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Volatile anesthetics cause conformational changes of bacteriorhodopsin in purple membrane

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We examined the effects of volatile anesthetics on the structure of the bacteriorhodopsin in the purple membrane by measurements of the absorption spectrum and the visible circular dichroism (CD) spectrum and assay of the retinal composition. As the concentrations of halothane, enflurane and methoxyflurane were increased, the absorption at 560 nm decreased but that at 480 nm increased with an isosbestic point around 510 nm. These anesthetic-induced spectroscopic changes were reversible. The CD spectrum showed the biphasic pattern with a positive and a negative band. As the concentration of halothane was increased from 4 mM to 8 mM, the negative band reversibly diminished more drastically than the positive band, and at 8 mM of halothane the postive band shifted to around 480 nm. These results show that halothane disturbed the exciton coupling among bacteriorhodopsin molecules. The retinal isomer composition was analyzed using high performance liquid chromatography. The ratio of 13-*cis*- to all-*trans*-retinal was 47:53, 34:66 and 19:81 at control, 7.4 mM and 14.9 mM enflurane, respectively. After elimination of enflurane, the ratio returned to the control value. These findings indicate that volatile anesthetics directly affect a bacteriorhodopsin in the purple membrane and induce conformational changes in it.

It has been debated whether volatile anesthetics act on either lipids or proteins in the neuronal membrane. Although volatile anesthetics certainly interact with membrane lipids [1–3], much less is known about their direct interaction with membrane proteins [4,5]. It is, therefore, a requisite for understanding the molecular mechanism of anesthesia to account for the direct effect of anesthetics on membrane proteins. In the present study, bacteriorhodopsin was used as a model system to investigate how volatile anesthetics affect the structure of membrane proteins.

Bacteriorhodopsin is an integral membrane protein in the purple membrane of *Halobacterium halobium* [6]. This protein forms a two-dimensional hexagonal crystalline lattice in the purple membrane [7,8]. The protein consists of a single polypeptide chain of 248 amino acids, the sequence of which has been determined by Ovchinnikov et al. [9] and Khorana et al. [10]. They revealed that this protein contains a high proportion of hydrophobic amino acid residues. Like a rhodopsin, bacteriorhodopsin contains as its chromophore a polyene aldehyde retinal which is bound via a Schiff-base linkage to the ε-amino group of the lysine-216 residue [11–13]. The purple color of

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this protein is due to the retinal and is influenced by the protonated Schiff base and the intramolecular interactions of the retinal with the surrounding amino acid residues [14,15]. Bacteriorhodopsin contains all-*trans*- and 13-*cis*-retinals in a 1 : 1 ratio in the dark at neutral pH [16,17]. The 13-*cis*-retinal can be rapidly converted to the all-*trans* form by illumination [16,17]. There is a close correlation between the conformation of bacteriorhodopsin and the configuration of the retinal [18]. The conformation change of bacteriorhodopsin readily affects the retinal-protein interaction, resulting in changes in the purples color and the retinal composition [18–20].

Purple membrane was isolated from *Halobacterium halobium* R1M1 according to the method of Oestherhelt and Stoeckenius [21]. Dark-adapted purple membrane was dispersed in 25 mM Tris buffer (pH 7.0). The employed volatile anesthetics were 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane), 2-chloro-1-difluoromethoxy-1,1,2-trifluoroethane (enflurane) and 2,2-dichloro-1,1-difluoro-1-methoxyethane (methoxyflurane) and those were added in the liquid form to the purple membrane suspension with a microsyringe. The suspensions were continuously stirred for 60 min for anesthetic-equilibrium before performing measurements. The anesthetic concentrations in the purple membrane suspensions were measured with a Shimadzu GC-4CM gas chromatograph equipped with a Shimadzu BGS-1-A gas sampler. The absorption spectra were determined with a Union SM-401 spectrophotometer (scanning rate 10 nm/s). The CD spectra were measured with a Jasco J500A spectropolarimeter. A 1 cm pathlength stoppered cell was used for these measurements. The temperature of the cell-holder was controlled by circulating water maintained at a temperature of 25°C. The retinal isomer composition was analyzed using a high performance liquid chromatography (HPLC) system (Yanaco L2000) with a silica gel column (Du Pont Zorbax Sil) after extraction of retinal from the purple membrane by the method of Groenendijk et al. [22,23]. The elution solvent was a mixture of hexane, diethyl ether (15%) and ethyl alcohol (0.1%), and the flow rate was 0.75 ml/min. The relative content of all-*trans*- and 13-*cis*-retinaloxime was calculated by integrating the 360 nm absorption peak.

Fig. 1 depicts the absorption spectra of purple membrane suspensions in the presence and absence of methoxyflurane. As the anesthetic concentration was increased, the absorption at 560 nm decreased, but that at 480 nm increased, with an isosbestic point around 510 nm. The methoxyflurane-induced spectroscopic changes were reversible. Enflurance and halothane showed the similar spectroscopic changes as methoxyflurane. In the presence of 16 mM halothane, however, the absorption bands at 560 nm and 480 nm and isosbestic point near 510 nm disappeared, and another peak appeared at around 380 nm (see dotted line). This spectroscopic change was irreversible. A free all-*trans*-retinal is demonstrated to have an absorption maximum at 380 nm. When it binds to a protein via a protonated Schiff base, the absorption maximum shifts to 440 nm [14,15]. The further red shift to 560 nm shown by bacteriorhodopsin indicates the existence of another specific intramolecular interaction between a retinal and a bacterioopsin [14,15]. Druckmann et al. [24] showed that at high pH, bacteriorhodopsin underwent a reversible transformation to a species absorbance at 460 nm, in which the Schiff base was deprotonated. So a species absorbing at 480 nm induced by volatile anesthetics also may be characterized by a deprotonated Schiff-base chromophore.

The present spectroscopic data indicate that volatile anesthetics change the structure of bacteriorhodopsin protein and affect the interaction between a retinal and bacterioopsin. Even though purple membrane suspensions were saturated with ether type anesthetics, i.e., enflurane, methoxyflurane and diethyl ether, the absorption around 380 nm did not appear and the spectral changes were reversible. In contradiction to ether-type anesthetics, the absorption peak at 380 nm appeared at extremely high concentrations of hydrocarbon-type anesthetics, i.e., halothane and chloroform. This reaction was not reversible. The absorption shift to 380 nm indicates a loosening of protein structure caused by hydrocarbon-type anesthetics, which would presumably lead to a hydrolysis of the Schiff base. Fig. 2 shows the visible CD spectra of purple membrane with and without halothane. The spectra of purple membrane was bilobed through asymmetric, consisting

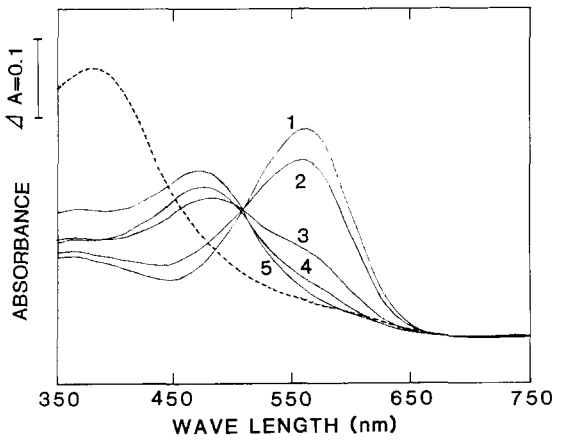

Fig. 1. The absorption spectra of the bacteriorhodopsin in the purple membrane in the absence and presence of methoxyflurane: control (1), 3 mM (2), 6 mM (3), 9 mM (4) and 14 mM (5). The dotted line shows the absorption spectrum in the presence of 16 mM halothane.

of a positive band around 530 nm and a negative band around 605 nm, as reported by others [25–28]. As the concentration of halothane was increased from 4 mM to 8 mM, the negative band diminished more drastically than the positive band, and at 8 mM halothane the positive band shifted to around 480 nm. At 16 mM halothane, the visible CD spectrum disappeared. In the range of halothane concentration from 4 mM to 8 mM, the changes in the CD spectra were reversible. The

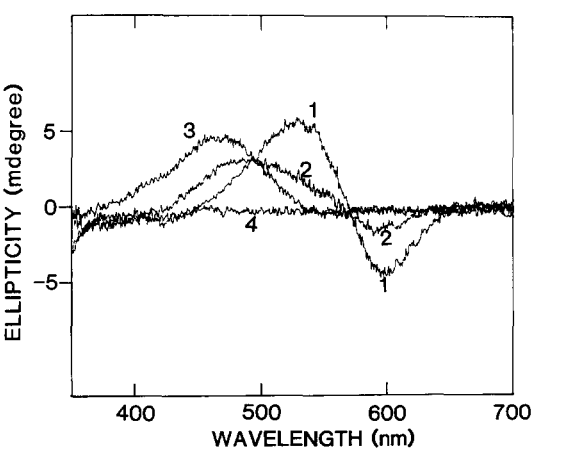

Fig. 2. The visible CD spectra of the bacteriorhodopsin in the purple membrane. The ellipticity of 15.9 μM bacteriorhodopsin is given with and without halothane: control (1), 4 mM (2), 8 mM (3) and 16 mM (4).

TABLE I

THE RETINAL ISOMER COMPOSITION OF THE BACTERIORHODOPSIN AT VARIOUS CONCENTRATION OF ENFLURANE IN THE DARK

Enflurane concentration (mM)	%composition	
	13-*cis*-retinal	all-*trans*-retinal
0	47	53
7.4	34	66
14.9	19	81

CD studies revealed that the negative CD band of the purple membrane indicates the existence of excition coupling among the bacteriorhodopsin molecules [25–28]. Disappearance of the negative band may indicate anesthetic-induced breaking of an excition coupling among bacteriorhodopsin molecules. Disappearance of the visible CD spectrum may indicate a loosening of Schiff-base binding between a retinal and protein moeity. The CD spectral change induced by volatile anesthetics suggests that volatile anesthetics affect the structure of bacteriorhodopsin and disturb the coupling among protein molecules. Table I depicts the concomitant chromophore analysis of dark-adapted bacteriorhodopsin at 7.4 mM and 14.9 mM of enflurane. The ratio of 13-*cis*- to all-*trans*-retinal was 34 : 66 and 19 : 81 at 7.4 mM of enflurane, respectively. After elimination of enflurance, the ratio returned to the control value, i.e. about 47 : 53. Isomerization of the from 13-*cis*- to all-*trans*-retinal, induced by enflurane, indicates the occurrence of a conformational change in bacteriorhodopsin. This result agrees with those of the present spectrophotometric and CD studies.

The present study clearly shows that volatile anesthetics affect the membrane protein and induce conformational changes in it. A few studies on the interaction of volatile anesthetics with water soluble proteins have been reported. Laasberg and Hedley-Whyte [29] demonstrated by optical rotatory dispersion measurement that halothane decreased reversibly the helicity of the beta-chain of human hemoglobin. We [30] have shown that diethyl ether increases the partial molal volume of bovine serum albumin. But there has been no report of direct interaction between volatile

anesthetics and the membrane protein. We [4] also have shown that volatile anesthetics affect the rhodopsin in the bovine rod outer segment (ROS) disk membrane and depress the light-induced proton uptake by rhodopsin. We speculated that volatile anesthetics might directly induce the conformational change in rhodopsin and depress its function. However, an effect of volatile anesthetics on the rhodopsin in the ROS membrane was observed only functionally in the previous study. The ROS disk membrane is too rich in lipids to confirm whether volatile anesthetics affect directly or indirectly the membrane protein. In the present study, the purple membrane was used because bacteriorhodopsin forms a two-dimensional hexagonal crystalline lattice in the membrane, such that it is hardly influenced by the properties of the surrounding lipids. In the dark-adapted purple membrane solubilized in detergent L-1690, the ratio of all-*trans*- to 13-*cis*-retinal was almost the same as that of native bacteriorhodopsin [18]. This indicates that the configuration of chromophore is hardly affected by the surrounding lipids. Therefore, anesthetic-induced isomerization of 13-*cis*- to all-*trans*-retinal indicates that volatile anesthetics directly affect bacteriorhodopsin and cause a conformation change in the protein near the chromophore.

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