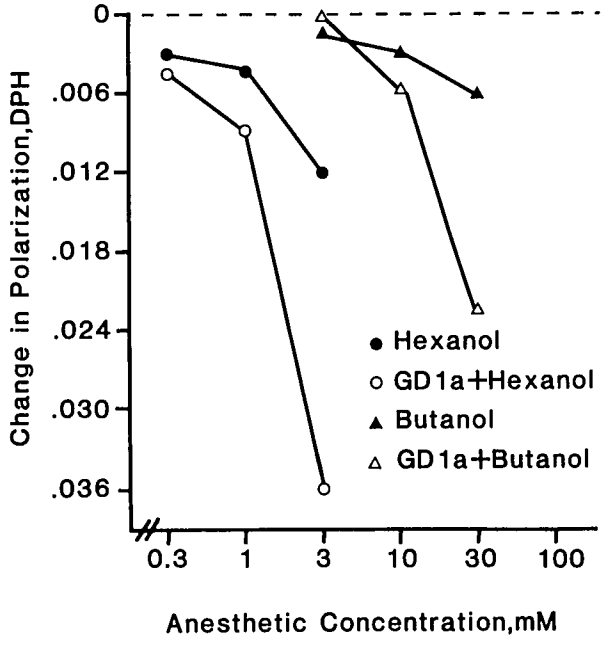

FIG. 2. Effects of n-hexanol (circles) and n-butanol (triangles) on the fluorescence polarization of DPH in vesicles of DMPC (solid symbols) or DMPC plus 10 mol% ganglioside (open symbols). Details as for Figure 1. Linear regression analysis of drug concentration *versus* change in polarization gave the following parameters (y-intercept, slope, correlation coefficient-r): hexanol, −.002, −.003, .99 (DMPC), +.002, −.013, .99 (DMPC + G_{D1a}); butanol, −.0006, −.0002, .90 (DMPC), +.0064, −.0009, .99 (DMPC + G_{D1a}).

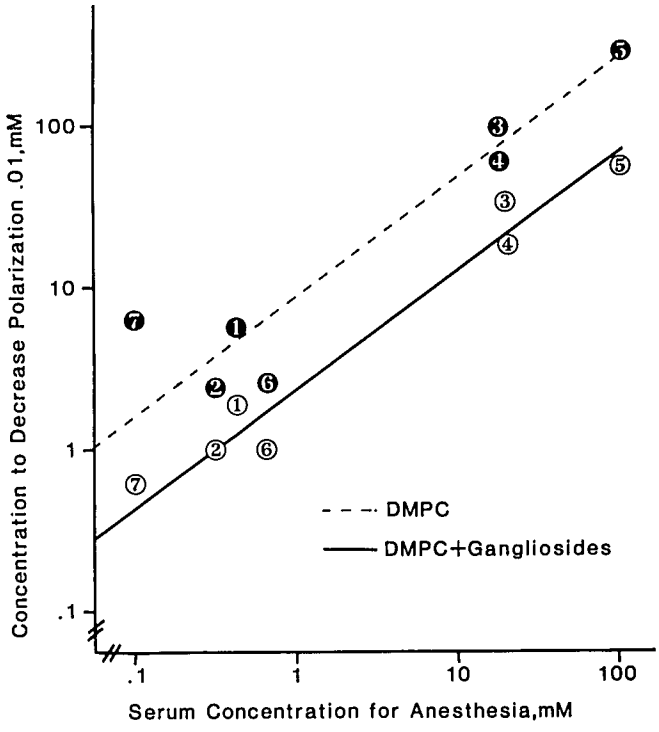

FIG. 3. Correlation of serum concentration for anesthesia (abscissa) with the concentrations required to reduce the fluorescence polarization of DPH in DMPC (solid circles) or DMPC plus 10 mol% ganglioside (G_{D1a}, open circles) by 0.01 (ordinate). For solid line, $r^2 = 0.96$, for dotted line, $r^2 = 0.88$. Numbers denote the following anesthetics: 1, enflurane, 2, chloroform; 3, diethylether, 4, n-butanol; 5, ethanol; 6, n-hexanol; 7, pentobarbital. Values of anesthetic potency are from Sturtvant[24] (compounds 5–7) and Janoff *et al.*[25] (1–4).

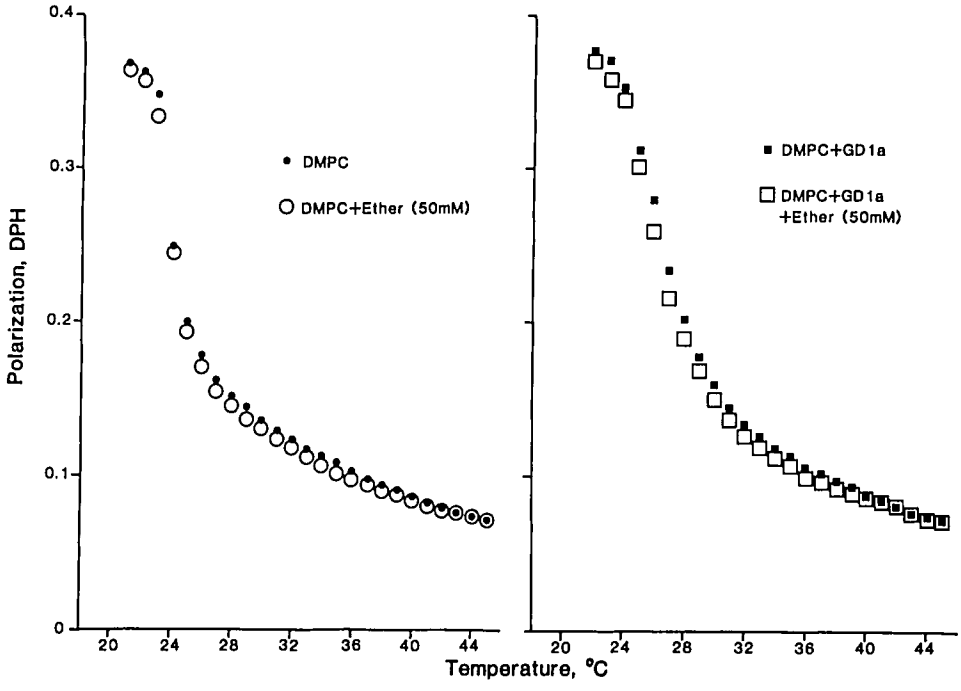

FIG. 4. Temperature dependence of the effects of diethylether (50 mM) (open symbols) on the fluorescence polarization of DPH in vesicles of DMPC (left panel) and DMPC plus 10 mol% ganglioside (G_{D1a}) (right panel). Values are the mean from three vesicle preparations. The SEM was 0.001–0.003.

phase transition of DMPC. The temperature dependence of the effects of ganglioside and diethylether was explored by determining fluorescence polarization while vesicles were cooled from 45 to 21° C. With vesicles of DMPC or DMPC plus ganglioside, a marked change in fluorescence polarization occurred at 24–28° C, indicative of a phase transition (fig. 4). In the presence of ganglioside, the change in polarization was more gradual and occurred at a higher temperature than in the absence of ganglioside. The decrease in polarization produced by diethylether, and the enhancement of this decrease by ganglioside, was more pronounced at temperatures near the phase transition than at temperatures above or below the transition (fig. 4).

Discussion

We previously found that gangliosides enhance the lipid-disordering effects of ethanol on vesicles of various types of PC as well as DMPC–cholesterol mixtures.[14] These present findings extend these results by demonstrating that gangliosides increased the actions of several chemically diverse anesthetic drugs on vesicles of DMPC. Interpretation of these results requires a brief discussion of fluorescence polarization. Numerous studies show polarization to be influenced by the mobility of the probe and the fluorescence lifetime.[17] Neither gangliosides nor anesthetic drugs (at the concentrations used in this study) alter fluorescence lifetime.[8,14,18] Thus, the decrease in fluorescence polarization produced by anesthetics can be attributed to an increased mobility of the probe. The motion of DPH in phospholipid vesicles has been studied in detail and appears to be hindered by the order of the acyl chains.[19,20] Because of restricted motion of the probe, steady state fluorescence polarization of DPH is difficult to relate to microviscosity of the lipids but instead appears to reflect the order of the acyl chains in the membrane core.[21] These considerations, together with results from ESR and NMR measurements,[18,22] indicate that anesthetics decreased fluorescence polarization because they disordered the lipid acyl groups.

With pure phospholipids or phospholipid–cholesterol mixtures, the lipid-disordering effect is small at clinical concentrations of anesthetics,[14,18] and its importance in anesthesia has been questioned.[6] The present results demonstrate that neuronal lipids such as gangliosides magnify the disordering effects of anesthetics and suggest that phospholipids may not provide an adequate model for the study of effects of anesthetics on the physical properties of neuronal membranes. Although gangliosides enhanced the effects of anesthetics, one must ask whether the disordering is sufficient to alter membrane function. This question cannot be answered definitively with information presently available, but we can note that in the present study clinical concentrations of anesthetics reduced DPH polarization by 0.01 to 0.02 units. In two other studies, the ability of membrane perturbants to increase brain synaptic Ca^{2+}-ATPase and inhibit voltage-dependent sodium influx was correlated with their potencies in decreasing DPH polarization.[3,5] In both of these studies, a change in polarization of 0.01 was associated with a significant change in membrane function. Changes in DPH polarization often are converted to changes in microviscosity (although this calculation assumes isotropic motion and may not be

valid, for the reasons discussed above) and a change of polarization from 0.17 to 0.16 (0.01 units) corresponds to a microviscosity change of 8.5%.[17] A decrease in microviscosity of 2–5% is associated with 20–50% change in ion permeability of lipid vesicles.[4] Taken together, these data indicate that the disordering effects of anesthetics on ganglioside–PC membranes observed in the present study may be sufficient to alter membrane function. The membrane disordering effects of anesthetics often are equated to increases in temperature.[6] In this context, the change in polarization produced by anesthetic levels of the drugs was equivalent to a change in temperature of 1–2° C.

There are several mechanisms by which gangliosides might enhance the lipid-disordering effects of anesthetics. An obvious possibility is that gangliosides could increase the partitioning of anesthetics into the lipid phase. Lipid composition is known to alter the partitioning of anesthetics,[23] and gangliosides increase the uptake of pentobarbital by DMPC vesicles.[14] These effects of lipids on drug partitioning are, however, rather small, and it is not clear that they can account for the observed changes in fluorescence polarization. A related possibility is that gangliosides increase the partitioning of anesthetics into lipids that are in a fluid phase but not those in a gel phase. This would explain why gangliosides did not alter the effects of anesthetics at temperatures below the phase transition and is consistent with thermodynamic arguments about the selective partitioning of anesthetics into fluid phases.[23,24] Another possibility is suggested by the observation that ethanol and butanol produce a larger depression of the lipid phase transition temperature than would be predicted from partition coefficients or thermodynamic parameters.[24] Thus, there may be some sort of cooperative interaction between anesthetics and lipids, and gangliosides may increase the degree of cooperativity. Regardless of the mechanism, the present results demonstrate that gangliosides enhanced the membrane-disordering effects of anesthetics and suggest that lipid composition may influence the action of anesthetics on cell membranes.

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