Effects of High Pressure on Polarity Change of the Water-liposome Interface Induced by Volatile Anesthetics

Ikuko TSUKAMOTO, Satoshi YOKONO and Kenji OGLI

The effects of high pressure on the interactions between volatile anesthetics and three kinds of phosphatidyl choline liposomes were investigated by fluorometry, using a thiacarbocyanine dye (3,3'-dioctadecyl-2,2'-thiacarbocyanine) which is sensitive to the environmental viscosity and dielectric constant. Seven general anesthetics, halothane, enflurane, isoflurane, methoxyflurane, sevoflurane, diethylether and chloroform were used. We have previously reported that these anesthetics decreased the phase transition temperature and increased the effective dielectric constant of the water-liposome interface using dipalmitoyl-phosphatidylcholine. In this study, it was confirmed that the effects of anesthetics on the effective dielectric constant were not altered by the use of a gel or liquidcrystal membrane, and were reversed by the application of high pressure (< 800 bars). The increase of the effective dielectric constant was attributed to the perturbation of hydrogen bonds at the liposomal interface. High pressure is considered to promote hydration. Our results obtained under high pressure supported previous observations made at ambient pressure, which suggested that the perturbation of hydrogen bonds at the water-liposome interface correlates with the mechanism of anesthesia. (Key words: anesthesia theory, high pressure, volatile anesthetics, polarity of interface, hydrated water)

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Pressure reversal of anesthesia was first demonstrated by Johnson and colleagues using luminous bacteria[1]. This phenomenon was confirmed with tadpoles[2] and was subsequently also shown to occur in newts[3,4] and mice[4]. If pressure antagonizes the effect of anesthetics *in vivo*, it should also be able to antagonize similar effects produced in any valid model of the site(s) of anesthetic action.

The high correlation of the potency of inhalation anesthetics with lipid solubility[5,6] suggests that the site of anesthetic action is in a lipid containing region of the nerve cell membrane. Therefore, many investigations of the molecular mechanisms of anesthesia has been performed using phospholipid liposomal membranes.

We have performed a series of experiments on liposomes in which

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a fluorescence probe was incorporated. From the spectral changes of the probe, which was very sensitive to both polarity and viscosity, we found that volatile anesthetics decreased the phase transition temperature of liposomes and increased the effective dielectric constant of the water-liposome interface[7]. It is well known that anesthetics depresses the phase transition temperature of phospholipid membranes[7–10] while high pressure increases it[9,10], but there have been no reports concerning the effects of high pressure on the changes in membrane surface polarity caused by anesthetics.

Ferguson reported that the biophase requires an amount of anesthetic which corresponds to a certain thermodynamic activity to cause anesthesia[11]. In view of this report, the effect of anesthetics on surface polarity would seem to be more strongly correlated with their anesthetic effect than the effect on phase transition temperature, since the former depends on activity while the latter depends of molar concentration. To confirm the correlation between the anesthetic effect and changes of the effective dielectric constant of the interfacial region, and to investigate the actions of anesthetics on a molecular level, we performed experiments at high pressure.

In order to assess differences in the effects of pressure between gel membranes and liquid-crystal membranes, three kinds of phosphatidylcholine liposomes were studied.

Experimental

The fluorescence probe 3,3'-dioctadecyl-2,2'-thiacarbocyanine (C18) was purchased from the Japan Research Institute for Photosensitizing Dye Co. Ltd. (Okayama, Japan). The *l*-α-dilauroylphosphatidylcholine (DLPC), *l*-α-dimyristoylphosphatidylcholine (DMPC) and dl-α-dipalmitoyl phosphatidylcholine (DPPC) were supplied by the Sigma Chemical Company. Halothane (2-bromo-2-chloro-1,1,1,trifluoroethane) was obtained from Takeda Chemicals (Osaka, Japan). Enflurane (2-chloro-1,1,2-trifluoroethyldifluoromethyl ether), isoflurane (1-chloro-2,2,2-trifluoroethyldifluoromethyl ether), and methoxyflurane (2,2-dichloro-1,1-difluoro-ethylmethyl ether) were purchased from Dainabot (Osaka, Japan). Sevoflurane (fluoromethyl-1,1,1,3,3,3-hexafluoro-2-propyl ether) was supplied by Maruishi Chemical (Osaka, Japan). Analytical reagent grade diethylether and chloroform were obtained from Wako Chemicals (Osaka, Japan). All reagents were used without further purification. Phospholipids and C18 were stored below 4°C as chloroform and methanol solutions, respectively.

The preparation of liposomes was performed in a similar manner to that described previously[7]. To verify the sonication, we subjected the liposome preparation to electron microscopy with 1% uranyl acetate for negative staining. The preparation was observed on a JEOL 1200EX transmission electron microscope (Tokyo, Japan) operating at 80 kV at a magnification of 40000. The diameters of the liposomes ranged from 40 to 100 nm, and were similar for DLPC, DMPC and DPPC.

Anesthetics were added as saturated aqueous solutions. The anesthetic concentration was expressed in terms of the dilution (%). The saturated molar concentrations are as follows: 512 mM diethylether; 64 mM chloroform; 17 mM halothane; 15 mM enflurane and isoflurane; 13 mM methoxyflurane; and 6 mM sevoflurane.

The absorption and fluorescence spectra were measured at 25 ± 1°C with a Hitachi U-3200 spectrophotometer and a Hitachi F-3000 fluorescence spectrophotometer, respectively.

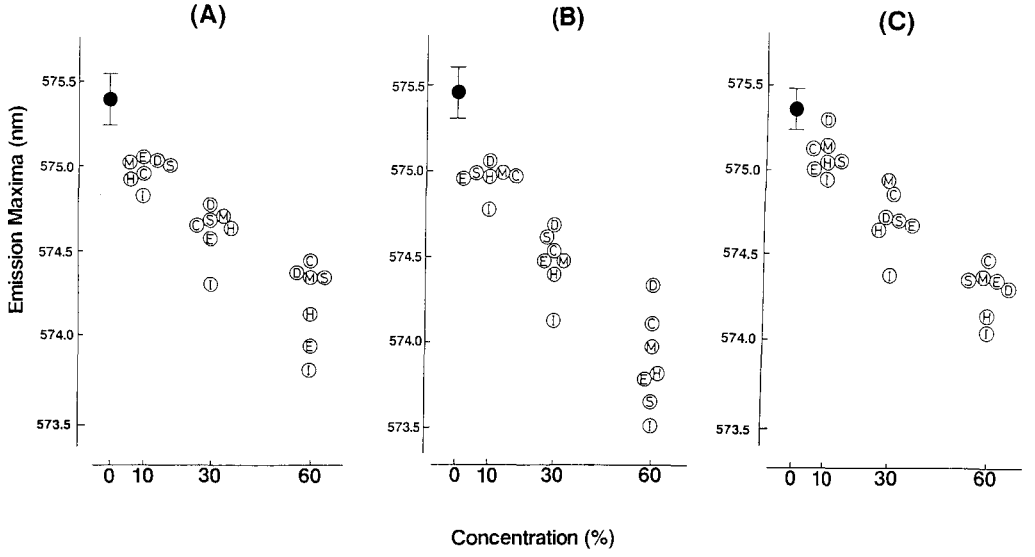

Fig. 1. Blue shift of emission maxima of C18 caused by anesthetics. [C18]=0.5 μM, [phospholipid (A): DLPC (B): DMPC (C): DPPC]=1.0 mM, 20 mM tris-HCl buffer solution (pH=7.0). Excitation 530 nm. H: halothane, E: enflurane, I: isoflurane, C: chloroform, D: diethylether, S: sevoflurane, M: methoxyflurane.

All measurements were made more than nine times and averaged. The standard deviations were less than 0.15 nm, which corresponds to the precisions of the spectrophotometers used.

For measurements at high pressure, a stainless steel high-pressure cell assembly with sapphire windows (Hikari Instrument Co.) was used. Hydrostatic pressure was applied by a hand-operated hydraulic pump.

In our previous paper[7], we reported that C18 is very sensitive to change of the dielectric constant of the surrounding environment, and can be used to evaluate the effective dielectric constant of the liposome which C18 is incorporated in. In a similar manner, the changes of the effective dielectric constants by anesthetics were estimated from the shifts of the emission maxima.

Results

The effects of anesthetics on the emission maxima of C18 in DLPC, DMPC and DPPC liposomes are shown in figure 1. All anesthetics induced blue shift in a dose-dependent manner. Taking into account the precision of the spectrophotometer used in this study ($\pm$ 0.2 nm), the blue shift caused by the various anesthetics was nearly the same.

Pressure induced red shift of the emission maxima both in the presence and in the absence of anesthetics. The emission maxima of C18 in DLPC and DPPC liposomes with and without halothane are shown in figure 2 and 3, respectively. Similar results were observed with all the anesthetics used. Data for DMPC liposomes were not obtained because of the phase transition induced by the application of pressure. As seen in figure 2 and 3, the blue shift induced by anesthetics was antagonized by pressure.

Discussion

Ferguson has proposed that the effect of drugs is related to thermo-

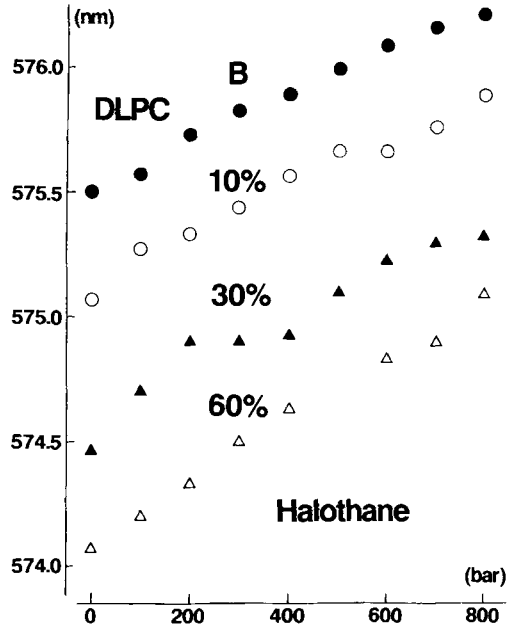

Fig. 2. Effect of pressure on the emission maxima of C18 in the DLPC-halothane system.Error bars (less than ± 0.15 nm) were omitted.

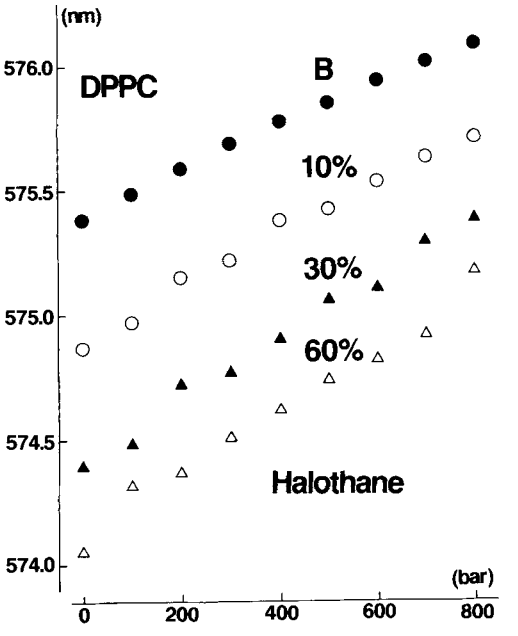

Fig. 3. Effect of pressure on the emission maxima of C18 in the DPPC-halothane system. Error bars (less than ± 0.15 nm) were omitted.

dynamic activity rather than molar concentration[11]. In the case of general anesthetics, the activity expressed by the ratio of isonarcotic pressure to the saturated vapor pressure is almost constant, despite the wide variation in isonarcotic pressures.

As the amount of liposomes used was very low compared with the amount of water (1 mM:55 M), the anesthetic concentration in the bulk water phase decreased very little after the addition of liposomes. Therefore, the dilution of each anesthetics indicates its thermodynamic activity in the solution. In figure 1, the shifts caused by anesthetics are similar at the same dilutions at which molar concentrations vary almost 100 times. Therefore, the effect seems to correlate with the physiological effect of an anesthetic.

From the relationship that the wavelength of the emission maxima of C18 decreased linearly with the increase of the dielectric constants of the solvents[7,12], the blue shift appears to indicate an increase of the effective dielectric constant of the microenvironment where the chromophore of C18 was located. The emission maximum of liposomal C18 in the absence of any anesthetic suggests that the effective dielectric constant is around 24. The chromophore of C18 is considered to be located in the interfacial region of the liposome.

DiPaolo and Sandorfy[13] have reported the hydrogen bond breaking potency of fluorocarbon anesthetics. The good correlation of this potency with their anesthetic potency has suggested that the breaking or perturbation of hydrogen bonds is an important step in the mechanism of anesthesia. Tsai et al. showed that volatile anesthetics attenuate phosphate-oriented hydrogen bonding by using Fourier transform infrared spectroscopy[14]. Yoshida et al.[15] and

Ueda et al.[16] also reported the release of bound water from the membrane interface by inhalation anesthetics. Since the dielectric constant of hydrated water is lower than that of bulk water[17], the perturbation of hydrogen bonds induces an increase of the effective dielectric constant of the liposomal surface. Thus, we attributed the blue shift of the emission maxima to an increase of polarity induced by the perturbation of hydrogen bonds.

The phase transition temperatures of DLPC, DMPC and DPPC are −2, 23, and 41°C, respectively. Considering that the measurement was performed at 25 ± 1°C, the data in fig. 1 were obtained from liquid-crystal phase and gel phase liposomes. The choline groups and the hydrated water molecules are the components which form interfaces, and are common to all the lipids used. Whether the liposomes were in the gel phase or the liquid-crystal phase, the effects of anesthetics on the emission maxima were nearly the same (fig. 1).

Mushayakarara et al.[18] reported that an increase of the external pressure decreases the O-H stretching frequency of dipalmitoyl glycerol monotonously from Fourier transform infrared measurements. This indicates that the hydrogen bond between the OH and the C=O groups of neighboring molecules is strengthened. The red shifts in figure 2 and 3 were thus attributed to the strengthened hydration which induced the decrease of the polarity.

The spectral changes at ambient and high pressure were respectively correlated with the perturbation and the promotion of hydration, and there are several other points remaining to be discussed. The degree of red shift induced by pressure was almost identical in the presence and absence of anesthetic. If C18 was moved into a more hydrophobic region (i.e., deeper inside the membrane) by pressure, an apparent decrease of the polarity would be observed. However we cannot confirm directly whether the red shift was caused by a decrease in polarity arising from the promotion of hydrogen bonding, or by a change of the location of C18.

Pressure reversal of anesthesia is a well known phenomenon observed *in vivo*. Accordingly, pressure must antagonize the effects of anesthetics in any valid model of anesthetic action. Pressure reversal *in vivo* is always observed in the range of 100–200 bars. In this study, the blue shift of the emission maxima caused by an anesthetic concentration of 10% was reversed by 200–300 bars (figs. 2, 3). Considering that the anesthetic concentration used was 2–5 times as large as the minimum alveolar concentration, the red shift was probably caused by antagonism of the anesthetic effect occurring at ambient pressure. In our previous paper, we suggested a relationship between the perturbation of hydrogen bonds at the water-liposome interface and the molecular mechanism of anesthesia. This perturbation was confirmed to be pressure reversible in the current study.

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