

*Annual Review of Biochemistry*  
**Mechanisms of General  
 Anesthesia**

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### Keywords

plasma membrane, ion channel, palmitoylation, cholesterol, signaling lipid, consciousness

### Abstract

Anesthetics are a chemically diverse collection of molecules that dictate neuronal excitability and form the basis of modern medicine. Their molecular mechanism of action is fundamental to understanding nerve excitability, mood, consciousness, and psychiatric disease. Sites of anesthetic action are located within ion channels and the plasma membrane. In the membrane, palmitate, a 16-carbon lipid, covalently links proteins and binds a lipid site to allow anesthetic sensitivity. In ion channels, anesthetics bind within an allosteric conduction pathway or compete for binding of regulatory lipids. Mechanisms of action arising from these binding sites share structural and functional characteristics with the classic anesthetic site in the enzyme luciferase. An update on the Meyer–Overton correlation is reviewed relative to each mechanism and placed in historical context with early theories. The review ends with a discussion of unresolved questions, including questions concerning endogenous anesthetics, anesthetic stereoselectivity, and aspects of a chain-length cutoff.

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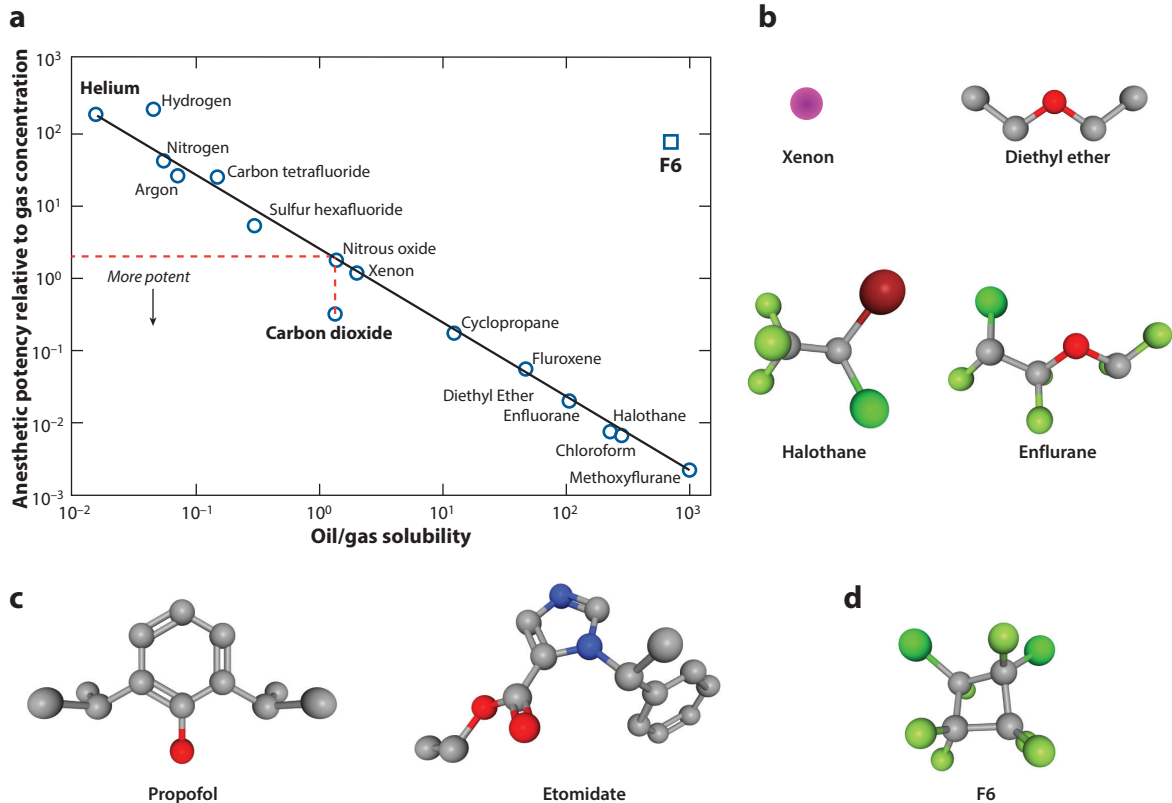
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## 1. INTRODUCTION

General anesthetics are hydrophobic molecules that induce reversible loss of consciousness (1). How anesthetics work has been a topic of intense debate for more than 100 years. Over the last decade, significant progress has been made in understanding the molecular mechanisms of anesthesia and identifying the sites within lipids and proteins where anesthetics act. This review is timely for its integration of membrane-mediated anesthesia, a topic that has been misunderstood for the last two decades.

The effect of anesthetic action was first established 200 years ago by Henry Hill Hickman in England. Hickman (2) found that nitrous oxide and carbon dioxide could reversibly suspend animation in small animals, a discovery he demonstrated to the king of France in 1824. Hickman died the following year. William Morton independently established diethyl ether as a suitable agent for abolishing surgical pain in humans 20 years later, thus ushering in the modern era of medicine founded on anesthesia.

Theories on the mechanism of anesthetic action began to be described well over 150 years ago with Claude Bernard (3). In the late 1800s, Meyer and Overton showed that anesthetic potency increases with lipid solubility, irrespective of the molecule's shape (**Figure 1a,b**). A lipid-solubility



**Figure 1**

The Meyer–Overton correlation. (a) The Meyer–Overton correlation illustrates the relationship between the anesthetic potency of various volatile compounds and their solubility in oil. Compounds that fall below the correlation line are more potent than predicted (e.g., carbon dioxide), while those less potent than expected (e.g., F6) fall above the line. The hypothetical potency is shown for F6 (blue square). (b) 3D structures of selected inhaled anesthetics, illustrating their chemical diversity and size. Xenon is a single atom and a reliable anesthetic. (c) Chemical structures of selected injectable anesthetics. (d) 3D chemical structure of F6, a rare outlier that does not cause anesthesia at the expected concentrations. The 3D structures were rendered in PubChem. Panel a adapted from Reference 157 (CC BY 4.0). Abbreviation: F6, 2-dichlorohexafluorocyclobutane.

hypothesis explained why chemically diverse structures cause anesthesia (4). Mullins (5) noted that adding volume improved the correlation (6), and Johnson & Flagler (7) demonstrated that pressure of approximately 100 bar could reverse anesthesia. These theories, generated in the absence of molecular-level knowledge, led researchers to suspect amorphous lipids as the targets of anesthetics (1).

Molecular theories began to be developed in the 1960s, starting with the discovery of the lipid bilayer (8). The membrane expansion theory grew to include physical expansion of the lipid bilayer and changes in fluidity and thickness of the bulk lipid membrane (9, 10). An anesthetic's hydrophobic nature allows it to accumulate in the membrane, increasing the volume of the bulk membrane. And by increasing the volume, and hence thickness, the anesthetics were speculated to change the conformation of transmembrane-spanning proteins to accommodate the change in thickness. For example, a helix positioned horizontally in the membrane could tilt normal to the membrane to accommodate the change in thickness.

Another theory, the lipid perturbation theory, proposed that anesthetics induce subtle changes in the order and dynamics of the lipid bilayer (11, 12). All these membrane theories relied on perturbations to membrane proteins, particularly ion channels. However, the precise mechanism by which the membrane affected ion channels remained unproven in a biological setting (1). At the same time,  $\gamma$ -aminobutyric acid receptor (GABA<sub>A</sub>R), a protein, emerged as a potential target for anesthetics, eventually leading to a debate regarding the importance of protein versus membrane sites, omitting the possibility that both could be targets.

Leading the efforts to show a protein target, Middleton & Smith (13, 14) in 1976 showed that anesthetics compete with aldehyde in the bacterial enzyme luciferase. This demonstrated anesthetics binding to a protein pocket, which was later shown to be consistent with the Meyer–Overton correlation (15). Structurally, the anesthetics bound nonspecifically and competed with the specific binding of the enzyme’s substrate, luciferin (16). This led to an understanding that anesthetic-binding sites could exist in proteins without specificity for the anesthetic’s structure. Over time this evolved into a proposed mechanism based on multiple binding sites in GABA<sub>A</sub>R, nicotinic acetylcholine receptor (nAChR), and glutamate receptors (GluRs), including *N*-methyl-D-aspartate receptor (NMDAR) (17).

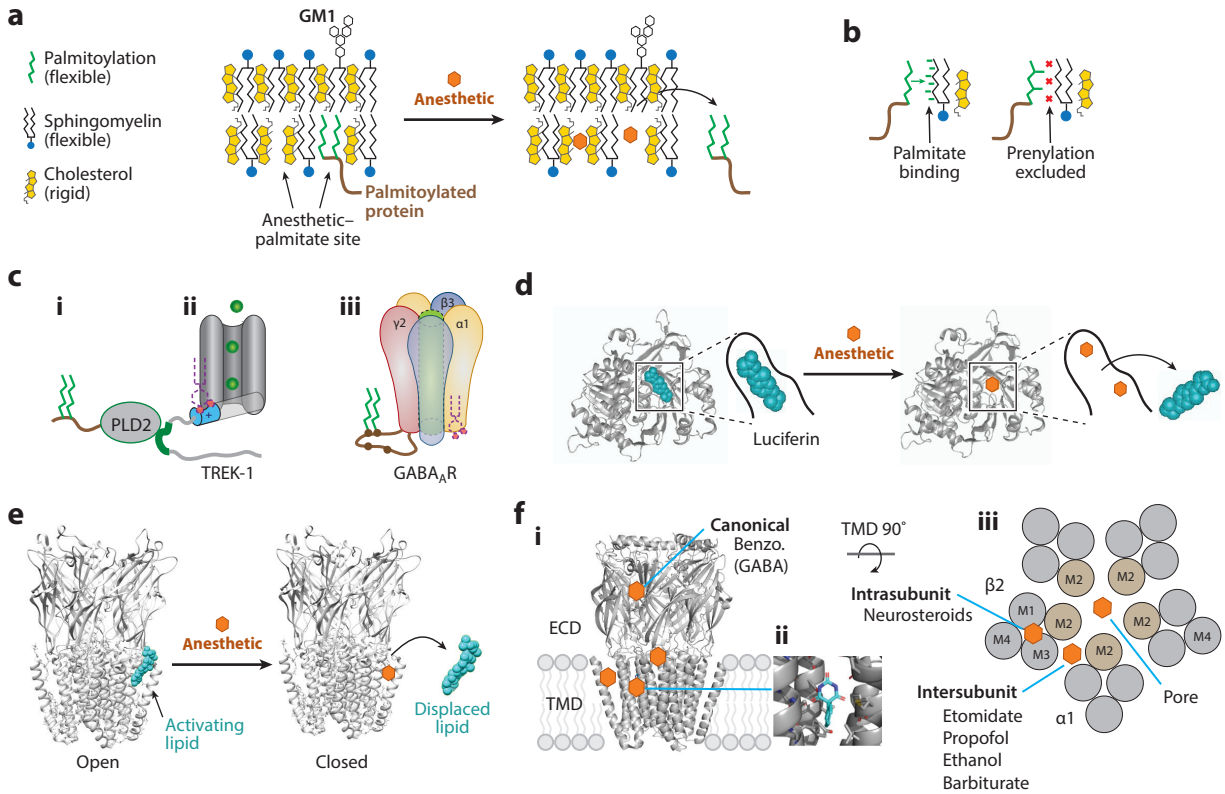
The possibility remained that any of a plurality of anesthetic-binding sites could be membrane mediated, although few have considered this the case over the last two decades. For the lipid sites, Anthony Lee (18) proposed a hypothesis based on the disruption of ordered lipids surrounding an ion channel. This was expanded by Richard Lerner (19) in 1997. The theory postulated that ordered lipids imbue functions to the channel that diminish once the lipids are disrupted.

However, the disruption of ordered lipids in Lee’s theory remained largely untested until 2020, when an anesthetic site emerged within the ordered region of the lipid membrane. Inhaled anesthetics compete for a binding site normally occupied by the lipid palmitate, a 16-carbon saturated lipid covalently attached to proteins (20) [hereinafter referred to as the anesthetic–palmitate (AP) site]. This lipid–lipid interaction depends on structural integrity and specificity, like the interaction between an anesthetic and the luciferase protein, although the ordered site is comprised only of lipids (**Figure 2a**).

This review focuses on inhaled and intravenous general anesthetics with an occasional reference to local anesthetics when some overlap in mechanism may exist. First, I discuss modern evidence for anesthetic-binding sites within lipids and proteins, including luciferase, and the more recently identified sites in ion channels and lipids. I compare the modern lipid and protein sites to the luciferase site and expand the multiple-site theory to include ordered lipids. Lastly, I contextualize the data from experiments designed to test previous membrane-mediated mechanisms by describing our understanding of the AP site and its function in membrane-mediated anesthesia. The review concludes with a discussion of unresolved questions, including those concerning endogenous anesthetics.

## 2. A MEMBRANE-BINDING SITE FOR ANESTHETICS

Identifying binding sites is the first step necessary for understanding the molecular basis of membrane-mediated anesthesia. Most modern lipid theories involve some type of disruption of lipid rafts (21), locating the sites to these lipids. Lipid rafts consist of saturated ceramide-containing lipids (e.g., the ganglioside GM1) and pools of cholesterol on the inner and outer leaflets of the plasma membrane. Cholesterol helps untangle the lipid acyl chains, allowing the lipids to pack tightly in an ordered manner, analogous to an ordered protein domain (22). This lipid order creates a binding site with a hydrophobic surface amenable to palmitate binding (23) on the inner leaflet (**Figure 2a**). The ordered domains span both leaflets of the plasma membrane. On



**Figure 2**

Anesthetic-binding sites. (a) Ceramide-containing saturated lipids form ordered structures in the membrane that selectively bind palmitate with high affinity. Inhaled anesthetics (orange hexagons) compete with palmitates (green lipids) covalently attached to proteins (brown lines) at the AP site. (b) Selected ligands for the AP site are shown. Saturated straight-chain palmitate (left) binds the AP site, while prenylation, a branched unsaturated lipid, does not (right). An arrow shows the displacement of the palmitate by the lipid. (c) Examples of palmitoylation in ion channel regulation: (i) The palmitoylated enzyme PLD2 binds to a disordered loop on (ii) TREK-1, and (iii) a palmitate is shown covalently attached to an intracellular loop of GABA<sub>A</sub>R. (d) Luciferin bound to the substrate-binding pocket of the enzyme luciferase (PDB ID: 5DWV). Anesthetics bind to the surface of the substrate pocket, competing with the substrate. (e) An activating lipid bound to a prokaryotic Cys-loop receptor (PDB ID: 3EAB). An anesthetic (orange hexagons) competes with the activating lipid, inhibiting the channel. (f, i) Anesthetics (orange hexagons) bound to allosteric binding sites in the ECD and TMD of GABA<sub>A</sub>R (PDB ID: 6X3W). (ii) Pentobarbital is shown tightly packed in the intersubunit site. (iii) TMD helices with anesthetic-binding sites indicated by orange hexagons. Structures were rendered in the software package PyMOL (159). Abbreviations: AP, anesthetic-palmitate; Benzo., benzodiazepines; ECD, extracellular domain; GABA<sub>A</sub>R,  $\gamma$ -aminobutyric acid A receptor; PDB ID, Protein Data Bank identifier; PLD2, phospholipase D2; TMD, transmembrane domain; TREK-1, TWIK-related potassium channel subtype 1.

the outer leaflet, glycosylphosphatidylinositol-anchored proteins bind to GM1-containing lipids (24).

Like ordered domains in proteins, the rigidity of the pocket has evolved specificity—unbranched saturated lipids bind tightly, while branched unsaturated (prenyl) lipids are excluded from the site (25) (Figure 2b). The exclusion is based on the ordered structure. Both types of lipids are covalently attached to proteins through processes called palmitoylation and prenylation, respectively. Once attached, the palmitate targets the protein to the ordered lipids within the plasma membrane. Conversely, prenylation inhibits a protein from associating with the ordered lipids (26).

**Table 1** Effects of anesthetics and cholesterol on ion channels

Channel	Anesthetic effect	Cholesterol effect	Palmitoylated subunit	Lipid raft localization	Anionic lipid binding	References
nAChR	–	+	a4, a7, b2	yes	PA	17, 88, 92, 147
5HT <sub>3</sub>	+	+	no	yes	ND	17, 148
NMDAR	–	+	NR2A/B	yes	PIP <sub>2</sub> <sup>a</sup>	17, 92, 149, 150
AMPA	–	+	GluR1–4	yes	PIP <sub>2</sub>	17, 92, 151
Kainate	+ <sup>b</sup>	+	GluR6	ND	ND	17, 92, 152
GABA <sub>A</sub> R	+	+/–	γ2	yes	PIP <sub>2</sub> <sup>c</sup>	17, 92, 147, 96
TREK	+	–	PLD2 <sup>a</sup>	yes	PIP <sub>2</sub> , PA	153, 20, 30, 101
K <sub>v</sub>	+/–, <sup>d</sup> +	+/–	K <sub>v</sub> 1, (Kcnp2–3 <sup>a</sup> )	yes	PA	84, 154–156

<sup>a</sup>Indirect.

<sup>b</sup>Barbiturates inhibit.

<sup>c</sup>Located at the α2 subunit.

<sup>d</sup>Anesthetics potentiate at low depolarizing potentials.

Abbreviations: 5HT<sub>3</sub>, 5-hydroxytryptamine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; GABA<sub>A</sub>R, γ-aminobutyric acid receptor type A; GluR, glutamate receptor; K<sub>v</sub>, voltage-gated potassium channel; nAChR, nicotinic acetylcholine receptor; ND, not determined; NMDA, N-methyl-D-aspartic acid receptor; NR2A/B, NMDAR2A/B subunit; PA, phosphatidic acid; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PLD2, phospholipase D2; TREK, TWIK-related potassium channel.

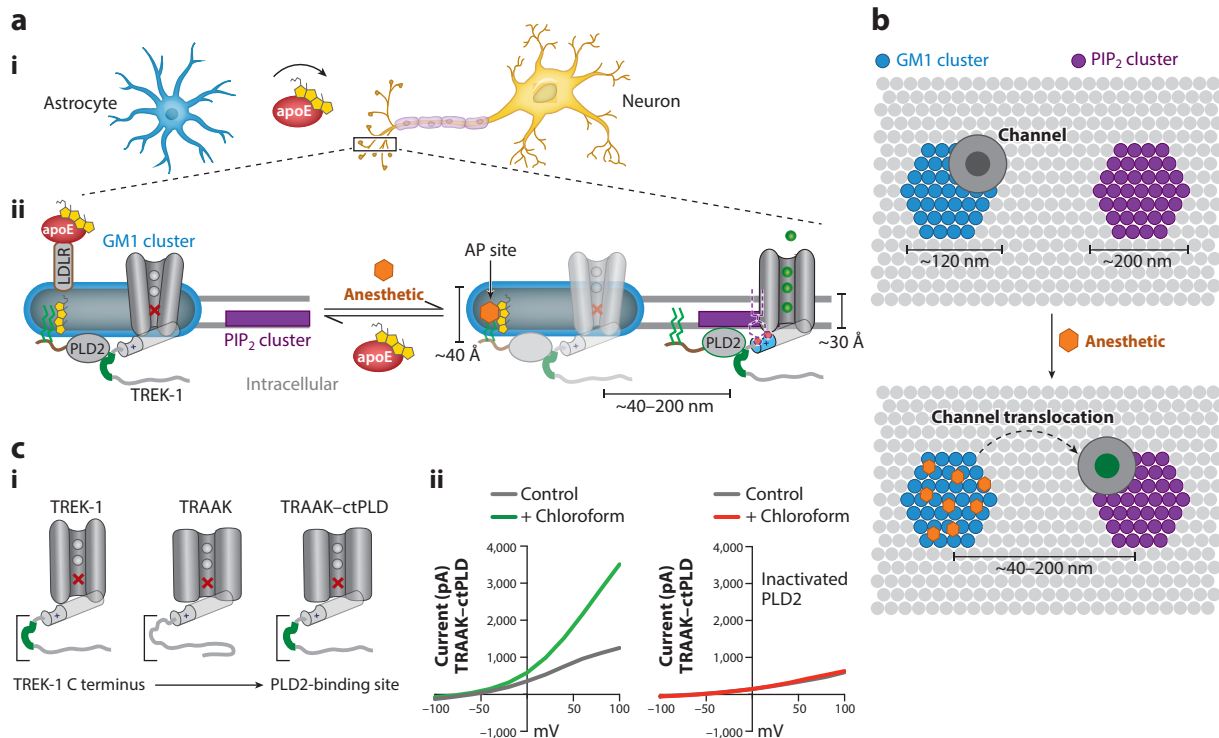
## 2.1. Regulation of the Anesthetic–Palmitate Site by Astrocyte-Derived Cholesterol

For anesthetics to have an effect at a binding site, they must alter the physiology at that site. In the case of ordered lipids, the physiology is regulated by cholesterol. As cholesterol increases in the membrane, palmitoylated proteins have increased affinity for the AP site, driving the protein to associate with ordered lipids (23) (**Figure 2a**). Many channels, including anesthetic-sensitive channels, are palmitoylated and regulated by cholesterol in this manner (see **Table 1**) (27, 28). Recent super-resolution imaging techniques have shown cholesterol's effect in intact cellular membranes, including its location-based effects on anesthetic-sensitive proteins at nanoscopic levels (20, 29). For example, uptake of cholesterol into neuronal and muscle cells drives TWIK-related potassium channel subtype 1 (TREK-1) and GABA<sub>A</sub>R to associate with ordered GM1 lipids as well as the anesthetic-sensitive enzyme phospholipase D2 (PLD2) (20, 30, 31).

The source of the cholesterol, and hence the source of the regulation, is astrocytes. In the brain, cholesterol is produced in astrocytes and shipped to neurons via apolipoprotein E (apoE) (32, 33) (**Figure 3a, subpanel i**). In the prototypic scenario, cholesterol is taken up by the low-density lipoprotein receptor (LDLR) (**Figure 3a, subpanel ii**) (34, 35). It is exported through the very-low-density lipoprotein receptor (VLDLR), a separate receptor that binds selectively to delipidated apoE (36). The balance between the import and export of cholesterol dynamically regulates palmitate binding and the function of the ordered-lipid domains (30, 32, 37, 38).

## 2.2. Displacement of Palmitate by Inhaled Anesthetics

Inhaled anesthetics displace palmitate from the AP site. This discovery is central to the membrane-mediated mechanism (21) (**Figure 2a**). The lipid domains remain intact and even increase in size, but the palmitate cannot bind to the lipid rafts (20, 39). The loss of affinity shifts the palmitoylated proteins away from the AP site, releasing them. This shifting of the protein between lipid domains is referred to as domain partitioning (40), meaning the partitioning of the protein into a different lipid domain. The distance between domains has been measured as 40–200 nm, depending on



**Figure 3**

Mechanisms of membrane-mediated anesthesia. (a) The membrane-mediated activation of TREK-1 perpendicular to the membrane. (i) In the brain, cholesterol is synthesized in astrocytes and transported to neurons via the cholesterol transport protein apoE. (ii) Uptake of cholesterol (yellow) through the LDLR (brown outline) and apoE induces a complex of TREK-1 and PLD2 (gray oval) to associate with ordered ganglioside GM1-containing lipids (lipid rafts; blue outline). The cholesterol-induced sequestration of the TREK-1–PLD2 complex deprives the channel and the enzyme of PIP<sub>2</sub>, thin lipids, and other factors that activate the channel. Competition between anesthetics and palmitate for binding to GM1 lipids displaces the complex from GM1. The complex partitions into PIP<sub>2</sub> clusters, where the channel is exposed to lipid activators and conducts ions (green spheres). (b) A simplified view of panel a orthogonal to the membrane. (c) Transfer of PLD2-induced anesthetic sensitivity from TREK-1 to TRAAK, an anesthetic-insensitive channel. (i) The PLD2-binding sequence in the disordered C terminus is transferred to the C terminus of TRAAK. (ii) Currents from whole-cell patch clamp showing that the TRAAK-ctPLD chimera is robustly anesthetic sensitive (left) but not when tested with catalytically inactive PLD2 (right). Panel c adapted with permission from Pavel et al. (20) (CC BY-NC-ND 4.0). Abbreviations: AP, anesthetic–palmitate; apoE, apolipoprotein E; LDLR, low-density lipoprotein receptor; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PLD2, phospholipase D2; TRAAK, TWIK-related arachidonic acid–stimulated K channel; TRAAK-ctPLD, C terminus of TRAAK; TREK-1, TWIK-related potassium channel subtype 1.

the cell type, the type of domains, and the proteins that are partitioned (31, 37). These distances are diffraction limited, i.e., they are too short to be seen effectively with confocal imaging. They are also difficult to monitor by Förster resonance energy transfer, which is limited to detecting distances <10 nm (41). Their observation in anesthesia was made possible by super-resolution imaging (20, 42).

In cultured N2a neuroblastoma cells and C2C12 muscle cells, all anesthetics displaced the palmitoylated protein PLD2 (20). The displacement activates PLD2 by substrate presentation, an activation mechanism based on spatial biochemistry (20, 31). The half-maximal concentration for PLD2 activation in cultured cells is near clinical concentrations (e.g., 1 mM for chloroform) (20). Fluid shear, present in vivo from blood flow, also disrupts lipid rafts and palmitoylated proteins

binding to the AP site (30, 31, 39). To date, fluid shear has not been tested in combination with anesthetics, which may shift the dose response of anesthetics to lower concentrations. Cellular composition also contributes to the sensitivity of the AP site to anesthetics, as the amount of PLD2 activation differed by cell type for unknown reasons (20).

The cholesterol and saturated fatty acids that comprise the AP site are robustly conserved in all animal cells. For example, anesthetic treatment of lipid rafts was shown to function similarly in flies and cultured mammalian cells (20). Plants, which also exhibit forms of anesthetic sensitivity, have sterols similar to cholesterol (43), but no studies have examined sterol-based localization of proteins with anesthetics, nor with the lipid rafts that are thought to exist in bacteria (44). Presumably, their lipid nanostructures function similarly.

The number of anesthetic-sensitive proteins known to respond through lipid rafts is limited but growing. As mentioned, TREK-1 in complex with PLD2 was the first ion channel shown to work through a membrane-mediated mechanism (20). Other proteins work similarly. For example, angiotensin-converting enzyme is displaced by the local anesthetic hydroxychloroquine (37, 45). Exogenous removal of cholesterol through apoE or methyl- $\beta$ -cyclodextrin (MBCD), a chemical mimetic of apoE, has the same effect on TREK-1, PLD2, and GABA<sub>A</sub>R as an anesthetic (i.e., these proteins leave lipid rafts). Many other channels treated with MBCD show profound changes in their function, but to date, they have not been tested for a change in domain partitioning with anesthetic. The testing of palmitoylated ion channels is particularly important for understanding membrane-mediated anesthesia.

### 3. PROTEIN SITES

Historically, the first molecular anesthetic-binding sites were established in proteins. The most important features of these sites are their consistency with the Meyer–Overton correlation, a remarkable observation that remains foundational to this day. Key anesthetic-binding sites have justifiably continued to be understood relative to the Meyer–Overton correlation.

#### 3.1. Luciferase Site

As mentioned, luciferase was the first protein site to establish anesthetic binding consistent with the Meyer–Overton correlation. The luciferase pocket is constrained and binds its substrate, luciferin, with specificity (16) (**Figure 2d**). The pocket also allows hydrophobic molecules to adsorb to hydrophobic regions; however, they compete mostly nonspecifically with luciferin, itself a hydrophobic molecule. Hence, the competitive displacement of the substrate inhibits the enzyme nonspecifically (16).

The action of anesthetics does not appreciably change the conformation of the enzyme, and thus anesthetic inhibition of luciferase does not establish an allosteric mechanism of action. But the presence of ATP does cause a small expansion of the pocket and a change in anesthetic potency (16). Presumably, the expansion provides better access to the anesthetics. However, the mechanism remains the same: competition between the anesthetic and the enzyme's substrate.

#### 3.2. Competition with Lipid-Binding Sites in Proteins

Ion channels often have specific regions where lipid molecules interact with the channel. These lipids bind within the transmembrane domain (TMD) and between the subunits of ion channels, modulating the channels' function (46, 47). For example, lipids can allosterically gate a channel (47, 48) or alter its rate of endocytosis or desensitization (49).

Due to their lipophilic nature, anesthetics also bind directly to lipid-binding sites within proteins. By occupying these sites, anesthetics and their metabolites compete with the lipid and

block its allosteric regulation (**Figure 2e**). For example, propofol, an injectable general anesthetic (**Figure 1c**), competes for a lipid-binding site on the outer leaflet of the prokaryotic *Gloeobacter* ligand-gated ion channel (GLIC). The acyl chains of the lipid extend into a cavity, and propofol binds competitively in the cavity (50, 51).

Molecular modeling has shown cholesterol binds to the same site as anesthetics in glycine receptors (GlyRs) (52). And a homolog of GLIC from *Erwinia* (ELIC) also has a lipid-binding site in the same region as GLIC that has been shown to be regulatory (53).

The affinity of lipids for their protein targets is specific to their function. Anionic lipids like phosphatidic acid (PA) and phosphatidylglycerol (PG) bind with relatively low affinity (10–100  $\mu\text{M}$ ), in contrast to phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ), which typically binds in the high nanomolar range (41, 54). Based on nonspecific surface binding predicted by luciferase and the Meyer–Overton hypothesis, the lipids with the lowest affinity would be affected by anesthetic competition first. Effective competition with an agonizing lipid inhibits a channel (50, 51), and competition with an antagonizing lipid presumably activates a channel (55).

In addition to regulatory lipids, annular lipids bind to anesthetic sites on ion channels. Recent structures of the  $\text{GABA}_A$ R show annular phosphatidylcholine (PC) lipids located at the subunit interfaces and in direct competition with two general anesthetic sites (56). In theory, competition with annular lipids could affect channel location and how the channel senses hydrophobic thickness and responds to ordered lipids.

### 3.3. Pore Block

Ion channels are composed of pores spanning the bilayer that allow ions to pass through the membrane. The pore of an ion channel often has hydrophobic stretches, some of which form the channel's gate. As expected, many structural studies have found anesthetics lodged in these pores (**Figure 2f**). When the binding site for the anesthetic resides in the conduction pathway, the pore is blocked, and the channel is thus inhibited. Anesthetic pore block has been demonstrated structurally for several channels, including the mammalian potassium channel TREK-1 (57), the chloride channel  $\text{GABA}_A$ R (58), the sodium channel NMDAR (59), and the prokaryotic cation channels ELIC and GLIC (60, 61).

The types of anesthetics involved in pore block are diverse and include propofol, isoflurane, and ketamine. These pore blockers also occupy allosteric sites. For example, the pore block of propofol observed in GLIC is accompanied by its binding to intrasubunit sites in GLIC and intersubunit sites in  $\text{GABA}_A$ R (50, 62).

### 3.4. Allosteric Sites in Proteins

$\text{GABA}_A$ R is among the most prominent proteins with allosteric sites for anesthetic binding. The understanding that anesthetics bind to  $\text{GABA}_A$ R dates to the 1960s. It was initially recognized as an unknown entity that altered nerve impulses (63), later identified as a protein with multiple binding sites (64), and eventually understood as a family of pentameric receptors composed of homologous subunits with known structure (65). Early studies from the Miller lab (66) demonstrated that radiolabeled pentobarbital bound to a saturable site on *Torpedo* acetylcholine receptors, expanding potential sites to the entire superfamily of Cys-loop receptors. Initially, volatile anesthetics were thought to work exclusively through a lipid mechanism, but rapid flux measurements showed that octanol and heptanol competed for a defined protein site in  $\text{GABA}_A$ R and ethanol binds to a protein site in PLD (67).

$\text{GABA}_A$ R was eventually overexpressed sufficiently to allow photolabeling studies to show that general anesthetics could occupy subunit interfaces in  $\alpha 1\beta 3\gamma 2$  receptors (68). Cryo-electron

microscopy (cryo-EM) has further characterized the occupation at these sites (62). Many reviews have detailed the binding sites on ion channels, including GABA<sub>A</sub>R and nAChR (e.g., 51, 68–71).

In general, at least three types of allosteric sites exist in Cys-loop receptors (**Figure 2f**): (a) the canonical site where the agonists bind, (b) intrasubunit sites located within a single subunit in the TMDs, and (c) intersubunit sites typically located between two subunits also in the TMDs.

While not universal for all ion channels, the sites in Cys-loop receptors are among the most important for anesthesia. They have been studied at the atomic level for decades and serve as a template for characterizing putative allosteric sites in ion channels.

The canonical site of the Cys-loop receptor family was first identified in an acetylcholine-binding protein (AChBP) (72, 73) and later confirmed in crystal structures of pentameric ligand-gated ion channels (pLGICs), as well as in native nAChR and GABA<sub>A</sub>R. In mammalian GABA<sub>A</sub>R, benzodiazepines bind in the extracellular domain (ECD), either in the canonical site for GABA or at subunit interfaces homologous with the canonical site (65, 74, 75).

Intrasubunit sites were first identified in GLIC. Anesthetics such as propofol, desflurane, and bromoform all bind within a subunit near the regulatory PC site (50, 51). Most anesthetics in mammalian systems bind to intersubunit sites located in the TMD (62) (**Figure 2f**). The prokaryotic channels are homopentamers, somewhat limiting their ability to recapitulate the complexities of binding sites between differing subunits in heterologous systems (51).

As noted by Goldstein and colleagues (51), 10–15% of proteins are photoaffinity labeled by anesthetics, and many anesthetic–protein interactions may not have relevant functional consequences. The promiscuity of anesthetic binding involves multiple sites within a single protein. For example, GLIC has 30 xenon molecules bound in the closed state and 21 in the open state (76). But it is highly improbable that all these sites are functional anesthetic sites. Larger anesthetics (**Figure 1b**) have fewer binding sites; nonetheless, deciding which of these sites is functional, and thus relevant, is a challenge.

TREK-1 channels are among the many channels functionally reconstituted and shown to be photoaffinity labeled by anesthetics (77). When tested directly for anesthetic activation in purified liposomes, there was, surprisingly, no response (20). In theory, all purified channels that bind directly to anesthetics should respond in a purified system, especially a protein like TREK-1 that has been functionally reconstituted by many labs (20, 78–80). Other channels, like GABA<sub>A</sub>R, GlyR, and nAChR, are not reliably reconstituted with native-like function in liposomes, so their direct binding cannot be reliably tested. Functional studies performed in whole cells are often used to compensate for a lack of a direct observation; however, cellular studies cannot demonstrate direct binding due to the complexity of the cell. For example, in TREK-1 channels, mutations that inhibit the binding of PLD2 could be mistaken for anesthetic-binding sites. Mutations that inhibit the binding of PA or PIP<sub>2</sub> to TREK-1 could also block anesthetic-induced activation of TREK-1 channels by PLD2. All channels that bind regulatory molecules have similar complexity.

#### 4. MECHANISMS OF ANESTHETIC ACTION

Ultimately, the identification of functional binding sites leads to a molecular understanding of general anesthesia. The key effects of general anesthesia are immobility and amnesia. Inhaled anesthetics depress excitatory neurotransmission and enhance inhibitory neurotransmission (17, 74). Currently, leaders in the field believe these effects are mediated through one or a few ion channels, particularly GABA<sub>A</sub>R, and through a plurality of binding sites (81). Plausible explanations for anesthetic action have continued to expand over recent decades. Two recent reviews on binding sites include thoughts on mechanisms of action (51, 69).

## 4.1. Membrane-Mediated Mechanism

Among the plurality of binding sites, the membrane-binding site within ordered lipids is the most recently identified; it is also the most unconventional. Understanding this mechanism of action is important and requires an understanding of the organization and signaling of lipids in the plasma membrane. The plasma membrane is organized into compartments defined by their chemical makeup, including the signaling lipids that reside in them and impart functional properties (41). As mentioned, the most prominent compartment is ordered and contains saturated GM1 lipids and cholesterol—this compartment (sometimes referred to as lipid rafts) contains the AP site (20, 24). GM1 clusters are separate from clusters of the signaling lipid PIP<sub>2</sub> (**Figure 3a**). PIP<sub>2</sub> typically forms its own clusters and resides within a domain of disordered lipids (31, 82, 83).

Many anesthetic-sensitive ion channels partition into domains with distinct lipid compositions, which dictates their exposure to regulatory lipids (41, 84). For many channels, a covalently attached palmitate lipid drives the sorting of the protein (23). For some proteins, the ion channel simply binds to a protein that is palmitoylated (20, 85). Palmitoylation controls lateral diffusion and exposes the channel to different lipid environments, such as high cholesterol. For some channels (e.g., excitatory Na<sup>+</sup> and Ca<sup>2+</sup> channels), the association with cholesterol and ordered lipids activates the channel, while for others (e.g., leak K<sup>+</sup> channels), it inhibits them (28, 30, 86–89).

As a specific example, the ion channel TREK-1 binds to PLD2, which is palmitoylated (31, 85) (**Figure 1c, subpanel i**). Localization to the AP site deprives TREK-1 of its agonizing lipids PA and PIP<sub>2</sub> (**Figure 3a,b**). Uptake of astrocytic cholesterol into HEK293T cells (**Figure 3a, subpanel i**) inhibits TREK-1, while depletion of cholesterol has the opposite effect (30). In addition to the change in signaling lipid concentration, the hydrophobic thickness near PIP<sub>2</sub>-containing lipids is thinner, and TREK-1 is optimally active in thinner lipids. Hence, the movement to thicker lipids may contribute to the inhibition of the channel.

Like TREK-1, the excitatory channel Piezo2 is downstream of PLD2 (55). Interestingly, PLD2 inhibits Piezo2 opposite its action on TREK-1. Hence PLD2, i.e., PA, decreases cellular excitability by inhibiting an excitatory channel and activating an inhibitory channel. This feature was predicted and speculated to contribute to the steep Hill slopes observed with anesthetics (39).

In a definitive experiment showing membrane-mediated anesthetic action, the ion channel TWIK-related arachidonic acid–stimulated potassium channel (TRAAK), which is not normally anesthetic sensitive, was rendered sensitive by adding the PLD2-binding domain from TREK-1 to its C terminus (**Figure 3c**) (20). TRAAK, like TREK-1, is activated by local production of PA (85). Previous theories regarding TREK-1 suggested that the TMD was responsible for activation of TREK-1 (77, 90). However, since the TMD remained the same, this definitively showed that the anesthetic-binding site is not in the TMD for TRAAK; rather, the enzyme PLD2 is the anesthetic-sensitive protein activating TRAAK. Early studies showed this same region in the C terminus of TREK-1 to be necessary for anesthetic activation (91), consistent with PLD2 being the anesthetic-sensitive entity in the TREK-1 channel as well.

Unlike TREK-1, most anesthetic channels are directly palmitoylated (92). GABA<sub>A</sub>R is palmitoylated at its  $\gamma$  subunit and binds PIP<sub>2</sub> (93–95). In the case of GABA<sub>A</sub>R, PIP<sub>2</sub> does not directly gate the channel (96); rather, it appears to provide a location for GABA<sub>A</sub>R to reside once displaced from GM1 lipids (97). Nonetheless, the channel's primary amino acid sequence contains components that permit regulation by the membrane AP site. Furthermore, the agonist GABA causes the channel to move from lipid rafts to PIP<sub>2</sub> domains (97).

**Table 1** shows the effect of cholesterol on select anesthetic channels. For the most part, cholesterol activates anesthetic-sensitive ion channels, increasing the total amount of ion permeability

(88, 98). At the same time, cholesterol inhibits select potassium channels, including TREK-1 and nonanesthetic-sensitive Kir channels (30, 89). In general, cholesterol drives a stimulated response to a stressed state (34), whereas anesthetics block the effect of cholesterol and drive a depressed state.

By opposing cholesterol localization, anesthetics have two major effects. First, opposing cholesterol reduces its overall ability to produce excitability (**Table 1**). Second, anesthetics selectively hyperpolarize the cell by inhibiting Na<sup>+</sup> and Ca<sup>2+</sup> channels and activating select K<sup>+</sup> and Cl<sup>-</sup> channels (39) (see **Table 1**). Importantly, the effects of diverse anesthetics on an individual channel are consistent across structurally diverse anesthetics. If one anesthetic activates a channel, almost all anesthetics, regardless of shape, activate the channel (17).

The membrane also contributes to an overall decrease in ion channel conductance through endocytosis (49). Receptor-mediated endocytosis initiates at GM1-containing lipids (99). When cholesterol drives too much signaling through lipid rafts, endocytosis pulls the channels off the membrane. Anesthetics have a functional effect similar to that of endocytosis, in that they remove ion channels from cholesterol domains; however, anesthetics leave the receptors on the surface rather than recycling them.

## 4.2. Protein-Mediated Mechanisms

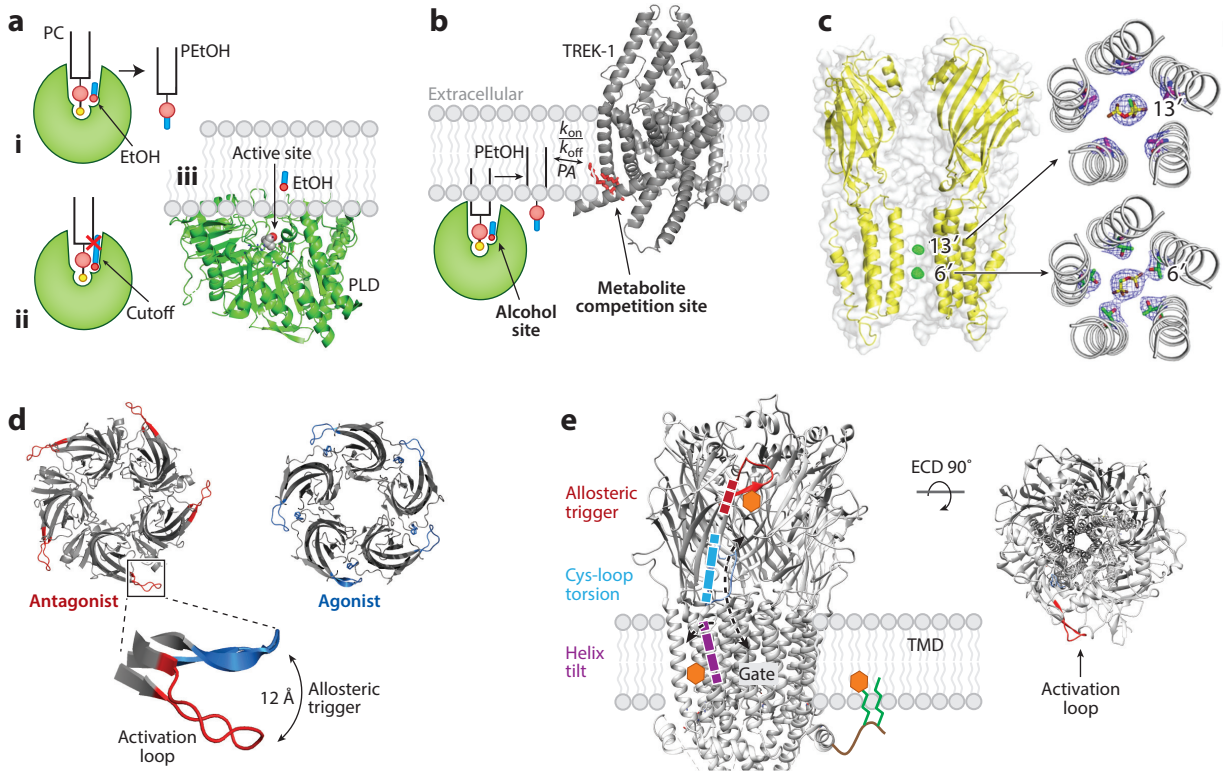
Most experts in the field, including myself, recognize the binding of anesthetics to sites on ion channels as a factor contributing to their effects in animals. At least three mechanisms have been described: (a) binding of anesthetics to allosteric regulatory sites (by far the most studied and accepted), (b) competition of anesthetics, and their metabolites, with regulatory lipids, and (c) pore block.

**4.2.1. Competition between anesthetics and regulatory lipids.** I first discuss the role of regulatory lipids. Many regulatory lipids have charged head groups that bind to channels and alter the conformation of the channel or flip a charged residue out of the conduction pathway (47, 48). Once displaced, the lipid no longer exerts its function on the channel. Anesthetic displacement of lipids from protein sites and their functional effect is largely inferred from structural studies showing anesthetics binding in the same location as the lipids. The specificity is contained within the endogenous lipid ligand, while the anesthetic simply blocks the function by preventing the binding of the lipid ligand (**Figure 2e**).

For example, in GLICs, a regulatory phosphatidylcholine lipid on the outer leaflet between transmembrane (TM) helices 1 and 4 (TM1 and TM4, respectively) is required to open the channel. In crystal structures, the binding site of propofol overlaps with the regulatory lipid site, meaning that propofol displaces the lipid and inhibits the channel (100). As expected, in cellular membranes, the anesthetic inhibits the channel (50, 51).

The putative displacement of a lipid depends on the relative affinities of the lipid and anesthetics for the channel. Few lipid-regulatory sites have been directly investigated for their interactions with anesthetics, largely due to the difficulty of studying the interaction between a hydrophobic molecule and a hydrophobic lipid competing for a hydrophobic binding site in a protein. Recently, sophisticated techniques using mass spectrometry and detergent-soluble channels have been developed to specifically evaluate the affinities of the lipids for the protein (41). PIP<sub>2</sub> species bind detergent-soluble potassium channels with affinities in the 0.1–1 μM range, and PA and PG bind in the 10–100 μM range (54, 101). Whether anesthetics compete for the lipid sites in these channels has not been tested with this method.

**4.2.2. Competition between ethanol metabolites and regulatory lipids.** Metabolites of anesthetics can also compete with regulatory lipids. This is exemplified by ethanol. Ethanol is a



**Figure 4**

Mechanisms of protein-mediated anesthesia. (a) Location of a binding site within the enzyme PLD2 that accounts for the chain-length cutoff effect. Alcohols are covalently attached to the headgroup of a glycerophospholipid. (i) The volume of the lipid-binding site accommodates ethanol (ii) but not a long-chain alcohol. (iii) A crystal structure of PLD2 (PDB ID: 6U8Z) shows the location of the hydroxylic oxygen in the active site (141), which indicates the location of the alcohol headgroup deep within a pocket of constrained dimensions. (b) A cryo-electron microscopy structure of TREK-1 (PDB ID: 8DE8) bound to its agonist PA is shown (158). PEtOH is shown poised to compete with PA, thus inhibiting the channel. (c) Pore block shown in a Cys-loop receptor. The amino acid numbers along the pore helix are labeled, and the location of the anesthetic is highlighted in green. (d) Allosteric trigger of a Cys-loop receptor, as shown by AChBP, a soluble homopentameric homolog of the nAChR. An activation loop caps the agonist pocket (*inset*). When an antagonist is present (PDB ID: 2BYP), the loop is in an extended conformation (*red*), and when an agonist is present, the loop adopts a compact conformation (*blue*; PDB ID: 2BYQ). (e) Description of the allosteric conduction pathway in the heteromeric nAChR (PDB ID: 6UWZ). Steps along the allosteric activation pathway are shown as colored dashed lines, beginning with the allosteric trigger in the extracellular domain (*red*), followed by torsion of the Cys loops (*blue*) and then tilting of the transmembrane helices (*purple*). Gating in Cys-loop receptors is initiated in the agonist-binding pocket when the activation loop (loop C; *red ribbon*) closes on an agonist. A rigid body movement impinges on the Cys loop. The pathway through the Cys loop connects to the transmembrane domain near TM3 (*purple dashed line*), causing tilting of the membrane-spanning helices. The tilting of the helices relative to the membrane is believed to gate the channel near the intracellular side of the membrane. The allosteric coupling pathway is shown as a black double-headed arrow. Anesthetics (*orange hexagons*) impinge on the allosteric coupling. Abbreviations: AChBP, acetylcholine-binding protein; Cys, cysteine; ECD, extracellular domain; EtOH, ethanol;  $k_{on}$ , association rate;  $k_{off}$ , dissociation rate; nAChR, nicotinic acetylcholine receptor; PA, phosphatidic acid; PC, phosphatidylcholine; PDB ID, Protein Data Bank identifier; PEtOH, phosphatidylethanol; PLD, phospholipase D; TMD, transmembrane domain; TREK-1, TWIK-related potassium channel subtype 1. Panels *a* and *b* adapted from Reference 67 (CC BY-NC-ND 4.0). Panel *c* adapted from Reference 61 (CC BY 4.0).

common but weak anesthetic. When taken up into a cellular membrane, PLD metabolizes ethanol into phosphatidylethanol, replacing the choline headgroup of phosphatidylcholine with ethanol (102) (**Figure 4a**). A similar reaction occurs for all primary *n*-alcohols with a chain length below 11 carbons (67).

Phosphatidylethanol then competes directly with the regulatory lipids PIP<sub>2</sub> and PA in potassium channels, including TREK-1 (**Figure 4b**). Once the regulatory lipid is no longer bound, its regulatory function on the channel is inhibited (67). This is particularly important for TREK-1 since the metabolic enzyme of ethanol forms a complex with TREK-1. Thus, the anesthetic-binding site for ethanol is within the metabolic enzyme associated with the channel, not the ion channel itself (**Figure 3a**). Nonetheless, the mechanism is the same as that of anesthetics competing directly for lipid regulatory sites, except that the metabolite is generated first.

Phosphatidylethanol also binds to nonanesthetic-sensitive TRAAK and inward rectifier potassium channels 2.1 (Kir2.1) (67). Presumably, localized production and/or high affinity for the lipid would dictate the channels' responses *in vivo*. More studies are needed to determine whether these additional channels contribute to some of the differences between ethanol anesthesia and inhaled anesthetics.

In addition to an allosteric change in the protein, competition of an anesthetic with regulatory lipids could alter the location of an ion channel. For example, PC localizes near PIP<sub>2</sub> in the disordered region. A channel that binds a lipid in the disordered region would be sequestered away from cholesterol and saturated fats. And in theory, competition of an anesthetic or anesthetic metabolite with a regulatory lipid could shift the channel into lipid rafts by outcompeting the regulatory lipid (**Figure 2e**).

**4.2.3. Pore block.** The last protein-binding site sits within the conduction pathway and blocks conduction. For a general anesthetic, the mechanism of action is twofold: Blocking conduction can decrease the permeability of the membrane, and it can decrease the amount of depolarization. For Na<sup>+</sup> and Ca<sup>2+</sup> ions, which depolarize the cell, inhibiting their conduction blocks depolarization. This model was first supported by structural and computational studies that found anesthetics lodging in the conduction pathway of prokaryotic cation channels (**Figure 4c**) (60, 61).

Ketamine is an NMDAR channel blocker used for anesthesia and, more recently, to treat major depressive disorder (103). S-ketamine binds to human GluN1–GluN2A and GluN1–GluN2B NMDARs in the conduction pathway at a constriction point near the extracellular leaflet (59). The atomic structure is consistent with pharmacology experiments and the proposed mechanism. Unlike allostery, after ligand binding, no change in ion channel conformation is necessary to establish the mechanism.

Pore block by anesthetics also occurs in potassium channels. Tetracaine and lidocaine inhibit TREK-1 channels through pore block (57). The mechanism is bimodal, with the anesthetics also inhibiting PLD2 directly. A bimodal mechanism also exists for GLIC: A binding site for propofol in the pore conduction pathway inhibits the channel. However, in contrast to pore block, binding to intrasubunit and intersubunit sites of GLIC is thought to activate the channel.

Some amount of pore block could also occur in the anesthetic-sensitive chloride channels like GABA<sub>A</sub>R and GlyR. However, at clinical concentrations, anesthetics activate GABA<sub>A</sub>R, so pore block is unlikely to be significant in this channel's mechanism of action. The reason for the selectivity is unclear. GABA<sub>A</sub>R and GlyR are homologs of cationic pLGIC.

**4.2.4. Allosteric regulation.** All modern theories of general anesthesia incorporate the concept of the allosteric action of anesthetics on ion channels. Allosteric regulation of ion channels by anesthetics is based on the ideas that proteins have multiple conformations and that the affinity for the ligand is higher for one conformation than another. This affinity is the sum of attractive and repulsive forces. The attractive forces at short distances require proximity to neighboring atoms, so the molecule must fit snugly, preferably within a pocket that maximizes interactions with multiple atoms (104). However, the pocket must also be larger than the anesthetic to avoid the strong repulsive forces of overlapping atoms. For these reasons, allosteric pockets typically

complement the shape of the allosteric ligand, which is borne out in sites identified by structural studies. These and additional insight into allostery and anesthetic action are discussed in Reference 104.

Allosteric activation of Cys-loop receptors, including GABA<sub>A</sub>R, GlyR, and nAChR, is coupled to the TMD through an agonist site in the ECD. Anesthetics presumably work along the same pathway. Cys-loop receptors serve as a good example for understanding allosteric activation. Agonist binding causes formation of a loop that caps the agonist site to close over it. This was first shown for a soluble homolog of the Cys-loop receptor ECD (73) (**Figure 4d**). Antagonists prevent the closure of the C loop covering the agonist site, and this is coupled to a twist in the ECD relative to the TMD. Similar changes in both the capping of the agonist site and the rotation of the ECD were shown in structures of full-length receptors (105, 106). The ECD movement couples to a tilting of the TMD helices that gates the channel.

The allosteric nature of Cys-loop receptor activation affords two mechanisms for allosteric regulation by anesthetics: (a) direct binding to the agonist site and (b) binding to a distinct site along the allosteric coupling pathway. By binding with high affinity to the open (for GABA<sub>A</sub>R and GlyR) or the closed and desensitized conformation (nAChR), anesthetic binding is thought to drive the receptors into their respective states.

Numerous ligands have been found bound to GABA<sub>A</sub>R, GlyR, and nAChR at sites within the coupling pathway and in the agonist-binding pocket (62, 69, 106–109). In several instances, compounds bind to multiple types of sites. For example, diazepam, a sedative, binds in the agonist pocket of GABA<sub>A</sub>R and in the TMD (62).

## 5. COMPARISON OF ANESTHETIC SITES

### 5.1. Anesthetic–Palmitate Site Versus Luciferin

Binding of anesthetics to the lipid ordered site is mechanistically similar to their binding to the luciferin site in luciferase. Both sites consist of a relatively large hydrophobic pocket that selectively binds a substrate. The AP site typically accommodates two palmitates (28 carbon atoms) or one palmitate and one myristate (26 carbon atoms). Luciferin consists of 18 carbon atoms, which is likely smaller but still sufficiently large to accommodate multiple anesthetics binding to the hydrophobic surface (16).

The most notable difference is that the ordered AP site is composed of lipids, while the luciferin site is composed of amino acids. Conventionally, only proteins are thought of as being structured. However, like nucleotides that were once thought to have no ordered function, lipids have ordered domains, and as mentioned, they are selective and have function (26). Hence, as when binding to luciferase, anesthetics bind to the surface of the ordered lipids nonselectively and compete with the endogenous substrate (palmitate). And like with luciferase, it is the competition with a selective molecule that provides the function, not the anesthetic itself. The ability of an anesthetic to compete depends on the relative affinities of the anesthetics for their ordered binding sites, whether lipid or protein. However, atomic structures of anesthetics bound to the palmitate-binding site within ordered lipids (110–112) await determination and may reveal additional similarities and differences to binding at the luciferase site.

### 5.2. Protein–Lipid Regulatory Sites Versus Luciferin

The binding of anesthetics to lipid regulatory sites within proteins also shares considerable similarities with their binding to the luciferin site in luciferase. Like palmitoylation, the regulatory lipids have two acyl chains. Hence, the volume and hydrophobicity of their binding sites in proteins are expected to be similar to the AP site. For example, the regulatory lipid overlapping with

anesthetics in the GLIC contains multiple carbon atoms, providing a comparable binding pocket to luciferin (50).

A competition mechanism with the lipid allows for a nonspecific interaction of an anesthetic with the channel to exert a specific effect through the lipid-binding site. Logically, if the lipid is regulatory and prevented from binding, its regulatory function is reversed. While speculative, a lipid competition model could help explain why some classes of channels are inhibited by anesthetics and other classes are activated. If the displaced lipids have an opposite effect on the channel, the displacement by anesthetics also has an opposite effect.

### 5.3. Allosteric Sites Versus Luciferase

The allosteric sites between ion channel subunits have the least similarity to luciferase. The large surface area found in the luciferin-binding pocket is not well represented in the allosteric sites of ion channels (62). As mentioned, allosteric sites often benefit from a tight fit that maximizes the interactions at short distances and results in specific chemical interactions, which is a mechanism distinct from that of luciferase and inconsistent with the Meyer–Overton hypothesis. **Figure 1f, subpanel ii** shows an example of a specific ligand–channel interaction exhibiting a snug fit. For the homolog of the nAChR, small species-specific differences in the agonist pocket, the best studied allosteric site, lead to large ligand-specific changes in affinity, up to four orders of magnitude (113).

A plurality of binding sites on channels can explain some of the chemical diversity of anesthetics, but evidence as to why potency correlates with hydrophobicity (**Figure 1a**) rather than shape is still lacking. And how an inhaled anesthetic like xenon (a single atom) (**Figure 1b**) could agonize GABA<sub>A</sub>R across many species and subtypes and then antagonize the homologous nAChR is also difficult to understand from allostery alone, absent the existence of endogenous anesthetics to select for that function.

## 6. FLUIDIZATION MODEL OF GENERAL ANESTHESIA

### 6.1. Historical Context

Aspects of membrane fluidity have contributed to theories of general anesthesia since the late 1800s. Here, I briefly revisit fluidization theories with an understanding of ordered lipids and the AP site. However, the ordered lipid as it relates to anesthetics (the AP site) is still relatively new, and the exact underlying biophysical properties are yet to be fully established. Hence, exact interpretations are not always possible. Nonetheless, a brief look back is important for recognizing the foundational work that is pertinent to our current understanding.

The original fluidization theory posited that the partitioning of anesthetics increases the fluidity of the lipid membrane, which in turn affects the membrane's function. How this fluidity could affect nerve function was not clear. Nonetheless, by 1977, it was known that membranes have regions of ordered and disordered lipids. Justin Trudell (12) offered a unitary theory of anesthesia based on lateral phase separations in nerve membranes and changes in fluidity and volume near ion channels residing within disordered lipids. Trudell's theory shared many features of the current model but relied on anesthetic perturbation to the disordered region of the membrane, not the ordered region.

The membrane theory was further refined to include changes in lateral pressure profiles, which are the differences in pressure a protein experiences based on its depth in the membrane (11). Changes in pressure profiles can cause changes in protein conformational states. However, when tested using chemically diverse anesthetics, this produced no meaningful changes to disordered lipids that would affect an ion channel (114). The lack of evidence does not prove lateral pressure profiles in disordered lipids play no part. Nonetheless over many years, the lack of a known

mechanism by which changes in a typical disordered membrane could affect an ion channel led many to believe there was no membrane target.

## 6.2. Lipid Raft Disruption Model

As predicted by Lerner, Lee, and colleagues (18–20), disruption of ordered lipids activates a protein. However, disruption does not require fluidization of the ordered lipids. As little as a 5% decrease in membrane cholesterol disrupts the function of lipid rafts, while their clustering remains largely intact (within 10% of the original apparent size) (20). Consistent with a functional disruption mechanism, the removal of 5% membrane cholesterol with MBCD mimics the effects of anesthetics, displacing PLD2 from lipid rafts and activating the TREK-1 channel (20). Similarly, fluid shear (3 dynes/cm<sup>2</sup>) or application of delipidated apoE, an endogenous cholesterol transport protein, removes cholesterol and disrupts lipid raft function (30, 39).

Once cholesterol is decreased, palmitoylated proteins dissociate from lipid rafts, which is identical to the effect of anesthetics. For mechanical force, this appears to include a chaotropic effect (30). For proteins inhibited by lipid rafts, such as PLD2 and TREK-1, disruption activates them (30, 31). For proteins activated by lipid rafts, such as  $\gamma$ -secretase and cytokine hydrolases, disruption inhibits them (32, 115).

Local anesthetics, as suggested by Lee (18), induce raft disruption. For example, in purified membranes, tetracaine and lidocaine disrupt lipid rafts (116). And in cellular membranes, as shown with direct stochastic optical reconstruction microscopy, they release PLD2 from GM1 clusters, similar to MBCD and delipidated apoE treatments (57). The intravenous anesthetic hydroxychloroquine also releases palmitoylated proteins from GM1 lipids, suggesting it utilizes a disruption mechanism (37).

The ability of local anesthetics to disrupt ordered lipids has been overshadowed by pore block (117). Consistent with direct binding and pore block, local anesthetics inhibit TREK-1 channels, including when purified in liposomes (57). As mentioned, this is the opposite of the effect of general anesthetics, which activate TREK-1 in cellular membranes and have no effect in purified liposomes (20). Nonetheless, many amphiphilic drugs targeting ion channels (~50% of those tested in one study) (118) also disrupt the membrane. The contributions of membrane disruption should be considered even when pore block is known to occur. The development of better drugs almost certainly requires an understanding of ion channel regulation by both the membrane and direct binding.

## 6.3. Anesthetic Effect on Phase Transitions

More than 30 years ago, studies on fluid changes within artificial membranes showed that lipids undergo phase transitions, which give rise to phase separation (119, 120). Studies on giant plasma membrane vesicles, blebs of cellular membrane lipids, showed that these phase separations exist in mammalian cellular lipids and that primary alcohols can lower the melting temperature of the phase (121–123). For lipid rafts, the phase transition occurs over more than 3°C (123), compared to disordered lipids, for which they occur over less than 1°C. Likewise, inhaled anesthetics applied to purified ternary lipids showed mixing and a decrease in ordered lipids compared to disordered lipids (124).

However, in both mammalian cells and whole insect brains, treatment with very high concentrations of chloroform (1 mM and saturating, respectively) does not lead to a decrease in phase separation, as would be expected from lipids undergoing a phase transition. Rather the lipid rafts increased in number and size (20). The anesthetics fail to drive membrane lipids to a miscible fluidity, although they could be close. Why the amount of lipid raft area increases in some instances

is still not known. Nonetheless, in contrast to the lipid rafts, the palmitoylated proteins become fluid.

The release of palmitoylated proteins suggests the palmitate may go through a phase transition, not the lipid raft (i.e., GM1 lipids). The melting temperature of the palmitate–sphingolipid interaction could be separate from the melting temperature of the ordered sphingolipids within the domain. Palmitoylation has two fewer carbons than typical sphingomyelin (16–18), which is common to lipid rafts. The shorter chain length may allow palmitoylated protein to reach a phase transition prior to the ordered domain, fluidizing the protein before the lipid raft structure becomes miscible.

In contrast to other anesthetics, ethanol in the membrane has little effect on the size or number of lipid rafts, nor does it release or activate PLD2. As mentioned, ethanol is a natural product that is metabolized, and organisms may have evolved to deplete it before it can appreciably lower the transition temperature of lipid rafts or palmitoylated proteins.

## 7. UNRESOLVED QUESTIONS

### 7.1. The Relative Contribution of the Membrane and Protein Sites

For decades, researchers have debated whether general anesthesia is mediated by proteins or membranes. Most debates have centered on the premise that only one mechanism is correct. However, the existence of a membrane site does not exclude a significant contribution from protein sites, nor vice versa. Research supporting a plurality of sites logically extends to include membrane sites, such as those in ordered lipids. A substantial number of anesthetics bind to multiple sites, and hydrophobic ligands generally perturb both ion channels and the membrane. Understanding anesthetic action likely requires accepting both types of sites and studying their combined effects.

The luciferase model is attractive because it accounts for the lack of chemical specificity combined with conserved function. Both use of the AP site in ordered lipids and the competition between anesthetics and regulatory lipids are modes of anesthetic action compatible with the luciferase studies. The competition of anesthetics with a regulatory lipid, suggested by PLD2 and GABA<sub>A</sub>R release from lipid rafts, provides a rationale both for the nonspecific potency observed in the Meyer–Overton correlation and for the selective regulation of inhibitory and excitatory ion channels. Individually, the data appear consistent for a membrane-mediated mechanism. However, the mechanisms by which allosteric sites are selective for ion channel types (e.g., inhibit nAChR and activate GABA<sub>A</sub>R) remain unclear.

Pore block is also an attractive model since ion channel pores contain regions of hydrophobicity that could bind anesthetics nonspecifically, as suggested by the Meyer–Overton correlation. However, pore block alone cannot explain the activation of GABA<sub>A</sub>R, which is an important component of *in vivo* anesthesia. Thus, any contribution from pore block is likely an overall decrease in permeability.

To date, few if any anesthetic channels have been reliably reconstituted into artificial lipids and shown to respond directly to anesthetics. TREK channels are an example of a channel that is functionally reconstituted into purified lipids and responds to its endogenous and exogenous agonist and antagonists, yet when tested with anesthetics, there is no response. GluRs are also functionally reconstituted into liposomes and can be activated by mechanical force (125–127), yet evidence for anesthetics inhibiting the channel in purified lipids is lacking. The lack of an anesthetic effect on ion channel function in purified lipids suggests the structures determined in these lipids may represent a nonfunctional or nonphysiological state, and some caution is warranted in an interpretation of their structures. Mutations in residues near a putative anesthetic-binding site are often tested in whole cells to claim a particular binding site is functional. But in whole

cells, the mutagenesis does not favor an allosteric mechanism over competition with a regulatory molecule.

## 7.2. Endogenous Anesthetics

As stated by Lerner (19, p. 13375), “when an exogenous natural product is found to have biological activity in that much activity is devoted to a search for the endogenous counterpart,” i.e., an endogenous anesthetic (128, 129). Recent efforts have identified ammonia (130) and ketones (131) as endogenous anesthetics. However, perhaps the most obvious and simple candidate is carbon dioxide (CO<sub>2</sub>).

In the 1820s, well before Morton’s public demonstration in the Ether Dome, Henry Hill Hickman demonstrated CO<sub>2</sub> and nitrous oxide anesthesia in small animals, a procedure he and presumably others at the time called suspension of animation (2, 128, 129). The mechanism was unknown, but the speculation of asphyxiation led to ridicule in the *Lancet* in 1824 (132). The asphyxiation model seemed to be supported by observation of the Bohr effect in the early 1900s; i.e., increased CO<sub>2</sub> decreases the affinity of O<sub>2</sub> for hemoglobin, allowing increased release of O<sub>2</sub> in metabolically active tissues. In theory, high CO<sub>2</sub> in the lungs could prohibit O<sub>2</sub> binding, consistent with asphyxiation.

However, well-controlled experiments in animals show the opposite. Hypercapnia (high CO<sub>2</sub>) delivered to the lungs of an animal leads to increased oxygenation of tissue, not decreased (133). These observations were also recorded in humans as early as the 1950s, when psychiatrists frequently rendered their patients unconscious with CO<sub>2</sub>—known as CO<sub>2</sub> therapy. Patients undergoing CO<sub>2</sub> therapy had increased cerebral blood flow and increased cerebral oxygenation (134). Hence, hypoxia is not the cause of CO<sub>2</sub> anesthesia in humans. Hypoxia was also dismissed as the mechanism in moths (135).

The first experimental studies on CO<sub>2</sub> as an inhaled anesthetic began as early as 1904. CO<sub>2</sub> was mixed with 20–27% O<sub>2</sub> and found to cause reversible anesthesia in guinea pigs, dogs, and birds (136). More recently, similar experiments were repeated for the optimization of CO<sub>2</sub> anesthesia in mice and other animals (135, 137). In mammals, the anesthetic properties of CO<sub>2</sub> are most like those of chloroform and less like those of diethyl ether (138) (**Figure 1a**).

CO<sub>2</sub> is the primary metabolite of cellular respiration and is used extensively by cellular pathways to estimate the cellular needs for O<sub>2</sub>. CO<sub>2</sub> directly controls the heart rate and induces vasodilation (139). High CO<sub>2</sub> in the lungs does affect the binding of O<sub>2</sub>, but this effect is minor compared to the driving force of O<sub>2</sub> partial pressure, i.e., the concentration of O<sub>2</sub>. As long as O<sub>2</sub> remains high in the lungs (20–30%), O<sub>2</sub> still binds in the presence of high CO<sub>2</sub>. The combined increase in cardiac output and vasodilation appears to more than compensate for the lower affinity of O<sub>2</sub> to hemoglobin (134).

## 7.3. Outliers to the Meyer–Overton Correlation

Of the thousands of molecules tested, a limited number are outliers to the Meyer–Overton correlation. For example, despite its high lipid solubility, 2-dichlorohexafluorocyclobutane (F6) (**Figure 1c**) does not produce anesthesia at concentrations predicted by the Meyer–Overton hypothesis. F6 is referred to as a nonimmobilizer because it does cause amnesia but fails to cause immobility (140), a key component of anesthesia, even at concentrations at which other, less-lipid-soluble, anesthetics would be effective.

The rarity of the outliers is remarkable and suggests that deviation relates to a factor other than shape. The Meyer–Overton correlation shows that, for almost all anesthetics, shape has no detectible effect on potency (**Figure 1a**). A few outliers do not negate the correlation. Nonetheless,

the existence of outliers highlights the need for a more nuanced understanding of anesthetic action that incorporates both lipid interactions and specific protein targets.

Long-chain saturated carbons are also considered outliers for their property known as the chain-length cutoff (5). Primary alcohols with a saturated short-chain carbon (e.g., ethanol with two carbons) cause anesthesia with increasing potency with longer chain lengths. The longer chain length increases the potency, consistent with the Meyer–Overton hypothesis. However, past a certain length, longer-chain alcohols, despite their hydrophobicity, lose potency. A metabolite was shown to contribute to their cutoff, as the long-chain carbons appeared to be too large to fit within the constricted hydrophobic catalytic pocket of the enzyme PLD2 (67). A recent structure of PLD1 shows a constricted hydrophobic catalytic pocket where an alcohol of limited chain length could bind (**Figure 4a**) (141). However, for straight chain alkanes, their site of action is not known, as they have no reactive headgroup. A pocket of circumscribed dimension has been theorized. But long-chain alkanes reduce the requirement for isoflurane, suggesting low vapor pressure may give rise to the apparent cutoff (142). Alkanes may also experience a cutoff effect in ordered lipids, where short alkanes disrupt the packing of the lipids and long-chain alkanes do not.

#### 7.4. Stereoselectivity

Anesthetics exhibit stereoselectivity, meaning that one isomer of a compound may have a slightly different potency than another. However, no anesthetic is stereoselective to the extent that one isomer is an effective anesthetic while another is not. This stereoselectivity has led to the theory that the primary targets of anesthetics must be proteins. Despite this, the origins of stereoselectivity in anesthesia remain unresolved, with at least four potential targets being considered, none of which are mutually exclusive: (a) ion channel sites, (b) ordered lipid sites, (c) anesthetic transport sites, and (d) molecular sinks (proteins or lipids).

Proteins are chiral compounds that often form ordered structures. Within these ordered regions, the chirality of amino acids can lead to the binding of ligands with specificity, meaning that one isomer may fit better into a binding site than another. This can be analogized to a left hand fitting more easily into a left-handed glove than into a right-handed glove. Classically, it is thought that a specific isomer may bind more tightly to an ion channel and induce an allosteric change that alters the gating of the channel. If one anesthetic isomer binds more tightly, this could indeed lead to a shift in potency. However, demonstrating this effect in a purified system without other proteins or ordered lipids has been challenging. As mentioned, while ion channels have been purified and reconstituted into lipids, their direct activation or inhibition by anesthetics has yet to be definitively shown for most channels.

The best experimental evidence for stereoselectivity comes from studies on the binding kinetics of anesthetics to albumin. While the affinities of the anesthetic isomers are the same, the rates at which they bind to and dissociate from the protein are stereospecific (143). Albumin, an abundant protein that transports anesthetics in the blood, could affect anesthetic potency *in vivo* by influencing the delivery rate of the anesthetic isomers. Unlike for ion channels, whose activity is measured by current, measuring the binding of anesthetics to albumin provides a direct assessment of the protein's function.

Lipids, like proteins, are also chiral compounds. In their ordered state, lipids can bind with structural specificity, such as binding palmitates over prenyl groups (**Figure 2b**). The binding of anesthetics to ordered lipids could exhibit chiral specificity, but this concept needs to be demonstrated experimentally.

Lastly, any protein or lipid sink within a cell could alter the concentration of anesthetic reaching its final target site. Anesthetics often have very steep dose–response curves (Hill slopes), so

even a small amount of stereospecific binding to a protein or lipid sink could alter the effective concentration at the anesthetic site and contribute to stereoselective differences in anesthesia.

## 7.5. Translating Binding Site to General Anesthesia in Animals

Presumably, a combination of binding sites translates into a biological function that creates anesthesia in an animal. The reduced sensitivity of a *pld* null fly to anesthetics helps establish the physiological relevance of the AP site in the context of general anesthesia (20, 144). As mentioned, PLD is a soluble enzyme upstream of TREK-1 in mammals. PLD2 is also upstream of the calcium channel Piezo2 (55), so by inhibiting calcium influx and activating potassium efflux, nerve excitability is reduced. Presumably, other proteins respond to PA in a similar coordinated manner.

Over the last 30 years, research has focused primarily on neuronal sites. But, as pointed out by Bernard in 1875 (10), anesthetics act on all cells in the body. Anesthetics depress breathing and blood pressure in the periphery (145). Loss of blood pressure alone causes loss of consciousness, i.e., fainting or syncope (146). How these phenomena relate requires a much broader understanding of anesthetic action.

The extent to which the AP site influences other anesthetic channels also awaits determination. Almost all anesthetic-sensitive channels are palmitoylated and regulated by aspects of the membrane-mediated mechanism, particularly direct regulation by cholesterol (see **Table 1**), suggesting its role could be broad. Many channels also bind PIP<sub>2</sub> (47), but whether anesthetics compete with cholesterol to release these channels is not currently known. Nor is it known whether their collective release can generate an anesthetic response in an animal. In theory, these are all testable questions.

Recently, GABA was shown to displace GABA<sub>A</sub>R from GM1 domains, presumably through the AP site. GABA<sub>A</sub>R is palmitoylated on an intracellular loop (**Figure 2c**). Application of GABA causes GABA<sub>A</sub>R located in lipid rafts to partition with PIP<sub>2</sub>, and this translocation correlates with function (97). Thus, membrane-mediated regulation is active in these channels.

In conclusion, binding sites are well defined for both protein and membrane sites. For proteins, the major challenge is functionally reconstituting the channels into lipids and showing direct activation or inhibition by an anesthetic. Presumably, all channels with direct sites respond in this way. For the membrane-mediated mechanism, identifying the proteins that are displaced from the AP site and their function is necessary to develop a comprehensive understanding. Lastly, the endogenous molecules that regulate anesthetic action need to be identified and characterized for their physiological function.

### SUMMARY POINTS

1. Anesthetics compete nonspecifically with palmitate for a binding site within ordered lipids (lipid rafts).
2. The competition of anesthetics with palmitate releases palmitoylated proteins from lipid rafts.
3. Anesthetics compete nonspecifically with regulatory lipids for binding sites within the transmembrane domain of proteins.
4. Anesthetics act primarily on ordered lipids, not disordered lipids.
5. Ordered lipids have structure with selectivity for regulatory lipids.
6. CO<sub>2</sub> is an endogenous anesthetic.

## FUTURE ISSUES

1. Many ion channels, as prepared for structural studies with anesthetics, are nonfunctional or in nonphysiological states. In order to advance from merely identifying anesthetic binding sites with very little evidence of function, the channels will need to be functionally reconstituted and shown to respond to an anesthetic in a purified system. Alternatively, methods are needed to distinguish an anesthetic's specific allosteric regulation from nonspecific competition with regulatory molecules binding to proteins.
2. To establish membrane-mediated mechanisms of general anesthesia, the spatial distribution of palmitoylated proteins, in particular ion channels, needs to be established at nanoscale levels in real time and eventually in living animals.
3. Ion channels may partition with nanoscopic lipid domains other than PIP<sub>2</sub>. Identifying the location of palmitoylated proteins after anesthetic release may require a better understanding of the various nanoscopic compartments in the membrane.
4. CO<sub>2</sub> causes anesthesia, but questions remain as to whether CO<sub>2</sub> functions the same as other inhaled anesthetics in competing with palmitate in ordered lipids or binding to protein sites.

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The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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With each new discovery, Richard's convictions about science, including general anesthesia, are reaffirmed. His intuition led him to believe that the body harbors its own secrets regarding anesthetics—endogenous molecules that regulate human consciousness. Though this idea was not obvious then, and remains elusive to many even now, in classic Lerner fashion, in the future it is likely to be recognized as truth. May Richard Lerner always be remembered for his kindness and selfless devotion to mentoring the next generation of scientists.

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