Clinical concentrations of chemically diverse general anesthetics minimally affect lipid bilayer properties

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General anesthetics have revolutionized medicine by facilitating invasive procedures, and have thus become essential drugs. However, detailed understanding of their molecular mechanisms remains elusive. A mechanism proposed over a century ago involving unspecified interactions with the lipid bilayer known as the unitary lipid-based hypothesis of anesthetic action, has been challenged by evidence for direct anesthetic interactions with a range of proteins, including transmembrane ion channels. Anesthetic concentrations in the membrane are high (10–100 mM), however, and there is no experimental evidence ruling out a role for the lipid bilayer in their ion channel effects. A recent hypothesis proposes that anesthetic-induced changes in ion channel function result from changes in bilayer lateral pressure that arise from partitioning of anesthetics into the bilayer. We examined the effects of a broad range of chemically diverse general anesthetics and related nonanesthetics on lipid bilayer properties using an established fluorescence assay that senses drug-induced changes in lipid bilayer properties. None of the compounds tested altered bilayer properties sufficiently to produce meaningful changes in ion channel function at clinically relevant concentrations. Even supra-anesthetic concentrations caused minimal bilayer effects, although much higher (toxic) concentrations of certain anesthetic agents did alter lipid bilayer properties. We conclude that general anesthetics have minimal effects on bilayer properties at clinically relevant concentrations, indicating that anesthetic effects on ion channel function are not bilayer-mediated but rather involve direct protein interactions.

anesthetic mechanisms | gramicidin channel | bilayer modification | amphiphiles | isoflurane

General anesthetics are essential drugs in modern medicine, yet their molecular mechanisms remain poorly understood, as it is unclear whether or not general anesthetics exert their effects by altering lipid bilayer properties. The Meyer-Overton correlation of anesthetic potency with lipophilicity, which does not identify a specific mechanism, led to lipid bilayer-based proposals for the mechanisms of general anesthesia that dominated the field until challenged in the 1970s (1–3). The seminal work of Franks and Lieb led them to conclude that "the lipid bilayer alone is not the anaesthetic site" (2). They subsequently showed that the Meyer-Overton correlation was preserved for inhibition of firefly luciferase, a soluble lipid-free model protein (4). This led to a search for critical protein targets for general anesthetics, which resulted in the identification of a number of plausible voltage-gated and ligand-gated ion channel targets (5, 6). The possible involvement of the lipid bilayer in the effects of lipophilic anesthetics on membrane proteins has not been excluded, however. Anesthetic concentrations in biological membranes are in the 10–100 mM range, making it difficult to exclude bilayer-mediated effects. Indeed, Cantor and colleagues have developed a modern, mechanistic lipid bilayer-based hypothesis, based on changes in the bilayer lateral pressure profile, to explain how general anesthetics could alter the function of membrane proteins (7, 8). An alternative bilayer-based mechanism for general anesthesia has been proposed based on anesthetic-induced changes in membrane domain organization (9, 10).

The concentrations of anesthetics at which effects are observed are critical for interpreting experimental data obtained in vitro. General anesthetics clearly alter lipid bilayer properties at high concentrations, which are irrelevant for clinical anesthesia but could contribute to toxicity at supratherapeutic doses (2, 3). This concentration dependence has clinical implications for the dose-dependent effects of general anesthetics: at low doses, the commonly used volatile anesthetic isoflurane causes only amnesia (11); at medium (therapeutic) doses it produces the desired clinical endpoint of hypnosis and immobility (5, 12); and at even higher supratherapeutic (toxic) doses it causes undesirable side effects such as cardiovascular and respiratory depression (13). Whereas isoflurane at anesthetic concentrations alters ion channel function with no discernible effect on lipid bilayer properties (14), it does alter lipid bilayer properties at supratherapeutic concentrations, supporting the idea that the lipid bilayer is not an important target for its therapeutic effects. The generality of this observation to other anesthetics has not been established, however.

To test the hypothesis that general anesthetics at clinically relevant concentrations do not produce marked changes in lipid bilayer properties, we tested a chemically and pharmacologically diverse panel of representative anesthetics using a functional assay sensitive to alterations in lipid bilayer properties. Our results show that general anesthetics have minimal, if any, effects on lipid bilayer properties at clinical concentrations. Thus,

Significance

General anesthetics are essential to modern medicine, yet their molecular mechanisms remain poorly understood. Whether general anesthetics primarily act by altering lipid bilayer properties or by interacting directly with specific membrane proteins is a longstanding controversy. We now show that diverse classes of general anesthetics do not alter bilayer properties at concentrations that induce clinical anesthesia. Although anesthetics can have bilayer-perturbing effects at supratherapeutic (toxic) concentrations, this has little pharmacological relevance. Our findings exclude indirect ion channel effects due to perturbations of lipid bilayer properties, supporting the notion that general anesthetics interact directly with therapeutically relevant membrane protein targets.

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general anesthetics effects on ion channel function involve direct rather than indirect bilayer mediated effects.

Results

The bilayer-modifying potency of anesthetics were examined using a fluorescence quench method (14, 15). Fig. S1 describes the conceptual basis for these experiments, and Fig. S2 shows fluorescence quench traces for a representative experiment with diethyl ether. The results for a group of chemically and pharmacologically diverse anesthetics are summarized in Fig. 1. Inhaled anesthetics, which are delivered as gases, are grouped as ethers or alkanes (Fig. 1 *A* and *B*). We also included the nonanesthetic compounds flurothyl and F6, which are predicted by the Meyer-Overton correlation to be anesthetics based on their lipid solubility, yet they do not produce immobilization in response to a painful stimulus (16). We tested inhaled agents at clinically relevant concentrations of 1 MAC (minimum alveolar concentration, defined as the concentration that prevents movement in response to a painful stimulus in 50% of subjects, comparable to EC_{50}) and 2 MAC, as well as a supratherapeutic (toxic) concentration (4 MAC). i.v. anesthetics represent a third group (Fig. 1*C*), the effects of which were tested at their EC_{50} for immobilization and multiples thereof.

At clinical concentrations (1 MAC), most of the anesthetics tested did not produce any effects on lipid bilayer properties detectable in the gramicidin-based fluorescent assay (Fig. 1 *B* and *C*). The ethers and F3 altered bulk lipid bilayer properties sufficiently to produce up to a 20% change in quench rate (Fig. 1 *A* and *C*), which produces minimal changes in membrane protein function (14), see also Fig. 2. The anesthetic ethers, F3, and the i.v. anesthetic etomidate increased the quench rate by > 50% at higher concentrations, indicating that they can alter lipid bilayer properties at these clinically irrelevant concentrations (see also (2, 3)). The nonanesthetics flurothyl and F6 did not significantly alter lipid bilayer properties.

The fluorescence experiments in single-component (1,2-dierucoyl-*sn*-glycero-3-phosphocholine, $DC_{22:1}PC$) large unilamellar vesicles (LUVs) are exquisitely sensitive to changes in lipid bilayer elastic properties due to the large hydrophobic mismatch between channel length (~2.2 nm (17, 18)) and bilayer thickness (~3.4 nm (19, 20)). Cellular membranes, however, are complex mixtures of many lipid species with lateral domain organization (21–23), and Veatch, Machta and colleagues (9, 10) have proposed that general anesthetics act by altering membrane domain organization. To explore this question, we also performed experiments with LUVs formed using an equimolar mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine ($DC_{18:1}PC$), cholesterol and brain sphingomyelin. This mixture is known to form membranes with immiscible liquid domains (24, 25).

None of the anesthetics tested in this system (isoflurane, halothane, ketamine and propofol) affected the quench rate at anesthetic or supratherapeutic concentrations (Fig. 1*D*), indicating that the anesthetics are unlikely to produce major changes in membrane domain organization under our experimental conditions. Experiments in planar bilayers, formed from the same "raft-forming" lipid mixture as was used here provide evidence for a single type of kinetically homogenous channel (26), which presumably form in the thinner, less ordered L_d phase. "Conventional" bilayer-modifying molecules, like alcohols, Triton X-100 and capsaicin, did produce increases in quench rates (Fig. 1*D*), albeit only by around 20–35% of the rates observed in $DC_{22:1}PC$ LUVs (14, 27, 28). (Fig. S3 shows amphiphile-induced changes in quench rates in

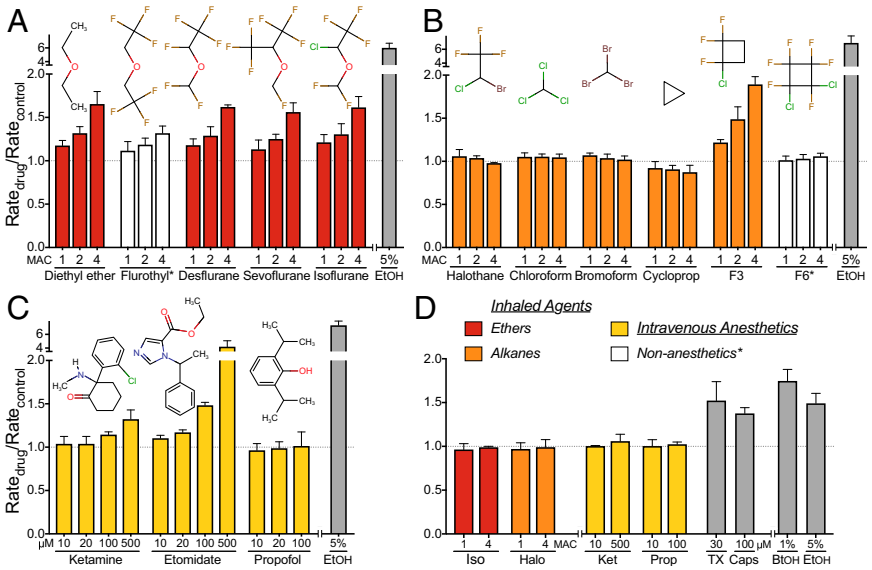

Fig. 1. Effects of general anesthetics on lipid bilayer properties. Normalized fluorescence quench rates of inhaled ether (*A*) and alkane (*B*) compounds at concentrations of ~1, 2, and 4 MAC (minimum alveolar concentration, defined as the concentration that prevents movement in response to a painful stimulus in 50% of subjects, comparable to EC_{50}), and of i.v. anesthetics (*C*) at 10–500 μM, using single-component lipid bilayer vesicles. White columns represent compounds that do not cause immobility (nonanesthetics*) that were tested at concentrations predicted to produce anesthesia based on their lipid solubility. A normalized quench rate ($Rate_{drug}/Rate_{control}$) of 1.0 indicates no significant effect on bulk lipid bilayer properties. Ethanol [EtOH] (gray columns), a known bilayer-modifier at 5% (~0.86 M), was included as a positive control. Data are expressed as mean ± SD, n = 3–5. [10 and 20 μM values for propofol are from (102).]. (*D*) Effects of anesthetics on lipid bilayer properties in multicomponent bilayer vesicles. Identical experiments (as in *A*–*C*) were performed using LUVs composed of an equimolar mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine ($DC_{18:1}PC$), cholesterol and brain sphingomyelin. Normalized fluorescence quench rates of select anesthetics (colored columns) representing each group at both low and high concentrations (1 or 4 MAC for isoflurane [Iso] and halothane [Halo]; 10 or 500 μM for ketamine [Ket]; 10 or 100 μM for propofol [Prop]). Conventional bilayer-modifying molecules (gray columns), such as 30 μM Triton X-100 [TX], 100 μM capsaicin [Caps] and alcohols (1% 1-butanol [BtOH] or 5% ethanol [EtOH]), were included as positive controls, which altered lipid bilayer properties even at low concentrations. Data are expressed as mean ± SD, n = 3–5.

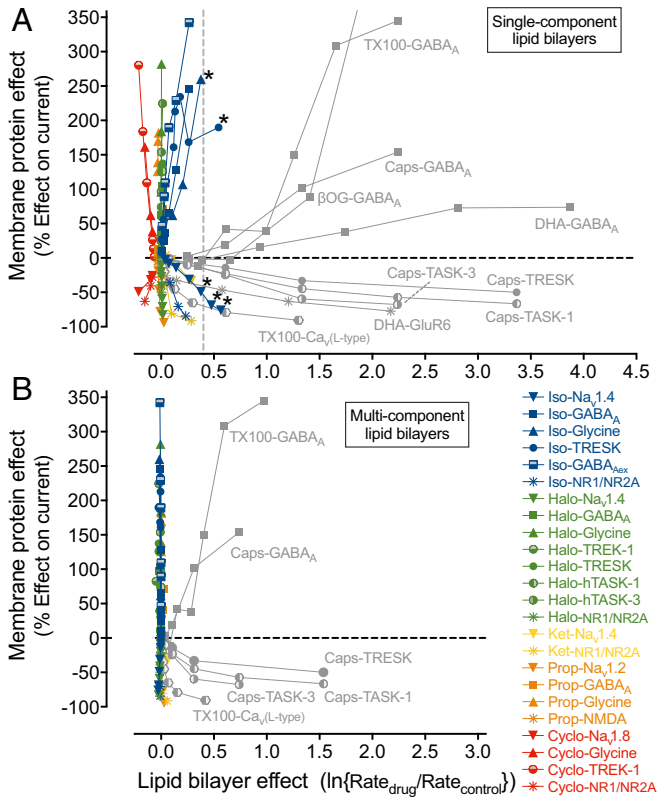

Fig. 2. Effects of general anesthetics and other amphiphiles on ion channel function compared with their lipid bilayer modifying properties. (*A*) Plot of anesthetic- and amphiphile-induced changes in specific ion channel function (as a percentage of potentiation or inhibition of ionic current) against changes in lipid bilayer properties measured (or extrapolated) from the fluorescence quench rate in single component $DC_{22:1}PC$ LUVs. The relation between bilayer-modifying effect and alteration of ion-channel function by five representative anesthetics (isoflurane [Iso], halothane [Halo], ketamine [Ket], propofol [Prop], cyclopropane [Cyclo], colored symbols) and other amphiphiles (Triton X-100 [TX100], β-octyl-glucoside [βOG], capsaicin [Caps], docosahexaenoic acid [DHA], gray symbols) is based on results from this study and from published studies on ion channels (30, 67, 75, 81, 98, 103–122). The horizontal dashed line denotes no change in ion-channel current, and the vertical dashed line shows the threshold for a significant effect on bulk lipid-bilayer properties. All five anesthetics have strong ion channel effects at concentrations at which they have minimal or no bilayer-modifying effects. Gray symbols represent amphiphiles known to strongly alter lipid bilayer properties. A few data points for isoflurane (Iso-$Na_v1.4$, Iso-Glycine and Iso-TRESK, denoted with asterisk) do reach or cross the vertical border, but these bilayer-modifying effects only occur at very high, supratherapeutic concentrations (>4 MAC). (*B*) Corresponding plot using the changes in fluorescence quench rates for multicomponent lipid bilayer LUVs for isoflurane, halothane, ketamine and propofol (abbreviation and color code as in *A*), as well as the known bilayer-modifying amphiphiles Triton X-100 and capsaicin (abbreviation as in *A*; gray symbols). In these multicomponent lipid bilayer experiments, the vertical alignment of the data (corresponding to anesthetics, colored symbols) is much more pronounced compared with the data for single component LUVs (*A*), confirming that clinical concentrations of anesthetics do not have any lipid bilayer altering effects.

single-component ($DC_{22:1}PC$) and multicomponent LUVs.) This presumably reflects the smaller hydrophobic thickness of the $DC_{18:1}PC$-rich liquid disordered (L_d) phase, as experiments with $DC_{18:1}PC$ LUVs show that amphiphiles produce little, if any, changes in fluorescence quench rates (29). We therefore do not observe qualitatively different results from experiments with single- and multicomponent bilayers, confirming the lack of any anesthetic membrane effects at clinical relevant concentrations.

Discussion

Our key finding is that a chemically and pharmacologically diverse panel of general anesthetics has no detectable effects on lipid bilayer properties *at clinically relevant anesthetic concentrations*. These results do not support a lipid bilayer-based mechanism for anesthetic effects on membrane proteins, but rather provide strong support for the hypothesis that general anesthetics exert their desired clinical effects through direct interactions with target membrane proteins.

Power of the Gramicidin-Based Approach. Our results show that the general anesthetics tested do not alter the gramicidin monomer↔dimer equilibrium. The experiments in single-component bilayers lend themselves to unambiguous interpretation: anesthetics do not alter the energetic cost of the local bilayer deformation, which is the bilayer contribution to the free energy of dimerization ($\Delta G^{M\rightarrow D}_{bilayer}$, where M denotes the monomer and D the dimer) associated with forming the bilayer-spanning dimer (30–32). $\Delta G^{M\rightarrow D}_{bilayer}$ varies as a function changes in bilayer thickness, intrinsic curvature, and the elastic bending and compression moduli, which in turn are determined by lateral interactions among membrane lipids (33–35). Since gramicidin channel function is altered by changes in lateral pressure (36), the lack of effects on gramicidin channel function indicates that general anesthetics have minimal, if any, effects on lipid bilayer elastic properties and lateral pressure.

These results do not exclude alterations in bilayer fluidity, i.e., changes in the lateral or rotational diffusion coefficients in the membrane. Such changes would have equal effects on the rate constants for channel formation and dissociation and therefore would not alter the monomer↔dimer equilibrium (nor the fluorescence quench rate) (37, 38). Similarly, changes in fluidity do not provide a causal mechanism for the bilayer regulation of membrane protein function (37, 39). Nor do our results with single-component $DC_{22:1}PC$ LUVs exclude changes in domain organization of multicomponent membranes, which could alter the lateral organization of channels in cell membranes (40, 41). We therefore did additional experiments using LUVs prepared with an equimolar mixture of $DC_{18:1}PC$, cholesterol and brain sphingomyelin, a generic raft-forming mixture (24, 25). In this system, the anesthetics tested were as inert as in the single-component LUVs, if not more so, whereas conventional bilayer-modifying compounds (ethanol, 1-butanol, Triton X-100 and capsaicin) increased the quench rate. Ethanol, 1-butanol, Triton X-100 and capsaicin also were less potent than in $DC_{22:1}PC$ LUVs, which we ascribe to a lesser hydrophobic thickness of the L_d domains in LUVs formed by the multicomponent mixture, which also could account for the lesser effect of isoflurane (4 MAC) and ketamine (500 μM) in the that system. The absence of anesthetic effects suggests that anesthetics do not alter domain organization under our experimental conditions.

Energetic Coupling Between Membrane Proteins and Host Bilayer. Membrane proteins are energetically coupled to their host bilayer through hydrophobic interactions (18, 42, 43). Changes in lipid bilayer properties can alter the equilibrium distribution among membrane protein conformational states and thus protein function (44–47) by altering the lipid bilayer contribution to the free energy difference for membrane protein conformational changes (e.g., between conformation I and II, $\Delta G^{I\rightarrow II}_{bilayer}$ (30)). This energetic coupling forms the conceptual framework for how drug-induced changes in bilayer properties can lead to changes in membrane protein function.

The classic studies of Meyer and Overton (48, 49) on the direct correlation between drug lipophilicity and anesthetic potency in vivo led to early lipid-based hypotheses of mechanisms of anesthesia. A number of compounds, however, do not conform to the predictions of the Meyer-Overton correlation: stereoisomers of anesthetics, for example, can have different anesthetic

potencies (50, 51) despite their identical partition coefficients (e.g., ref. 52). The fluorinated nonanesthetics (flurothyl and F6), despite being very lipid soluble and predicted to be anesthetics, do not produce anesthesia in vivo (16) or altered lipid bilayer properties at their predicted anesthetic concentrations (Fig. 1 *A* and *B*). The chemically similar anesthetic F3 has minimal bilayer effects at anesthetic concentrations, though it is bilayer-active at higher concentrations. These differences in bilayer-modifying potencies could reflect agent-specific distributions of the different anesthetic and nonanesthetic agents within the bilayer. Anesthetic compounds tend to localize within the hydrocarbon core close to the membrane/solution interface based on NMR (53–55) and molecular dynamics (56) studies in contrast to the nonanesthetics F6 and hexafluoroethane, which localize within the midbilayer hydrocarbon core (53, 55, 57).

Such differential bilayer localizations could in principle explain the different effects of F3 and F6 within the framework of the mechanistic hypothesis of anesthetic effects based on changes in membrane intrinsic curvature (44) or lateral pressure profile (7). This mechanism does not invoke direct drug binding to "target" proteins (8). Rather, it is proposed that compounds accumulate at the aqueous-bilayer interface affecting bilayer properties and thereby modulating membrane protein activity. This hypothesis, however, is difficult to reconcile with the lack of changes in gramicidin channel function at clinically relevant concentrations (14), as well as the observation that the partition coefficient of halothane does not vary as a function of aqueous concentration or mole-fraction in the bilayer (58), a result that excludes large changes in lateral pressure within the bilayer.

Membrane Proteins as Anesthetic Targets. The notion of the lipid bilayer as the primary target for general anesthetic action was challenged in the late 1970s (2, 3, 59, 60), which stimulated a search for protein targets involved in producing anesthesia. Inhaled general anesthetics were known to bind to globular proteins (61), and alter the function of cytosolic proteins (1, 62), and many ion channels were also found to be affected by anesthetics (63–65). Subsequent studies have identified potential protein targets that include a range of ligand-gated ion channels such as $GABA_A$-receptors (66) and NMDA-type glutamate receptors (67, 68), two-pore domain K^+ channels (69, 70), and voltage-gated Ca^{2+} (71, 72) and Na^+ (14, 73) channels.

There is wide consensus that proteins are the most likely targets for i.v. anesthetics. The i.v. anesthetics propofol, thiopental and etomidate produce their effects primarily through potentiation of inhibitory $GABA_A$ receptors (74, 75), whereas ketamine inhibits excitatory NMDA-type glutamate receptors and HCN channels (76, 77). Point mutations of $GABA_A$-receptors that result in anesthetic insensitivity markedly reduce propofol and etomidate anesthesia in knock-in mice in vivo (78–80). It remains unclear whether their effects at higher concentrations are due solely to direct effects on non-$GABA_A$ proteins targets, or involve contributions from the lipid bilayer, as has been demonstrated for a number of other drugs (26, 28, 30, 81–83). For volatile anesthetics, however, the evidence is not as clear. Halogenated ether anesthetics, for example, fail to produce anesthetic resistance in knock-in mice with $GABA_A$-receptors engineered for resistance to these compounds – suggesting a role for additional or alternate mechanisms, thus raising the question of lipid bilayer contributions. Indeed the range of plausible membrane protein anesthetic targets suggests that a shared (so-called "unitary") mechanism of action that could include alterated membrane properties might be important for this drug class (84, 85). It is therefore important to know if anesthetics at clinically relevant concentrations alter lipid bilayer properties sufficiently to produce changes in membrane protein function.

Anesthetic Effects on Membrane Protein Function Do Not Correlate with Changes in Bilayer Properties. Membrane protein function can be modulated by changes in lipid bilayer composition (86–90). Similarly, membrane protein function is regulated by small, membrane-active compounds at concentrations that also alter lipid bilayer properties (26, 28, 30, 81–83, 91–98). It is therefore important to examine changes in membrane protein function in relation to bilayer-modifying effects for a wide range of anesthetics, and thus test the generality of the observation that fluorobenzene anesthetics and isoflurane do not affect bulk membrane properties at clinically relevant concentrations (14).

To highlight the relationship between general anesthetic effects on specific membrane proteins and their lipid bilayer-perturbing effects, we compared our lipid bilayer modification results in LUVs prepared using either single component $DC_{22:1}PC$ or a more complex multicomponent (i.e., ternary) mixture, with published concentration-response studies of anesthetic effects on a variety of anesthetic-sensitive ion channels. We then compared these results to those for conventional bilayer-modifying compounds (Fig. 2).

It is evident that conventional amphiphiles alter ion channel function at concentrations where they perturb lipid bilayer properties (right two quadrants in Fig. 2*A*), which suggests that they do so by altering lipid bilayer properties. In contrast, anesthetics do not alter lipid bilayer properties at clinically relevant anesthetic concentrations (left two quadrants in Fig. 2*A*), and there is no clear relation between bilayer-modifying and anesthetic potencies. At supratherapeutic and potentially toxic concentrations, anesthetics can alter lipid bilayer properties, as illustrated by isoflurane effects on $Na_v1.4$, glycine receptors and TRESK (Fig. 2*A*, denoted with asterisk), in which isoflurane is a bilayer modifier at concentrations above 4 MAC (14). These bilayer effects, however, are unlikely to be relevant for desired anesthetic effects.

The membrane mole-fractions of general anesthetics at 1 MAC (Table S1) are similar to the mole-fractions at which many amphiphiles alter lipid bilayer properties (14, 26, 27, 30, 99, 100). In single-component bilayers, a conventional bilayer modifier, Triton X-100 for example, produces a fourfold increase in the fluorescence quench rate at 30 μM, corresponding to a membrane mole-fraction of ~0.1 (15). The modest membrane effects of the inhaled anesthetics at 4 MAC, where (except for F3) mole-fractions in the membrane are 0.1 or above, raises the question of why are clinical anesthetics are so inert as lipid bilayer modifiers? Our results do not provide insight into this question, but we note that anesthetics and their nonanesthetic counterparts are located within the bilayer hydrophobic core, with the anesthetics localizing closer to the bilayer/solution interface than the nonanesthetics (53–57), whereas conventional amphiphiles are anchored to the bilayer/solution interface (101).

Conclusions

We used a well-characterized model system to explore the bilayer-modifying potencies of general anesthetics. Anesthetics are weak bilayer modifiers at clinically relevant anesthetic concentrations, whether tested in bilayers formed by a single phospholipid or by a multicomponent lipid mixture that is known to exhibit domain immiscibility. Clinical anesthesia is therefore unlikely to involve alterations in lipid bilayer properties. Our results support the notion that general anesthesia involves specific anesthetic-membrane protein interactions, which in turn alter central nervous system properties to produce the characteristic features of general anesthesia.

Materials and Methods

Details are in *SI Materials and Methods*. Anesthetic and nonanesthetic solutions were prepared as described (14), and their lipid bilayer perturbing effects were tested using a gramicidin-based fluorescence assay (GBFA).

Single component large unilamellar vesicles (LUVs) composed of 1,2-dierucoyl-*sn*-glycero-3-phosphocholine ($DC_{22:1}PC$) or multicomponent LUVs composed of equimolar amounts of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine ($DC_{18:1}PC$), cholesterol and brain sphingomyelin were filled with the Tl^+ quenchable fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS). Changes in fluorescence decay were measured using a stopped-flow spectrophotometer followed by offline analysis as described (14, 15).

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