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Saturable and unsaturable binding of a volatile anesthetic enflurane with model lipid vesicle membranes

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Presence of specific receptors for volatile anesthetics has recently been proposed (Evers, A.S. et al. (1987) Nature 328, 157–160) by a finding that halothane uptake by the rat brain was characterized, in part, by saturable binding. We report here that volatile anesthetics bind model lipid membranes also with saturable and unsaturable kinetics. Binding of enflurane to dipalmitoylphosphatidylcholine vesicle membranes was measured by gas chromatography. At low anesthectic concentrations, comparable to the clinical level, the interaction was saturable. After reaching a temporary saturation, a sudden increase in the anesthetic binding to the membrane occurred, when the anesthetic concentration in the aqueous phase exceeded 2.7 mM, or $6.3 \cdot 10^{-2}$ atm partial pressure in the gas phase in equilibrium with the aqueous phase. The secondary binding was linear to the aqueous anesthetic concentrations and was unsaturable to the limit of this study. We also found that enflurane self-aggregated in water above 4 mM. When the aqueous concentration exceeded 6 mM, the aggregation number was about 8. We conclude that the saturable binding indicates adsorption onto the vesicle surface, and the unsaturable binding indicates multilayer stacking of the enflurane molecules, where the initially adsorbed molecules provide the binding sites to the succeeding molecules according to the multilayer condensation kinetics. The tendency of enflurane to self-aggregate in water promotes the multilayer stacking at the surface of the membrane.

Introduction

Action of volatile anesthetics is generally considered to be nonspecific, not involving specific receptors (see, for instance, the review Ref. 1). Recently, however, receptors for anesthetics have been proposed in an enzyme, luciferase, by Franks and Lieb [2], and in the rat brain by Evers et al. [3]. The latter group used ^{19}F nuclear magnetic resonance spectroscopy (^{19}F-NMR) to measure the halothane uptake by the rat brain. From the analysis of the spin-lattice relaxation times, T_2, Evers et al. [3] reported that the anesthetic uptake by the rat brain occurred in two distinct modes; saturable and unsaturable. The saturable binding was characterized by a short T_2 and unsaturable binding was characterized by a long T_2. They proposed that the saturable binding represents receptor occupancy and the unsaturable binding represents nonspecific partition into lipid parts in the brain [3].

We also found two different modes of halothane interaction with surfactant micelles by ^{19}F-NMR spectroscopy [4]. At low concentrations, halothane solvated onto the micellar surface with tendency to saturate. The binding became temporarily saturated when the halothane concentration was about one-half of the surfactant concentration. When halothane was added further, the anesthetic started to bind micelles above the temporary plateau line.

With a planar lipid bilayer (BLM), we showed that volatile anesthetics (halothane, enflurane, and chloroform) increased the capacitance [5] and conductance [6] of the BLM in two distinct modes; saturable and unsaturable. At clinical anesthetic concentrations, the binding was saturable. The unsaturable binding started after the capacitance reached a plateau level of about 0.58 $\mu F/cm^2$. The secondary binding was proportional to the bulk anesthetic concentrations and was unsaturable. The order of the partial pressures of the volatile anesthetics in the gas phase at the beginning of the secondary binding correlated to that of clinical potencies expressed by the minimum alveolar concentrations that induce surgical depth of anesthesia (MAC values)

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[7]. When the membrane capacitance reached about 0.7 $\mu F/cm^2$, the membrane ruptured.

These bimodal interactions indicate the complexity of anesthetic–macromolecule interactions. It is important to clarify the anesthetic binding process to membranes. There have been a number of reports that estimated the binding of volatile anesthetics in the form of membrane-buffer partition coefficients [8–12].

In this communication, the binding mode of enflurane to phospholipid membranes is investigated by 'total-sampling' gas chromatography. The results revealed saturable and unsaturable binding of volatile anesthetics to phospholipid vesicle membranes, in agreement with our previous reports on the electrical properties of BLM [5,6] and ^{19}F-NMR binding to surfactant micelles [4].

Materials and Method

Enflurane (2-chloro-1,1,2-trifluoroethyldifluoromethyl ether) was a product of Anaquest (Madison, WI). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma. Triply distilled water was used throughout. The DPPC vesicles were prepared by ultrasonic irradiation by a Branson Sonifier W185E (Danbury, CT) under nitrogen gas at temperatures above the main phase transition. The DPPC concentration was $2.0 \cdot 10^{-3}$ molal. The vesicles were fused at 4°C to a uniform size of about 800 Å in diameter [13].

The anesthetic binding to DPPC vesicles was measured at 37°C and ambient pressure by a custom-built reaction chamber (Fig. 1) for the total-sampling gas chromatography. It consists of a glass cylinder (A),

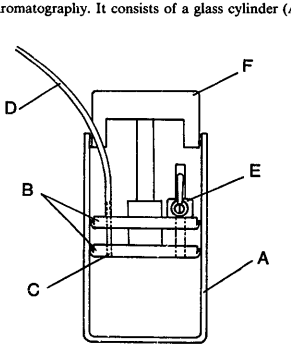

Fig. 1. Block diagram of the total-sample cell for gas chromatography. A, glass cylinder; B, Teflon piston; C, stainless steel hypodermic needle (internal diameter 0.1 mm); D, Teflon tube; E, two-way metal cock; and E, guide for the piston movement.

42 mm internal diameter and 75 mm length, to which a piston with double Teflon discs (B) is inserted. Teflon seal-rings are attached to the piston discs to prevent gas leaks. A hypodermic needle (C), 0.1 mm internal diameter and 0.25 mm external diameter, pierces the Teflon piston discs. The needle is connected to a Teflon tube (D) 0.2 mm internal diameter. At the end of the Teflon tube, a hypodermic needle is attached, which is used to inject gas samples to the intake port of a gas chromatograph. A metal two-way valve (E) is affixed to the piston. The valve is opened when the outside air is introduced into the chamber. A guide (F) is attached to insure linear piston movement. The piston is lowered by a constant-speed squeeze-out system with a rate of 7.5 mm/min (10.4 cm^3/min). The constant-speed system is composed of a micrometer (Toyoshima Co., Tokyo) with 50 mm maximum length, and a Reaction Motor 23K4GK-A (Oriental Motor, Tokyo). Two microswitches determine the upper and lower position of the piston drive. The total-sampling gas chromatography uses the total volume of the gas phase rather than a fraction of the volume for determination.

The anesthetic concentration in the squeezed-out gas was measured by a Hitachi Model 023 (Tokyo) gas chromatograph with a flame ionization detector. A glass column 3 mm internal diameter and 50 cm length, packed with Shimadzu Sillicone DC-550, was used at 60°C.

The experiment was performed in the following order. A measured amount of water or the liposome was introduced into the chamber, to which liquid enflurane was added by a microsyringe. The amount of added anesthetic was measured by weighing the cylinder by an analytical balance. The piston position in the cylinder was set so that the volume of the air was about equal to the aqueous volume, then the metal valve was closed and the chamber was immersed into a waterbath maintained at 37.0 ± 0.1°C. The anesthetic mixture was rigorously mixed by a magnetic stirrer for 6 h. Fig. 2 shows the time course of the enflurane distribution ratio between water and gas phases. The ratio reached an equilibrium at about 4 h.

After the mixing, the total volume of the gas phase was introduced into the gas chromatograph by the constant-speed squeeze-out system. From the micrometer readings on the start and finish of the micrometer travel, the gas phase volume was estimated. After the enflurane signal was recorded on the chromatograph, the metal valve of the piston was opened and the piston was returned to the initial position, introducing the outside air into the chamber. Then the valve was closed, and the content was stirred to transfer the remaining enflurane in the aqueous phase to the gas phase. The gas phase was transferred into the gas chromatography again by the squeeze-out system. These procedures were repeated until the enflurane peak was no longer de-

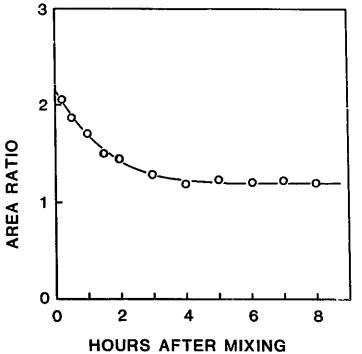

Fig. 2. Time course of the enflurane solvation into water and 37 °C. 50 µl of liquid enflurane was added to the reaction chamber filled with equal volumes of gas and water phase. After the initial vigorous shaking, the water phase was mixed by a magnetic stirrer. Ordinate is the area ratio in the gas chromatogram between the water and gas phases. The equilibrium was reached at about 4 h after the initial mixing.

tected by chromatography. These secondary transfers of enflurane into the gas phase do not require gas-liquid equilibrium.

The initial peak area in the chromatogram indicates the enflurane mass in the gas phase, and the sum of the areas obtained after the second chromatography indicates the mass of enflurane in the aqueous phase.

Results

Total enflurane mass in the system (gas and aqueous phases) is designated by A_t (mol), the volume of the gas phase by V (cm^3), the volume of the aqueous phase by W (cm^3), the enflurane area in the gas chromatogram by S_g and S_s (cm^2), where subscripts g and s signify gas and solution, respectively. The enflurane concentration in the gas phase, A_g (molarity), and that in the aqueous phase, A_s (molarity) are expressed as follows.

$$A_g = A_t\left(\frac{S_g}{S_g + S_s}\right)\frac{1000}{V} \quad (1)$$

$$A_s = A_t\left(\frac{S_s}{S_g + S_s}\right)\frac{1000}{W} \quad (2)$$

Because the increase in the aqueous volume by the addition of $1 \cdot 10^{-2}$ M enflurane is negligible, W indicates the volume of the solvent (water).

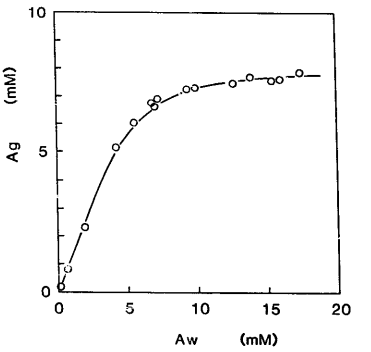

Fig. 3. Partition of enflurane between water and gas phases at 37 °C. Ordinate: enflurane concentration in the gas phase, A_g. Abscissa: enflurane concentration in water, A_w. The anesthetic concentrations are expressed in 10^{-3} M.

The enflurane distribution between the gas and aqueous phases in the absence of phospholipid vesicles is depicted in Fig. 3, and that in the presence of $2.0 \cdot 10^{-3}$ molal DPPC vesicles in Fig. 4. As expected, the enflurane content in the liposome solution was larger than in the control at the same A_g values.

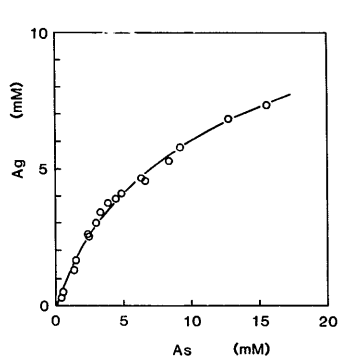

Fig. 4. Partition of enflurane between dipalmitoylphosphatidylcholine vesicle suspension and gas phase at 37 °C. Ordinate: enflurane concentration in the gas phase, A_g. Abscissa: enflurane concentration in the vesicle suspension, A_s. The phospholipid concentration was $2.0 \cdot 10^{-3}$ molal.

Discussion

Enflurane in water: self-aggregation at high concentration

In Fig. 3, when the enflurane concentration in the water phase was less than 4 mM, a linear $A_w - A_g$ relationship was demonstrated, where A_w is the anesthetic concentration in water. Above this enflurane concentration, however, the enflurane concentration in the gas phase started to level off to approach a constant value. Mori et al. [14] reported that the plot between the partial molal volume versus aqueous concentration of enflurane also showed a break at about 4 mM. Apparently, the state of enflurane in the aqueous phase changes around this concentration.

We assume that when enflurane concentration exceeds a limiting value in water, it forms aggregate of n molecules, whereas it exists as monomer in the gas phase, then,

$$\frac{A_w}{(A_g)^n} = K_c \tag{3}$$

where K_c is the water-gas partition coefficient, and

$$\log A_w = n \log A_g + \log K_c \tag{4}$$

The slope of the plot between $\log A_g$ and $\log A_w$ indicates the aggregation number. Fig. 5 shows such plotting. The plot was rectilinear with a curve at the enflurane concentration range between 4 to 6 mM.

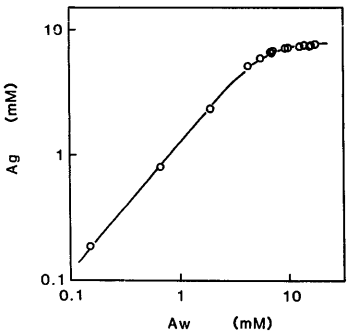

Fig. 5. Enflurane solubility in water, replotted from Fig. 2 on logarithmic scale. At the low concentration range, enflurane was dispersed as monomer. At the high concentration above 4 mM, enflurane started to aggregate. Above 6 mM the aggregation number was estimated from the slope. The value was 7.9.

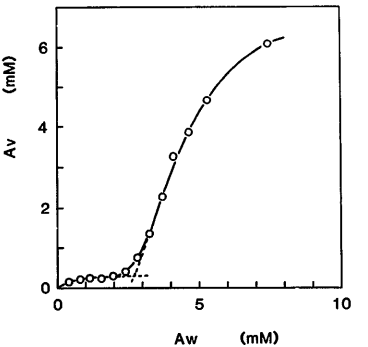

Fig. 6. Enflurane binding to dipalmitoylphosphatidylcholine vesicle membranes, A_v, as a function of enflurane concentration in the water, A_w. Enflurane concentrations are expressed by 10^{-3} M.

At the low enflurane concentration range, the slope was unity; enflurane was dispersed as a monomer. From the A_w intercept of the straight line in this range, the water-gas partition coefficient was estimated. The value was 0.814, in agreement with 0.82 (37°C) provided by the manufacturer. From the slope of the linear part in the high concentration range above 6 mM, the aggregation number of enflurane in water was estimated to be 7.9. Apparently, the enflurane molecules start to aggregate each other at about 4 mM in the aqueous phase and form octamer when its concentration exceeds 6 mM.

Enflurane in DPPC liposome: bimodal binding

The concentration of enflurane molecules that were bound to the vesicle membranes, A_v, was estimated from the difference between the enflurane concentrations in water, A_w, of the water-gas system (Fig. 3) and in the liposome solution, A_s, of the liposome-gas system (Fig. 4) at the identical enflurane concentrations in the gas phase, A_g.

In Fig. 6, the obtained A_v values were plotted against A_w. The curve reached plateau at about 2.7 mM enflurane concentration in water, then the adsorbed amount suddenly increased. This value translates to the partial pressure of $6.3 \cdot 10^{-2}$ atm in the gas phase in equilibrium with the solution.

Because phase-transition temperature of lipid membranes decreases when anesthetics are present, there is a chance that the sudden increase in the enflurane binding may represent binding to the liquid-crystalline membrane caused by the phase transition. To depress

the phase-transition temperature of dipalmitoylphosphatidylcholine vesicle membranes to 37 °C (the present experimental temperature), we [12] have shown that the bulk enflurane concentration should be in the range of about 7 mM. Because the linear secondary increase in binding started at 2.5 mM, the change in the state of the membrane at the higher end of the enflurane concentration is not a problem for the present analysis.

As described in the Introduction, similar bimodal interactions have been observed with halothane binding to surfactant micelles [4] and other volatile anesthetic binding (halothane, enflurane, and chloroform) to planar lipid bilayer membranes [5,6].

The initial enflurane binding site

The initial part of the interaction of enflurane with the liposome was characterized by a Langmuir-type curve that reached a limiting constant value at about 2 mM enflurane in the aqueous phase (Fig. 6). The data at the low enflurane concentration range were replotted in Fig. 7 according to the linearized double-reciprocal Langmuir equation between the reciprocal of membrane-bound enflurane concentrations and the reciprocal of the enflurane concentrations in water. A straight line, that conforms to the Langmuir adsorption isotherm, was obtained. The binding constant was found to be $1.6 \cdot 10^3\ M^{-1}$.

The initial saturation mode suggests that the anesthetic molecules occupy the binding sites of the lipid vesicle surface. This surface adsorption model is supported by our study with ^{1}H-NMR [15] on the thermotropic phase transition of dipalmitoylphosphatidylcholine vesicle membranes. The thermotropic phase transition in the absence of anesthetics was characterized by a simultaneous decrease in the half-height linewidth of the proton signals of the choline head and the lipid tail. On the other hand, the anesthetic-induced isothermal phase transition occurred in two stages. Clinical concentrations of volatile anesthetics decreased only the linewidth of the choline protons when the temperature was below the phase transition. When the anesthetic concentration was increased above the clinical level, the linewidth of the lipid protons started to decrease. Apparently, anesthetics bind lipid membranes in two distinct modes, and interfacial adsorption predominates at clinical concentrations.

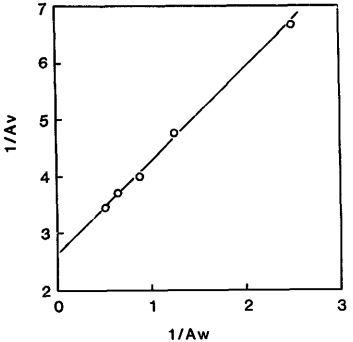

Fig. 7. Double-reciprocal plot of the saturable part of the enflurane binding to DPPC vesicle membranes at the enflurane concentrations below 2.0 mM. The reciprocal of the vesicle-bound enflurane concentrations A_v is plotted against the reciprocal of the enflurane concentrations in the aqueous phase A_w. The straight line conforms to the Langmuir adsorption isotherm.

From the chemical shift of proton signals in ^{1}H-NMR, we showed that the proton of the $CHCl_2$ end of the rod-like methoxyflurane ($HCCl_2$-CF_2-O-CH_3) molecule did not lose contact with the aqueous phase when bound to surfactant micelles [16]. The interfacial adsorption of volatile anesthetics was further supported from the binding of the counterion, estimated by ^{23}Na-NMR [17], and from the behavior of water molecules in reversed micelles, estimated by ^{1}H-NMR [18]. By Raman spectroscopy, Craig et al. [19] demonstrated halothane interacts with the head-group region of dipalmitoylphosphatidylcholine vesicle membranes. Our study [20] with Fourier transform infrared spectroscopy also showed that the anesthetic interaction site on lipid membranes was limited to the hydrophilic moiety with no effect on the C-H stretching of the hydrocarbon tail or on the C = O stretching of the ester linkage.

The secondary enflurane binding site

The region for the secondary binding site remains to be clarified. Two mechanisms are possible: penetration into the lipid core, or multilayer stacking where the initially bound anesthetic molecules provide binding site for the next molecules.

To simulate the core property, we [4] used liquid *n*-decane and showed that the chemical shift of decane protons in ^{1}H-NMR spectra changed at halothane : decane mole ratio of 1 : 10. The dielectric constant of decane is about 1.9 which is almost identical to the dielectric constant of the bilayer core. The presence of 10% halothane in the core region should be detectable by this technique. When halothane was added to sodium dodecylsulfate micelles to a mole ratio of 1 : 1, the chemical shift of the proton peaks of the surfactant did not change. If halothane penetrated into the micelle core, the proton signal of the surfactant tail would be affected. We concluded that halothane molecules reside near the micelle surface. This does not mean that anesthetics do not mix with core region. The probability

of finding dipolar anesthetic molecules is statistically highest at the interface. Lipid vesicle membranes and micelles are not directly comparable, but the result suggests multilayer condensation at the surface is a possibility for the secondary increase in the binding. The present result that enflurane tends to self-aggregate in water supports the multilayer adsorption model where initially bound enflurane attracts the subsequent enflurane molecules. A statistical mechanical theory for such surface condensation mechanism according to the Bose-Einstein statistics has been described by Eyring et al. [21]. Schöpflin et al. [22] demonstrated with Fourier transform infrared spectroscopy with ATR (attenuated total reflection) technique that tetracaine binds phosphatidylcholine membranes by the multilayer condensation.

We conclude that enflurane interacts with DPPC vesicles by the surface adsorption at low concentration and by the multilayer condensation at high concentration. In this two-stage adsorption model, the Langmuir-type adsorption ends at $A_w = 2.7$ mM, where A_v reaches 0.25 mM. When A_w is further increased, the condensation mechanism [21] sets in, where adsorbed enflurane becomes the binding site for the next enflurane molecule.

Estimation of membrane-buffer partition coefficients

The interaction of volatile anesthetics with vesicle membranes has generally been expressed by the membrane-buffer partition coefficients [8–12]. For comparison, the membrane-water partition coefficient, K, of enflurane is estimated. The partition coefficient is written by expressing DPPC with m_L in 1000 g (55.5 mol) water,

$$K = \frac{A_v}{m_L + A_v} \Big/ \frac{A_w}{55.5 + A_w} \tag{5}$$

where A_v, A_w, and m_L are expressed by molality. At the dilute solution condition, molality in water can be approximately substituted by molarity.

At $A_w = 2.7$ mM where the first stage adsorption appears to be complete, A_v is 0.25 mM from Fig. 6, and the membrane-water partition coefficient, K, of enflurane is estimated to be 2300, according to Eqn. 5. At $A_w = 5.0$ mM where the adsorption is multilayer, $A_v = 4.3$ mM and $K = 7600$.

The DPPC membrane-water partition coefficient of enflurane, estimated from the depression of the main phase-transition temperature, was 1680 [12]. This is an apparent value, K_{app}, that ignores the anesthetic interaction with the gel membrane. The true partition coefficient is expressed [23]

$$K_{app} = (1 - k)K \tag{6}$$

where k is the gel-liquid partition coefficient of the anesthetic, and the value is estimated to be about 0.3 [11]. Then the value of K translates to 2400. The membrane-water partition coefficient of enflurane, estimated from the electric capacitance of a planar lipid bilayer was 2760 [5]. These values are in reasonable agreement with the present result.

Saturable and unsaturable bindings

The present study demonstrated that the enflurane binding to model phospholipid membranes consists of two modes. When the enflurane aqueous concentration was lower than 3 mM, the binding was characterized by saturation kinetics. When the enflurane concentration was higher, the binding was unsaturable to the extent of the present experimental condition and appeared to be multilayer condensation. The adsorbed enflurane molecules reside in two different environments: the lipid surface and the enflurane multilayer aggregates. The presence of two states for bound anesthetic molecules agrees with the result by Koehler et al. [24] who reported that the regions of the bilayer where anesthetics partition at lower concentrations are different from the regions where they partition at higher concentrations.

The rotational freedom of the anesthetic molecules adsorbed directly onto the membrane is expected to be more restricted than those aggregated in the multilayer. Then, T_2 of the anesthetic molecules in the first layer may be shorter than T_2 of those in the multilayer. Because the first layer adsorption is Langmuir and follows the saturation kinetics, the shorter T_2 enflurane binding will saturate. The present result is qualitatively similar to that reported by Evers et al. [3], where the binding of halothane molecules with shorter T_2 followed saturation kinetics.

Acknowledgements

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