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Molecular mechanisms of anesthesia

Issaku Ueda

Anesthesia Service and Research, VA Salt Lake City Health Care System, University of Utah School of Medicine, Salt Lake City, UT, USA

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Abstract. Anesthesia was a blessing to humankind. It is a miracle that simple molecules such as chloroform ($CHCl_3$), diethyl ether ($CH_3 \cdot CH_2 \cdot O \cdot CH_2 \cdot CH_3$), or nitrous oxide ($N_2O$) induce a state of unconsciousness where patients can tolerate surgery. The diversity of the structures of these molecules indicates that there are no common receptors. The action of anesthetics is nonspecific and physical. After the demonstration by Meyer and Overton that anesthetic potencies correlate to their solubility into olive oil, the nonspecific lipid theories monopolized anesthesia theories for almost a century. The dominance of lipid theories invited repulsions against the nonspecificity idea. Protein theories that stress receptor bindings became the top mode. Nevertheless, the wide varieties of anesthetic molecules and the wide varieties of responding systems are difficult to reconcile with the specific interaction concept. This article discusses the recent progress and controversies on the molecular mechanisms of anesthesia. Anesthetics are unique drugs in pharmacology. They affect all macromolecules. The only comparable drugs are disinfectants. Both are nonspecific drugs. We use alcohols and phenols to wipe off the injection sites. We do not use penicillin or any other antibiotics for this purpose, because they are specific binders. Interestingly, these two nonspecific drugs opened the window for the modern medicine. (Keio J Med 50 (1): 20–25, March 2001)

Key words: anesthetics, molecular mechanisms, firefly luciferase, pressure effect, anesthetic-protein interaction

The Meyer-Overton Rule

The year 2001 commemorates the centenary of the publication of Charles Ernest Overton[1] on the correlation between the potency of anesthetics and their olive oil solubility. Together with the work by Hans Meyer,[2] the lipid theory of anesthesia dominated the anesthesia research for more than one hundred years. The lipid theories are often misinterpreted to mean that the anesthetic action site is lipid membranes. This includes the original authors. There is a major difference between solvation into unstructured (isotropic) olive oil and binding to structured lipid membranes. There are no units for hydrophobicity. When dielectric constant is used as a measure for hydrophobicity, olive oil is about 10, water is 80, and the core of lipid bilayer is 1.8.[3] Though the lipid theories of anesthesia assume lipid membranes as the anesthetic action site, there is no similarity between olive oil and lipid membrane. Nevertheless, the correlation that covers 5-orders of magnitude is just amazing. The potency of trichloroethylene to anesthetize 50% of the population (ED_{50}) is 0.5 volume percent when expressed by the gas phase concentration, or 0.005 atm when expressed by the partial pressure. The ED_{50} partial pressure of nitrous oxide is 1.01 atm. Nitrogen has anesthetic potency and the ED_{50} is about 50 atm. The correlation covers 0.005 to 50 atm. The eminent contribution of Meyer and Overton was to find olive oil from a milliard of organic solvents.

When the Fluid-Mosaic Model of lipid membrane was proposed by Singer and Nicolson[4] in 1972, the membrane fluidity suddenly ran through the world like

Reprint requests to: Dr. Issaku Ueda, Anesthesia 112A DVA Medical Center, Salt Lake City, UT 84148, USA, e-mail: issaku.ueda@m.cc.utah.edu

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wild fire. Though the membrane fluidity has never been clearly defined, the fluidity soon became the fad in membrane biology. As a backlash to the dominance of lipid theories of anesthesia, protein theories became the top fashion. Protein theories often assume specific receptors.

Anesthetics and Ion Channels

Huge amounts of reports on volatile anesthetic interaction with neurotransmission systems have accumulated. The articles presented at the New York Academy of Science Symposium[5] in 1991 included the nicotinic acetylcholine receptor, $GABA_A$ receptor, $GABA_B$ receptor, NMDA receptor, non-NMDA glutamate receptors, glycine receptor, peptide receptor, G-protein-linked systems, α_2-adrenergic receptor, Na^+ channel, K^+ channel, and Cl^- channel, to name a few. In addition, volatile anesthetics affect the presynaptic transmitter release and a number of enzymes, e.g., protein kinase C, phospholipase C, inositide turnover, mitochondrial electron transport, transport ATPases, luciferases, etc. Actually, almost all channels are affected by volatile anesthetics when the concentrations are raised. To this author, this means nonspecific interaction.

Channels are highly organized lipoproteins. It is impossible to isolate active channel proteins in the lipid-free form. Surfactants are necessary to isolate the channel proteins. The isolated protein is covered by surfactant layers. It is difficult, if not impossible, to distinguish anesthetic interaction with proteins from the supporting lipid bilayer or surfactant layers.

Claude Bernard

In 1875, Claude Bernard[6] published an essay on the mechanisms of anesthesia. He emphasized that anesthetics are not a special poison to neuronal systems and that all tissues are affected. Using an animal muscle model, he reported the application of the aqueous solutions of chloroform or diethyl ether, or exposure to their vapor, made the muscle unresponsive to electrical stimuli. At the same time, the muscle became rigid and the transparency was decreased. When the anesthetics were removed by washing or evaporation, the muscle returned to the normal transparency and responded to electrical stimuli. He interpreted these observations as a physicochemical phenomenon and discussed the similarity between this phenomenon and that of snails losing transparency and activity when placed in arid conditions, returning to normal appearance and activity when adequate humidity is regained.

In modern terminology, Claude Bernard envisioned anesthesia as a reversible equilibrium state of protein colloid in which the conformation and the state of the hydration changed. He also emphasized the advantage of introducing a suitable model that can be used successfully to analyze drug effects. His wisdom probably encompasses the whole present concept of anesthetic-protein interactions.

Firefly Luciferase

I graduated medical school in 1948 and spent about seven years in biochemistry, studying ATP degradation intermediates. Then, the Fulbright Grant started. When I passed the examination, the committee advised me to apply for clinical positions. Medical schools are suffering a shortage of residents, and the positions are wide open. I started my anesthesia residency in July 1957. In the 1950s, the mechanisms of the firefly bioluminescence and the action of Na^+-K^+ ATPases were getting established. Both are ATP-dependent enzymes. Because my background in biochemistry concerns ATP, these reports excited me. However, it was impossible to catch fireflies in Utah. I was determined to work with this enzyme when I returned to Japan. The chance came earlier than I expected. The United States returned one of its occupied hospitals to Japan. The Welfare Ministry of Japan decided to start the first National Center under direct control of the Ministry. I was called back to Japan to manage the anesthesia department.

The National Cancer Center was equipped with top anesthesia machines, monitors, CO_2 analyzers, etc., plus the research building with an ultracentrifuge, spectrophotometers, etc. To my surprise, there were firefly suppliers in Tokyo for entertainment. I prepared cell-free luciferase from firefly tails and added anesthetics. When the anesthetics inhibited the ATP-induced light output at subclinical concentrations, I was ecstatic.[7]

Firefly tail contains an enzyme luciferase and a substrate luciferin, and addition of ATP in the presence of molecular oxygen induces a flash of light.[8]

$$\mathrm{FFL} + \mathrm{Luciferin} + \mathrm{ATP} \rightleftharpoons \mathrm{FFL\text{-}Luciferyl} \cdot \mathrm{AMP} + \mathrm{PPi}$$

$$\mathrm{FFL\text{-}Luciferyl} \cdot \mathrm{AMP} + \mathrm{O_2} \rightarrow \mathrm{FFL} + \mathrm{Oxyluciferin} + \mathrm{AMP} + \mathrm{CO_2} + \mathrm{Light}$$

where FFL is firefly luciferase and PPi is pyrophosphate. The light intensity is stoichiometrically proportional to the amount of added ATP.

In 1967 we[9] studied the anesthetic effect on the Na^+-K^+ ATPase. The enzyme was purified from the cerebral microsomal fraction of rabbits. The tissue was homogenized and the enzyme was obtained by ultracentrifugation with sucrose gradient. The result was disappointing. Anesthetics inhibited ATPase activity only

when the concentration exceeded the clinical range tenfold. The enzyme also failed to respond to sedatives, such as hydroxyzine, haloperidol, and diazepam,[10] at concentrations equivalent to the clinical use. The enzyme responded to these drugs when the concentrations exceeded one-order of magnitude over the clinical doses. Firefly luciferase turned out to be the most sensitive enzyme to anesthetics.[7] I was extremely lucky.

Firefly Luciferase and Nonspecificity of Anesthetic Effect

The detailed anesthetic action mode on firefly luciferase was evaluated by Ueda and Kamaya[11] in 1973 according to the rate-process theory of Eyring. The dose-response curves of FFL bioluminescence are transformed into linear form according to the Johnson-Eyring equation.[12]

$$\frac{I_1}{I_2} - 1 = K[\mathrm{X}]^r$$

$$\ln\left(\frac{I_1}{I_2} - 1\right) = r\ln[\mathrm{X}] + \ln K = r\ln[\mathrm{X}] - \frac{\Delta G}{RT}$$

where I_1 and I_2 are the control and the inhibited light intensities, respectively, X is the mole concentration of the inhibitor, K is the association constant of the anesthetic and FFL, and r is the number of bound ligands. When the logarithm of the inhibited portion $(I_1 - I_2)$ and the uninhibited portion (I_2) is plotted against the logarithm of the inhibitor concentrations, the r value is obtained. When the left side is plotted against the reciprocal of absolute temperature, ΔG is obtained. The r value is equivalent to the n_{H} of the Hill plot. From the temperature-dependence of the light intensity, we estimated the free energy changes, ΔG, of the enzyme by volatile anesthetics. The enthalpy change, ΔH, values were in the range of 90 kcal/mol. The entropy change, ΔS, values were in the range of 300 cal/mol/deg. This means that the anesthetics induce a large conformational change and relax the structure nonspecifically. From the entropy change, Eyring, *et al.*[13] estimated about 50 water molecules are released from the firefly luciferase by clinical concentrations of volatile anesthetics. Incidentally, the equation is supported by a rigorous thermodynamic argument and a convenient procedure to analyze dose-response curves.

Disputes on Firefly Luciferase

Eleven years later, Franks and Lieb[14] objected to our 1973 conclusion,[11] that anesthetics unfold firefly luciferase. They claimed that anesthetics inhibit luciferase by competing with the substrate luciferin. However, it is inconceivable that the uncharged volatile anesthetics compete with the negatively charged luciferin. The charge-induced dipole interaction (luciferin binding) is stronger than the dipole-induced dipole interaction (anesthetic binding). It is difficult to displace stronger binders by weaker binders, except in overtly excess concentrations. They also claimed that the size of the luciferin binding cavity discriminates between anesthetics and nonanesthetics.

Their opposition has an underlying story. They were outraged about our article[15] in the Molecular Pharmacology which pointed out the error in their article[16] in the Nature about anesthetic interaction with lipid bilayer. We showed that their definition of the partial molal volume of anesthetics in lipid bilayers is incorrect.

The story of luciferin-anesthetic competition was a result of erroneous use of the Lineweaver-Burk plot. The Lineweaver-Burk plot analyzes one-substrate one-inhibitor reaction. Even with a one-inhibitor one-substrate system, the analysis of the reaction rate is highly complicated.[17] Firefly luciferase is a three-substrates (luciferin, ATP, and oxygen) enzyme with two product inhibitors: one at the initial stage by pyrophosphate and the other at the final stage by oxyluciferin. The plot is inapplicable unless supported by a proper theory. Without rigorous derivation, the double reciprocal plot only measures whether the inhibition is surmountable or insurmountable by the infinite increase of the substrate. It does not prove or disprove ligand-substrate competition.

The bioluminescent reaction starts with the acylation of ATP to form the high-energy acyl-AMP complex, followed by oxidation of luciferin to boost the luciferin-luciferase complex to the exited state. Transition back to the ground state produces light. Anesthetics can interact in any one of these processes. It is wrong to conclude that anesthetics compete at the first stage (association of luciferin and luciferase) from the final reaction (light emission).

In addition, Franks and Lieb[14] used the initial flash intensity to construct a Lineweaver-Burk plot. The rapid kinetics of the transition state theory[18] shows that when the rate of the initial reaction to form the first product is faster than that of the final reaction to form the last product in a multiple stage reaction, the first product accumulates rapidly. The accumulation is represented by a peak. The peak is designated as the pre-steady-state burst. The use of the non-steady-state initial flash by Franks and Lieb violates the basic rules of inhibition kinetics.

Real Competitive Inhibitor of Firefly Luciferase

Ueda and Suzuki[19,20] found that long-chain fatty acids are 1000-times stronger inhibitor than anesthetics.

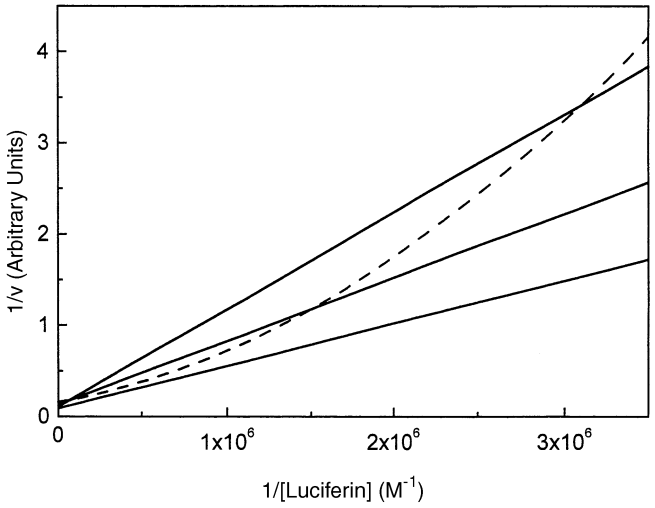

Fig. 1 The Lineweaver-Burk plot. The broken line is halothane 1.0 mM, and the solid straight lines are, from the bottom, control without additives, myristic acid 1.0 and 2.0 μM, respectively.

The 14-carbon fatty acid, myristate, inhibited firefly luciferase at the micromolar range, compared with the millimolar range of anesthetics. The Lineweaver-Burk plot produced linear plots with luciferin, whereas those of anesthetics were nonlinear (Fig. 1). The curved broken line is halothane 1.0 mM, and the solid straight lines are, from the bottom, control without additives, myristic acid 1.0 and 2.0 μM, respectively. The difference between our curved Lineweaver-Burk plot[19] and the linear plot by Franks and Lieb[14] was caused by the steady-state condition and unsteady-state condition, respectively.

It is not surprising that long-chain fatty acids are strong luciferase inhibitors. Luciferin is a hydrophobic carboxylic acid, and long-chain fatty acids are also hydrophobic carboxylate. Both are negatively charged. Luciferin acylates ATP by firefly luciferase to form high-energy acyl-AMP. Fatty acids also acylate ATP by acylCoA synthetases and form high-energy acyl-AMP. Suzuki and coworkers[21] reported significant homology among these enzymes.

Anesthetics inhibit bacterial luciferase,[12] but myristic acid does not.[22] Rather, myristic acid activates certain bacterial luciferase.[22] This proves clearly that the myristate action is specific but anesthetic actions are not. Myristate increased the thermal transition temperature of luciferase, whereas anesthetics decreased it.[19,20] The report by DeLuca[23] that 2-(*p*-toluidino)-6-naphthalenesulfonate, TNS, and 1-anilino-8-naphthalene sulfonate compete with luciferin indicates that negative charges are required to compete with the luciferin binding.

High Pressure Reversal of Anesthesia

High pressure antagonism of anesthesia is well known. However, Moss, *et al.*[24] reported that high pressure did not show any effect on the light intensity of firefly luciferase in the absence or presence of anesthetics. This is highly strange because thermodynamics dictates that all reactions are the function of temperature and pressure. Thermodynamics is the law of the universe. Every reaction must obey. Besides, the pressure reversal of anesthesia was first discovered with bacterial luciferase by Johnson, Eyring and coworkers[25–27] in 1942. Hydrostatic pressure about 10 MPa reversed the anesthetic effects on bacterial luciferase. It opened a new era for the understanding of the molecular mechanism of anesthesia. The criticism that bacterial light intensity has no relevance to anesthesia was countered by Johnson and Flagler[27] nine years later by showing pressure reversal of anesthesia in tadpoles. Tadpoles anesthetized by ethanol and staying quietly at the bottom of the container started swimming again when 100 atm pressure was applied.

There are sporadic negative reports on pressure reversal. Smith, *et al.*[29] reported that high pressure did not reverse anesthesia in freshwater shrimps. They proposed that crustaceans lack strychnine receptors: high pressure reverses anesthesia by stimulating the strychnine receptor. The deviation of crustaceans from others originates from their anomalous response to high pressure. High pressure suppresses activity of shrimps even in the absence of anesthetics. When both anesthetics and pressure inhibit the activity, the analysis of the combined effect becomes complicated. Simon, *et al.*[30] reported that pressure antagonized anesthesia in brine shrimps. They came to this conclusion by comparing the anesthetic effect with the control in the absence of anesthetics. We[31] described a method of analyzing the combined effect of pressure and anesthetics when both are inhibitory. Above all, strychnine does not reverse anesthesia. Most importantly, however, the shrimp studies demonstrated that the pressure effect is unrelated to the pain or discomfort associated with compression.

The difference between the negative result with FFL bioluminescence by Moss, *et al.*[24] and the positive result with the bacterial bioluminescence by Johnson, Eyring, *et al.*[25–27] is caused by the difference between the continuous light intensity (steady-state condition) of luminous bacteria and initial flash intensity (unsteady-state condition) firefly enzyme. By a high-pressure stopped-flow study, Ueda, *et al.*[32] confirmed that pressure did not affect the FFL light intensity when measured by the initial flash intensity. When the light output of the first 10 sec was integrated, however, pressure did antagonize the anesthetic action.[32] Or, when studied at a quasi-

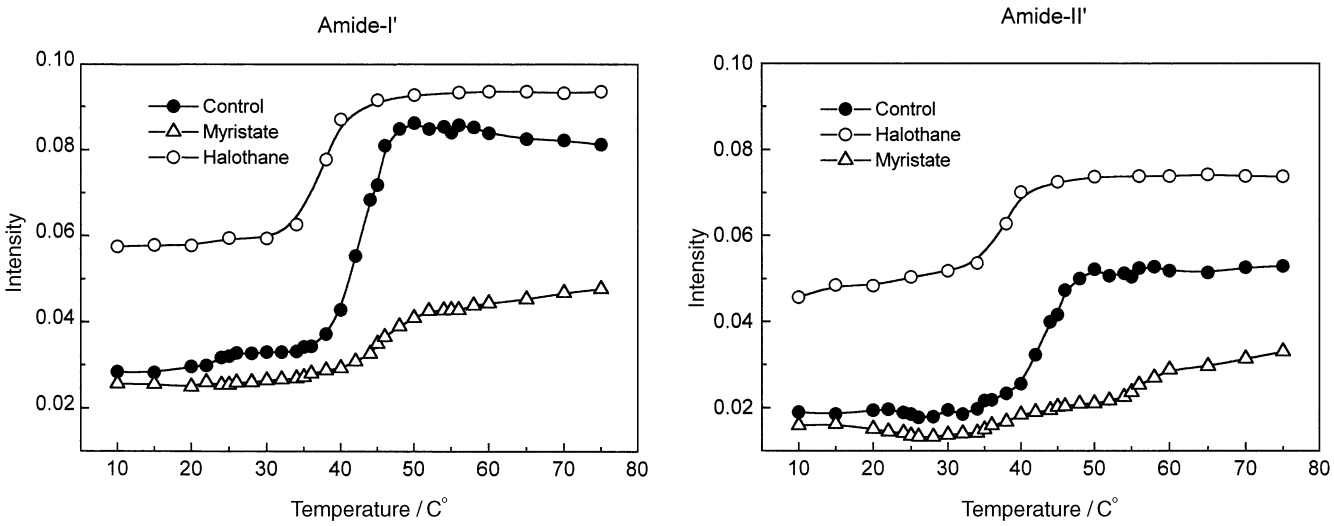

Fig. 2 The thermal effect on Fourier-transform infrared spectroscopy. Temperature elevation relaxes the protein structure demonstrated by the increase of intensity. Ethanol increased the size of the peak intensity of Amide-I' and Amide-II' region whereas myristate decreased the peak intensity. Alcohol relaxed the secondary structure of FFL, and myristate tightened it.

steady state under deceleration of the reaction rate with pyrophosphate, pressure also antagonized the anesthetic effect.[33] Pressure always antagonizes anesthesia.

The pressure reversal of anesthesia means only one thing: the volume of the anesthetized state is larger than the awake state. It indicates that an increase of the partial molar volume of the system is the crucial condition for anesthesia.

The Enthalpy of Anesthetic Effect

Dickinson, *et al.*[34] contested our 1973 thermodynamic study.[11] They argued that the anesthetic action on firefly luciferase is not nonspecific conformational change and our ΔH values were wrong. They reached their conclusion by measuring the initial peak light intensity again. From the anesthetic effect on the flash intensity, they constructed the Lineweaver-Burk plot again and supported the 1981 erroneous report by Franks and Lieb.[14]

From the temperature dependence of the inhibitor constant, they estimated ΔH and obtained negative small values. The averaged value for halothane, methoxyflurane and diethyl ether was −3.87 kcal/mol. Our result[11] was +80 kcal/mol. They argued that our value is the result of using the old kinetic scheme, and insisted on the idea that our conclusions are wrong. They further proposed that the anesthetic interaction with firefly luciferase should be characterized by the negative free energy. The article was accompanied with an editorial[35] designating it as an enthalpic model of anesthesia.

These authors[34,35] did not recognize that the sign is positive with ours and negative for theirs. Their values correspond to the transfer of anesthetic molecules (molecular weight about 300) from water to the protein. Our values represent the transfer of firefly luciferase (molecular weight 62,000) from water to the anesthetic solution. The change of the enthalpy of anesthetic molecule has little significance to the mechanism of anesthesia. What is important is the change of energy level of the enzyme when anesthetized. The large positive ΔH and ΔS represent relaxation of the protein structure by anesthetics.

We[19,20,36] demonstrated the structural relaxation of firefly luciferase by the effect on the thermal transition[19,20] of the protein, and by Fourier transform infrared spectroscopy[36] at the Amide-I' and Amide-II' region (Fig. 2). The infrared spectra showed that the temperature elevation relaxed the secondary structure of firefly luciferase. Ethanol increased the structural relaxation, whereas myristate antagonized the relaxation.

Conclusion

Biophysical studies attempt to find physicochemical mechanisms that dictate the interaction of anesthetic molecules with proteins and lipids. I believe there is no system that is uninfluenced by anesthetics. The crux of anesthetic action is the pressure reversal. Anesthetized state is larger than the awake state. Whatever decreases the volume antagonizes anesthesia. Our study showed that anesthetics unfolded and expanded firefly luciferase whereas myristate tightened the structure. Because

myristate may antagonize the anesthetic effect by decreasing the volume, we measured the effect of myristate on the anesthesia potencies in goldfish. Myristate successfully antagonized anesthesia in goldfish. The halothane partial pressure that induces anesthesia in half of the population increased 300% in the presence of 40 μM myristate.[37] Myristate has no action on channels proposed to be the targets of anesthetic actions. In this context, it is futile to pinpoint one neurotransmission system for the specific anesthetic target site. We found that the Hill number of anesthetizing goldfish averages about five. The large Hill coefficients in intact animals indicate that multiple neurotransmission systems respond to anesthetics with high cooperativeness. There is no single button that can be pushed to induce anesthesia.

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