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Effects of volatile anesthetics on bacteriorhodopsin in purple membrane, *Halobacterium halobium* cells and reconstituted vesicles

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Abstract

In this study, we have investigated effects of volatile anesthetics on absorption spectra, proton pumping activity and decay of photointermediate M of bacteriorhodopsin (bR) in differently aggregated states. Anesthetics used in this study are ether-type general anesthetics; enflurane and sevoflurane. The observed effects on bR depend not only on variety or concentration of anesthetics but also strongly on the aggregation state of bR molecules in the membrane. In purple membrane (PM), bR having maximum light absorption at 567 nm (bR_{567}) is formed in the presence of sevoflurane or a small amount of enflurane, while a species absorbing maximally at 480 nm (bR_{480}) is formed upon the addition of large amounts of enflurane. X-ray diffraction studies show that the former species maintains crystallinity of PM, but the latter does not. In reconstituted vesicles where bR molecules exist as monomer, even sevoflurane forms bR_{480}. Flash photolysis experiments show that bR_{567} contains a shorter-lived M intermediate absorbing maximally at 412 nm in the photoreaction cycle than bR does and that bR_{480} contains at least two long-lived M intermediates which seem to absorb maximally near and at lower than 380 nm. The measurements of light-induced pH changes of the whole cells and of the reconstituted vesicles in the presence of the anesthetics indicate that bR_{567} has a enhanced proton pumping efficiency, while bR_{480} has a quite low or no activity. No significant difference was observed in the anesthetic action between two inversely pumping vesicles. These observations suggest that on the formation of bR_{480}, anesthetics enter into the membrane and affect the protein–lipid interaction.

Keywords: Bacteriorhodopsin; Purple membrane; Anesthetic–membrane interaction; Flash photolysis; Proton pump; *Halobacterium halobium*

1. Introduction

General anesthetic affects functions of the cell via cell membrane. Various investigations have been done to elucidate the mechanism of the anesthetic action [1–7]. However, it is for instance still obscure whether the anesthetics act on membrane proteins directly or via surrounding lipids and whether there are binding sites for anesthetics on the protein molecules or not.

In this study, we have examined the effects of volatile anesthetics on properties and functions of

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bacteriorhodopsin (bR). Bacteriorhodopsin is the only protein contained in the purple membrane (PM) of *Halobacterium halobium* [8]. It has the following properties: (1) bR trimers together with lipids form a well-ordered two-dimensional hexagonal array in PM [9]; (2) bR undergoes a photochemical reaction cycle through several photointermediates (K, L, M, O, etc.) each of which has a characteristic absorption spectrum [10]; and (3) during the photocycle, bR functions as a light-driven proton pump from the inside to the outside of the cell through the membrane [11]. Therefore, PM is an appropriate model system for investigating the effects of anesthetics on structures and functions of cell membranes.

In a previous paper [6], Nishimura et al. reported that PM in saturated solution with certain kinds of volatile anesthetics showed (1) a blue shift in absorption maximum of bR from 560 nm to 480 nm, (2) disappearance of the biphasic band in the visible part of the CD spectrum, and (3) alteration in the ratio of the retinal isomers in the dark-adapted state from all-*trans*:13-*cis* ~ 1:1 to 4:1. Their observations indicate that anesthetics induce conformational changes of bR in the vicinity of retinal to affect the apo-protein-chromophore interaction and disturb the crystalline array in PM. Also, Henry et al. [7] reported by the examination on the photocycle of bR absorbing maximally at 480 nm that the M intermediate showed the maximum absorption at 380 nm instead of that at 412 nm (M_{412}) which was observed in intact PM, and that one-third of M-intermediates directly returned to the initial state but the rest did via O-intermediate.

We have studied the effects of volatile anesthetics, enflurane and sevoflurane on bR by measuring absorption spectra, proton pumping activity and decay of M-intermediate, both in crystalline array (PM, whole cells and DMPC vesicles containing bR) and in monomeric state (three kinds of reconstituted vesicles). When anesthetics act on bR in crystalline array at low concentrations, the absorption maximum of bR shifted slightly to 567 nm, the decay of M_{412} became fast and the proton pumping efficiency of bR was enhanced. On the other hand, if anesthetics were added to bR in its crystalline array state in higher concentrations or to bR in its monomeric state, the absorption maximum of bR shifted drastically to 480 nm. In addition slowly decaying M-intermediates ($15 < \tau < 120$ ms) absorbing maximally near or at lower than 380 nm occurred in the photocycle and the proton pumping activity reduced. X-ray diffraction from PM pellets showed the disappearance of the crystal state in the presence of an excess amount of enflurane, suggesting that the anesthetics entered into the membrane.

2. Materials and methods

2.1 Sample preparation

Growth of *H. halobium* S9 and isolation of PM were done by standard procedures [12]. PM was suspended in 150 m*M* KCl, 10 m*M* Bis–Tris buffer (pH 6.0) The whole cells were washed twice and resuspended in basal salt solution (BSS; 250 g NaCl, 2 g KCl, 20 g $MgSO_4 \cdot 7H_2O$, and 3 g Na-citrate per liter). The chloride pumping activity of halorhodopsin in the cell was inhibited by preincubation at 65°C for 5 min (the heat treatment) [13]. The proton influx by ATPase in the cell was inhibited by preincubation with 1 m*M* *N,N'*-dicyclohexylcarbodiimide (DCCD) dissolved in ethanol (0.1% (v/v) final concentration) for at least 5 h [14].

Four kinds of reconstituted bR vesicles were prepared by the following procedures: (1) the octylglucoside dilution procedure as described by Racker et al. [15] was used for the mixture of liposome of soybean phospholipid (SBPL) and bR solubilized with 2.5% octylglucoside (weight ratio of 60:1 lipid/bR). The liposomes were prepared by sonication in a water/ice bath using ultrasonic disrupter UD200 (TOMY) equipped with a microtip. (2) The freeze–thaw sonication procedure as described by Casadio and Stoeckenius [16] was used for liposome of dimyristoylphosphatidylcholine (DMPC) with PM (weight ratio of 5:1 lipid/bR). (3) The vesicles containing bR in right-side-out orientation (right-side-out vesicles) were prepared by sonication of liposome of SBPL with dicetylphosphate and blue membrane (weight ratio of SBPL : dicetylphosphate : bR = 100:2:1)

in deionized water at pH 3.0 as described by Ihara and Mukohata [17]. The blue membrane was prepared by passing PM through a column of cation exchanger AG50X2 (BioRad). (4) The vesicles containing bR in inside-out orientation which consist of the same composition as right-side-out bR-SBPL vesicle (inside-out vesicle) were prepared by sonication of the mixture of liposome and PM in 50 m*M* KCl at pH 7.0. The obtained vesicle suspension was centrifugated (200,000*g*, 12 h) on sucrose density gradient (0–40% w/v) and broad single purple band was collected. This treatment reduced the light scattering by vesicle suspension in absorbance measurement. The obtained vesicles in procedures (1)–(4) described above were suspended in 150 m*M* KCl (pH 6.0).

2.2 Measurement of proton pumping efficiency of bR

Volatile anesthetics used through this study were ether type anesthetics: enflurane and sevoflurane. Whole cells or reconstituted bR vesicles were put into a quartz 1-cm pathlength spectrophotometer cell. The anesthetics were added in liquid form to the sample the suspension with microsyringes. A pH electrode (Horiba 6028) was inserted into the sample cell. The gap between the electrode and sample cell was sealed with utility wax (G-C Dental Industrial). The mixture was stirred continuously with a magnetic stirrer. After equilibrium was reached (3–8 h for the whole cells, 1 h for the reconstituted bR vesicles), light-induced pH change was measured by a pH meter (Horiba F-7) for 10–30 min. The actinic light was provided by a 500 W tungsten lamp through a 500 nm high-pass yellow glass filter (Toshiba Y-50) and 10 mm path of 10% (w/v) $CuSO_4$ solution. The sample temperature was kept at 20 °C during the experiment by circulating water through the brass sample cell holder.

2.3 Absorption spectrum and flash photolysis experiments

Absorption spectrum was measured by spectrophotometer UV-200S (Shimadzu). Flash photolysis experiments were performed by the apparatus previously described [18]. The samples were put into the quartz spectrophotometer cell with quartz stopper. In the case of the reconstituted bR vesicle, an electrode was inserted into the sample cell and used as such for the measurement of the proton pumping efficiency following the experiment of absorption. Before measurement, each sample was light-adapted by the light of a 100 W halogen lamp passing a 500 nm high-pass yellow glass filter and 10 mm path of 10% $CuSO_4$ solution. The sample temperature was kept at 20 °C. The actinic light was provided from a strobo flash unit Auto zoom 4800 (Sunpak) through a 500 nm high-pass filter and blue glass filter (Toshiba IRA-05). The intensity change of transmitted light through the sample after flash was detected by a photo-multiplier tube R106 (Hamamatsu photonics) and stored in a digital oscilloscope (Nicolet model 3091), then transferred to a personal computer (NEC PC-98XA).

Five sets of data were summed, transformed and smoothed to yield the absorbance change. The transient absorbance change after a period of 1 ms following a flash was fitted by a polynome of exponential functions. In order to equally weight each component, the data were sampled at points of equal periods on a logarithmic time scale as described by Nagle [19]. The fit was obtained using SALS, a non-linear least-squares fitting program system (copyrighted by SALS group) [20], with a supercomputer (ACOS system 2000, the Computation Center of Osaka University).

2.4 X-ray diffraction experiments

PM pellets in distilled water were sealed in a thin-wall glass capillary (1 mm diameter) together with an excess amount of volatile anesthetics in the liquid form and incubated more than 20 h for saturating with anesthetics before X-ray diffraction measurements. A fine-focus rotating anode X-ray generator, RU-100 (Rigaku Denki), was operated at 40 kV and 25 mA. Copper K_α radiation ($\lambda = 1.542$ Å) was monochromatized and focused at the detector plane by a point-focusing optical system which consists of a bent Ge monochromator and Ni-coated bent glass mirror. The diffraction patterns were recorded on Sakura cosmic ray film. Sample-to-detector distance was 110 mm.

The experiments were done at room temperature (~ 23°C).

2.5 Chemicals

Enflurane (2-chloro-1,1,2-trifluoroethyl difluoromethyl ether) was obtained from Abbott Laboratories. Sevoflurane (fluoromethyl-1,1,1,3,3,3-hexafluoro-2-propyl ether) was purchased from Maruishi Chemicals. DCCD, SBPL (type IV-S), DMPC and dicetylphosphate were obtained from Sigma Chemicals.

3. Results

3.1 Absorption spectra

3.1.1 Purple membrane

Figure 1 shows the changes in the absorption spectra of PM suspension after the addition of enflurane or sevoflurane. At the saturated concentration of sevoflurane, the absorption maximum of light-adapted bR (bR_{570}) shifted from 569 nm to 567 nm with a 3% decrease of extinction coefficient (Fig. 1b). We call this new species bR_{567}.

In the presence of enflurane concentrations up to ~ 6 m*M*, changes in the absorption spectrum were similar to those observed in the case of sevoflurane. In accordance with previous reports by Nishimura et al. [6] and Henry et al. [7], upon the addition of enflurane in concentrations larger than ~ 6 mM, the absorption at 567 nm decreased and a new species absorbing maximally at about 480 nm having a 40% smaller extinction coefficient appeared with an isosbestic point in the 505–510 nm range (Fig. 1a). We call this last species bR_{480}. These observations indicate that enflurane acts on PM in two different manners depending on the concentration and that at low concentrations the effects of enflurane on the interaction between retinal and the apo-protein (bacterioopsin) are similar to those of sevoflurane.

3.1.2 Reconstituted bR vesicles

The absorption spectra of inside–out bR–SBPL vesicles produced by sonication were measured in the presence of volatile anesthetics. Results are shown in Fig. 2. Since vesicles were reconstituted from the mixture of high lipid-to-bR ratio (initial weight ratio 100 : 1), most of bR should exist as monomers [21]. In the vesicle, the absorption maximum of light-adapted bR was at 560 nm. In the presence of enflurane in concentrations up to ~ 10 m*M*, the absorption around 480 nm increased with the isosbestic point being in the 505–510 nm range (Fig. 2a). Further addition of enflurane caused a small blue shift (~ 10 nm) of the absorption maximum from 480 nm with no apparent isosbestic point.

In contrast to PM suspension, sevoflurane reduced the absorption of the vesicle suspension at

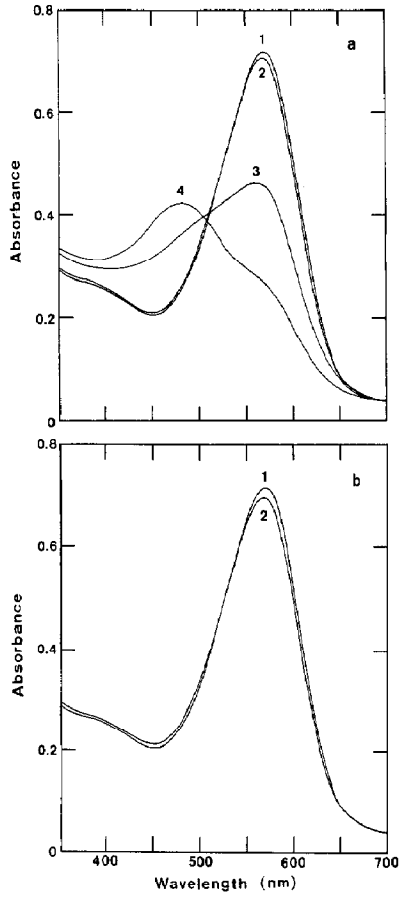

Fig. 1. Absorption spectra of PM in the absence and presence of enflurane (a) or sevoflurane (b). (a): 0 m*M* (1), 5.9 m*M* (2), 13.2 m*M* (3), and 20.6 m*M* (4). (b): 0 m*M* (1), and 10.8 m*M* (2). Sample: 150 m*M* KCl, 10 m*M* Bis–Tris buffer (pH 6.0), at 20 °C.

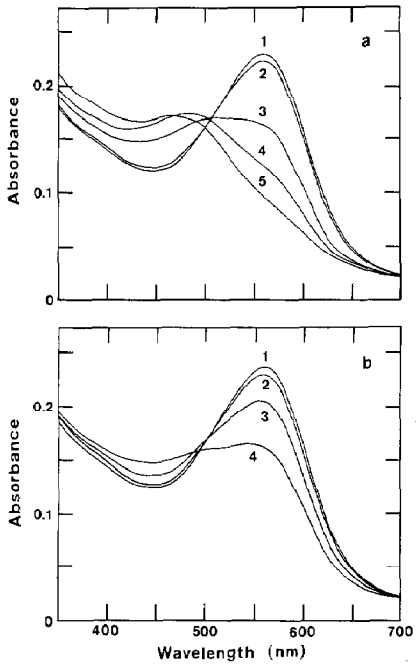

Fig. 2. Absorption spectra of the reconstituted inside-out bR-SBPL vesicle prepared by sonication in the absence and presence of enflurane (a) or sevoflurane (b). (a): 0 m*M* (1), 0.7 m*M* (2), 6.2 m*M* (3), 10.1 m*M* (4), and 15.8 m*M* (5). (b): 0 m*M* (1), 0.7 m*M* (2), 4.1 m*M* (3), and 10.8 m*M* (4). Sample: 150 m*M* KCl, pH 6.0, at 20 °C.

560 nm by ~50% of the decrease observed in saturated enflurane solutions (Fig. 2b). This observation indicates that sevoflurane is also able to interact with bR to yield bR_{480} in the reconstituted vesicle but with crysallinity lacking.

The isosbestic point between 505–510 nm seen at low concentrations (< 4 m*M*) of sevoflurane also disappeared at higher concentrations. Nishimura et al. [6] reported that the addition of large quantities of halothane induced a further blue shift in the absorption maximum of PM, from 480 nm to 380 nm, and a loss of the isosbestic point near 510 nm. This might suggest that only part of bR was transformed into 380 nm species in the reconstituted vesicle at high concentrations of enflurane or sevoflurane.

3.2 Proton pumping efficiency

The effects of volatile anesthetics on the proton pumping efficiency of bR were examined for the whole cell and the four kinds of reconstituted vesicles described in Section 2.1.

3.2.1 Whole cell

Figure 3 shows the dependence of the light-induced pH changes on the concentration of anesthetics in whole cell suspensions. The halorhodopsin was inactivated by the heat treatment [13]. Due to the high salt concentration and thereby high osmotic pressure of the BSS, the water/gas partition coefficient of both anesthetics remarkably decreased [22]. Consequently, the cell suspension was already saturated at ~2 m*M* for the case of enflurane and ~1 m*M* for the case of sevoflurane which was about ten times lower than that for PM or reconstituted bR vesicle suspension containing 150 m*M* KCl. With the saturated solutions of anesthetics, the light-induced pH changes increased by a factor of about 7 to 8 for enflurane and of about 6 for sevoflurane, respectively. These observations indicate that the proton pumping efficiency of bR is substantially enhanced by the action of anesthetics in whole cells containing bR arranged in a crystalline order. However, the increase in pH change was also observed to be a factor of 4 to 5 without anesthetics when treating the cells with 1 m*M* DCCD in order to inhibit the activity of ATPase [14]. Upon the addition of anesthetics until saturation, the change in solution pH increased further by a factor of 2 to 3. DCCD inhibits the proton influx both through ATPase

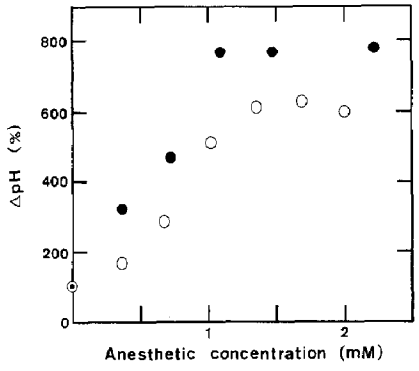

Fig. 3. Light-induced pH changes of the whole cell suspension after the heat treatment as the concentration of enflurane (●) or sevoflurane (○). ΔpH of control sample, 0.07. Sample: BSS, initial pH 5.9–6.0, at 20 °C.

and halorhodopsin [23], while the heat treatment does only the latter. Hence, the experiments with heat-treated cells suggest that anesthetics inhibit the ATPase activity in addition to the enhancement of bR activity.

3.2.2 Reconstituted bR-SBPL vesicle

The effects of volatile anesthetics on the light-induced pH change were examined for three kinds of reconstituted bR-SBPL vesicles. In these vesicles, bR should not be in the aggregated state owing to the high lipid/bR ratio. Results are shown in Fig. 4. Since bR is the only protein contained in the reconstituted vesicles, the observed pH changes should directly reflect the alteration in the proton pumping efficiency of bR. In contrast to the whole cell, the activity of bR in these vesicles reduced with increasing the concentration of anesthetics. Also, the reduction was similarly observed for both inside-out vesicles and right-side-out vesicles. This observation indicates that there is no specific mode in the action of anesthetics, depending on the orientation of monomeric bR molecule in the vesicles.

3.2.3 Reconstituted bR-DMPC vesicle

Cherry et al. [24] showed by X-ray diffraction that bR molecules aggregated each other to make crystalline array in bR-DMPC vesicle of low lipid/bR ratio below the phase transition temperature of DMPC ($\sim 23°C$). In bR-DMPC vesicle at 6°C, the proton pumping efficiency of bR was enhanced, in contrast to the other vesicles, maximally by a factor of ~ 1.5 upon the addition of sevoflurane up to ~ 3 mM, while the further addition of sevoflurane decreased the activity gradually (Fig. 4b). It indicates that sevoflurane enhances the activity of bR in crystalline array which seems to be maintained at low concentration of anesthetics.

3.3 Flash photolysis experiments

Effects of the volatile anesthetics on the decay of photointermediate M were examined by flash photolysis. Henry et al. [7] reported that the species absorbing at 480 nm (bR_{480}) induced by the saturated enflurane passed through the M intermediate of the photocycle absorbing maximally at 380 nm, that we call M_{380}. Therefore, we have examined the decay of M by measuring the changes in the absorptions at 370 nm and 420 nm after the flash.

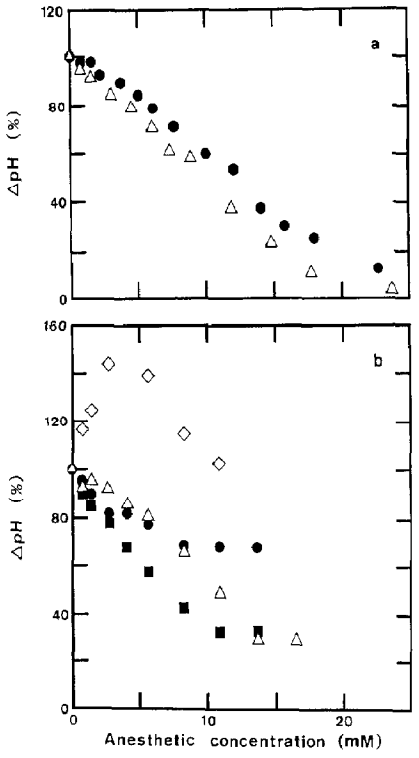

Fig. 4. Light-induced pH changes of the reconstituted bR vesicle suspensions as the concentration of enflurane (a) or sevoflurane (b). Symbols: bR-SBPL vesicle prepared by octylglucoside dilution (△), inside-out bR-SBPL vesicle prepared by sonication (●), right-side-out bR-SBPL vesicle prepared by sonication (■), bR-DMPC vesicle (◇). ΔpH of control sample, 0.1–0.2. Samples except for bR-DMPC vesicle: 150 mM KCl, initial pH about 6.0, at 20°C. bR-DMPC vesicle: 150 mM KCl, initial pH about 5.8 at 6°C.

3.3.1 Purple membrane (PM)

Figure 5 shows the time course of the absorbance change of PM after actinic flash in the absence and presence of anesthetics. Data of the absorbance change were analyzed by fitting with a sum of exponentials in a least-squares method as described in Section 2. The estimated half time of M decay component and population of M_{412} were plotted against the concentration of anesthetics in

Fig. 6. In the presence of sevoflurane, the decay of M_{412} became fast with increasing the concentration of anesthetics. Upon the addition of enflurane up to ~ 6 m*M*, the transition of bR_{570} to bR_{480} did not occur (Fig. 1a) and the decay of M_{412} became fast like sevoflurane. In contrast, at high concentration of enflurane, the absorbance change at 370 nm showed that additional two long-lived decay components ($\tau \sim 18$ ms and $\tau \sim 100$ ms) appeared according to the transition to bR_{480}. The occurrence of the slowest decay component ($\tau \sim 100$ ms) could be assured by the absorbance change at 420 nm which had negative values. It agrees with the result obtained by Henry et al. [7] based on the difference spectrum between bR_{480} and M_{380}. Therefore, if can be presumed that the slowest decay component corresponds to M_{380} which has an absorption maximum near 380 nm. The decay component with $\tau \sim 18$ ms is not apparent in the absorbance change at 420 nm. This can be explained by the assumption that this component has an absorption maximum at lower than 380 nm and therefore, the flash-induced absorbance change is significantly observed at 370 nm but not at 420 nm.

3.3.2 Reconstituted bR-SBPL vesicle

Figure 7 shows the transient absorbance change of inside-out bR-SBPL vesicle prepared by sonication in the presence of enflurane. In this reconstituted vesicle, slowly decaying components were already observed even in the absence of anesthetics. At low concentration of enflurane, in contrast to PM, the life time of the fast decay component M_{412} seems to be almost unchanged. At high concentration of enflurane, the absorbance showed

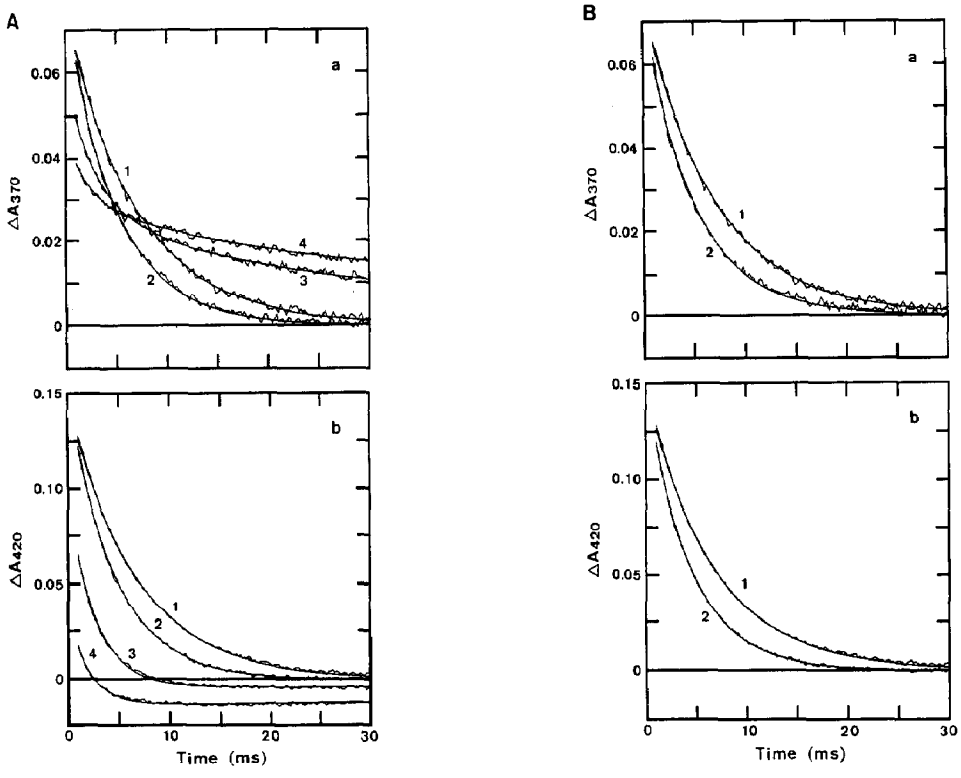

Fig. 5. Time course of the observed and multiexponentials-fitted absorbance changes of PM at 370 nm (a) and 420 nm (b) after the flash in the absence and presence of enflurane (A) or sevoflurane (B). (A): 0 m*M* (1), 5.9 m*M* (2), 13.2 m*M* (3), 20.6 m*M* (4). (B): 0 m*M* (1), 10.8 m*M* (2). The fittings were done to the observed data after a period of 1 ms following the flash. Samples were the same as those in Fig. 1.

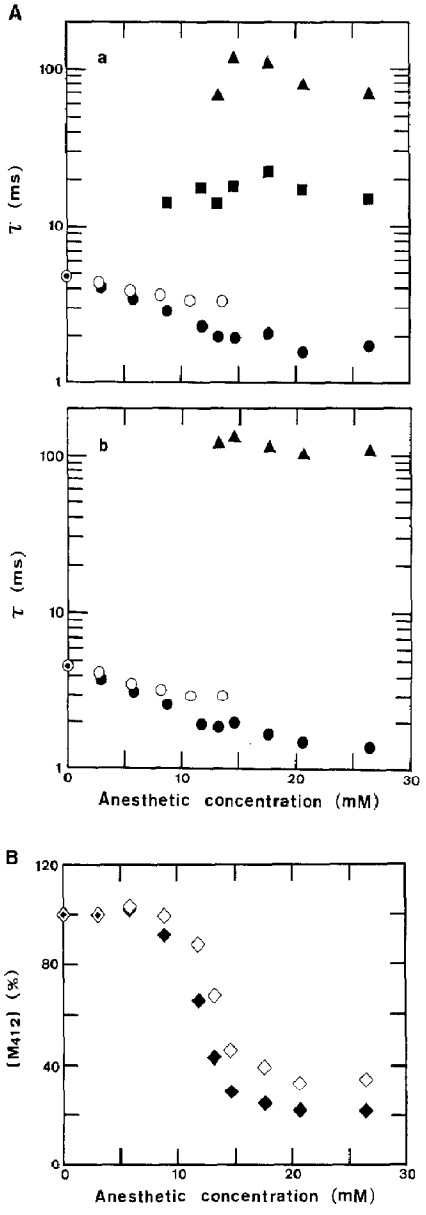

Fig. 6. Plots of half time (τ) of the M decay components (A) and population of the fastest decay component M_{412} ($[M_{412}]$) (B) calculated from the multiexponentials fit to the data in Fig. 5, against the concentration of anesthetics. (A): enflurane (●, ■, ▲) and sevoflurane (○) at 370 nm (a) and 420 nm (b). (B): enflurane at 370 nm (◆) and 420 (◇). $[M_{412}]$ was the value obtained by extrapolation to 0 ms and normalized by that without anesthetics.

positive change at 370 nm and negative one at 420 nm, indicating the occurrence of M_{380} with long lifetime. The analysis of the data will be presented in more detail later.

3.4 X-ray diffraction patterns

Figure 8 shows X-ray diffraction photographs from the pellets of PM saturated with anesthetics. The native PM pellet gives rise to Bragg reflection rings in diffraction patterns from two-dimensional hexagonal arrays of bR and lipids. At the saturated concentration of enflurane, the color of PM pellets turned to red, indicating the transition of bR_{570} to bR_{480}. The X-ray diffraction from this sample (Fig. 8A) shows that the Bragg reflection rings disappear, corresponding to the loss of crystalline array. However, there are diffuse peaks in Fig. 8A,

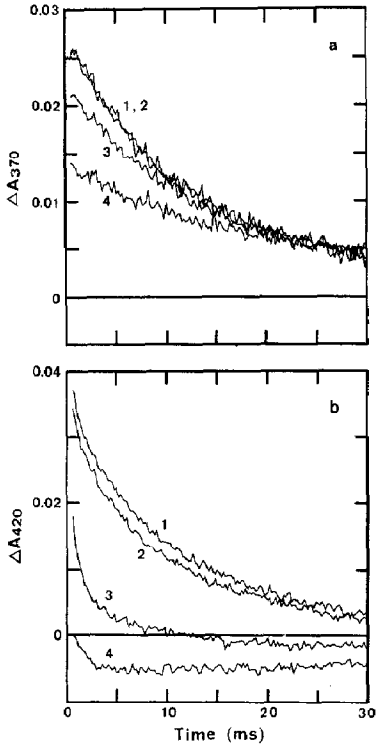

Fig. 7. Time course of absorbance changes of inside-out bR-SBPL vesicle prepared by sonication at 370 nm (a) and 420 nm (b) after the flash in the absence and presence of enflurane. Curves: 0 mM (1), 0.7 mM (2), 6.2 mM (3), 15.8 mM (4). Samples were the same as those in Fig. 2a and Fig. 4a (●).

which suggests that the trimeric structure of bR molecules remains as discussed about diffraction pattern from PM above the phase transition temperature by Hiraki et al. [25]. These changes in diffraction were reversible. On the other hand, the diffraction pattern from PM saturated with sevoflurane (Fig. 8B) indicates that the crystallinity of PM is perfectly retained. These results suggest that in PM saturated with the anesthetics, enflurane molecules enter into the membrane and alter the environment of bR significantly, while sevoflurane molecules are not able to enter into the membrane.

4. Discussion

In the present study, we have investigated the effects of volatile anesthetics on bR in whole cell, PM and reconstituted vesicles. As already known, addition of volatile anesthetics induces changes in the absorption spectra of bR [6,7]. The absorption maximum of bR shifts to 567 nm or 480 nm in the presence of enflurane or sevoflurane. The 567 nm pigment (bR_{567}) appears upon incorporation of sevoflurane or small amounts of enflurane into the PM suspension. The increase in the concentration of enflurane results in the formation of a new species absorbing maximally at 480 nm (bR_{480}). The 480 nm pigment is also observed upon addition of sevoflurane or enflurane to the reconstituted vesicles, irrespective of the concentration. The difference in the conditions for forming new species relates to the aggregation state of bR. X-ray diffraction shows that crystallinity of the PM is maintained in the presence of the saturated sevoflurane. However, on the other hand, the sharp reflection rings disappear in the X-ray pattern of PM containing bR_{480} under the saturated enflurane. Then, the visible CD spectrum lost a biphasic band due to the exciton-coupling among bR trimers as shown by Nishimura et al. [6]. These results may suggest that the anesthetics interact with bR on its hydrophilic surface without disruption of crystalline structure and yield bR_{567}. But, in the condition of forming bR_{480}, the anesthetics may enter into the PM and disorder the bR arrangement. As bR does not form a crystalline packing in the reconstituted bR-SBPL vesicle, the anesthetics could be easily solubilized into the membrane. Therefore, bR_{480} seems to occur when the anesthetics enter into the membrane and affect the lipid–protein interaction. The difference in the behavior between enflurane and sevoflurane could be attributed to their solubilities into the water and lipid, i.e. the water/gas partition coefficient

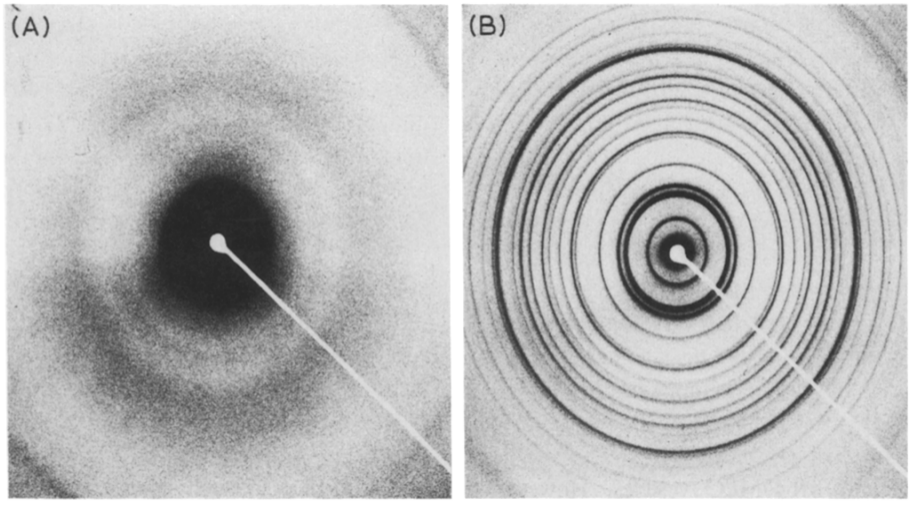

Fig. 8. X-ray diffraction photographs from PM pellet saturated with enflurane (A) or sevoflurane (B). Collection time: 21 h. Temperature: about 23°C.

of enflurane is twice that of sevoflurane and the lipid/gas partition coefficient of enflurane is 3 times greater than that of sevoflurane.

The effect of the anesthetics on proton pumping depended also on the aggregation state of bR. While the anesthetics increased greatly the efficiency of proton translocation in whole cells, it decreased gradually with increasing concentration in reconstituted bR-SBPL vesicles. In the latter, bR_{480} has appeared as shown in Fig. 2. The above difference does not relate to the orientation of bR within membranes, because both right-side-out and inside-out vesicles show the similar decrease in proton pumping efficiency. These results suggest that the effect of anesthetics on the efficiency of proton pumping relates to the aggregation state of bR. In whole cells, bR is arranged in crystalline structure and bR_{567} induced by the anesthetics may enhance proton pumping. However, bR_{480} appears and the efficiency of proton transport decreases in bR-SBPL vesicles. To confirm this point, we have investigated the effect of anesthetics on the bR-DMPC vesicle at 6 °C. In which bR are thought to form the crystalline domains [24]. Indeed the efficiency of proton pumping increased upon the addition of sevoflurane, though it tended to decrease at the high concentration. Though the absorption spectrum could not accurately be obtained due to the large light scattering, the appearance of bR_{567} which has truly an absorption maximum at 560 nm in reconstituted vesicles and the shift to the 480 nm form were discerned as the concentration of sevoflurane increased. The different behavior observed between bR-DMPC vesicles and whole cells may be attributed to the differences in lipid compositions and the packing mode of bR and lipid.

Next, we have investigated the effect of anesthetics on the lifetime of M intermediate to elucidate the changes in the mechanism of proton pumping. Figures 5 and 6 shows that the M_{412} originated from bR_{567} decays faster than the M_{412} in bR_{570} photocycle. The appearance of the short-lived M_{412} form may be closely correlated with the increase of proton pumping efficiency in whole cells and bR-DMPC vesicles. However, more quantitative data is needed to discern whether the enhancement of efficiency can be simply explained by the shortening of the lifetime leading the increase of turnover rate of proton pumping cycle. When bR_{480} appeared, the occurrence of two long-lived intermediates was observed in PM, as described in Section 3. The one is thought to absorb maximally at 380 nm and the half time of its decay is about 100 ms. The other is thought to have the absorption maximum at shorter wavelength than 380 nm and $\tau \sim 18$ ms. Though, in bR-SBPL vesicles, it was clear that the long-lived intermediates were induced by anesthetics, the accurate determinations of the number of intermediates originated from bR_{480} and the lifetimes of them were difficult. The reason for this is that the long-lived components already exist without anesthetics and the data of flash photolysis suffers from large light scattering by vesicles. Therefore, we have attempted to calculate the amount of M_{412} against the concentration of anesthetics by fitting two exponentials to the absorbance change at 370 nm. Further, in this analysis, we have fixed the half time of M_{412} decay to the value in PM without anesthetics ($\tau = 4.88$ ms). The results are presented in Fig. 9. It can be seen that the amounts of M_{412} decrease monotonically and in the similar manner to proton pumping efficiency with increasing the enflurane concentration (Fig. 4a). The present results suggest that bR_{567}, containing the short-lived M intermediate (M_{412}) in the photocycle, functions actively as a proton pump but bR_{480} has very low efficiency of proton pumping or not proton pumping activity.

A 480 nm absorbing species was also shown to result from the treatment of PM with 40–60% dimethyl sulfoxide (DMSO) [26,27] or to form upon solubilization of the delipidated bR in laurylsucrose at pH > 7.5 [28]. Drukmann et al. [29] reported the similar blue shift of the absorption maximum at pH above 11. Recently, Pande et al. [27] have reinvestigated the effect of DMSO–water mixtures on bR by X-ray and the visible CD spectrum, and showed that the crystalline arrangement of bR retained in 60% DMSO. Since DMSO is a very strong hydrogen-bond acceptor, it may affect the lipid–protein interaction near membrane surface in such a way as to cut off the hydrogen-bond between protein and lipid hydrophilic groups. On the other hand, enflurane mole-

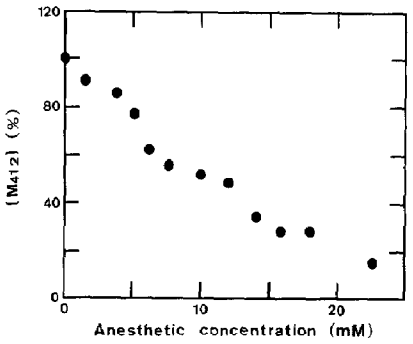

Fig. 9. Population of the fast decay component M_{412} ($[M_{412}]$) in inside-out bR-SBPL vesicle against the concentration of enflurane. To obtain the data, two-exponentials were fitted to the absorption change at 370 nm shown in Fig. 7a after a period of 1 ms following the flash, in which the lifetime of M_{412} was fixed to that in the native PM, 4.88 ms. $[M_{412}]$ presents the values extrapolated to 0 ms and normalized by that in the absence of enflurane.

cules may enter into membranes due to their lipid soluble properties and change the lipid–protein interactions, presumably by breaking the hydrogen-bond between them. Concomitantly, the crystalline structure of PM was destroyed by the inclusion of enflurane. The changes in the interactions between protein and lipids may induce the alteration of protein structure around the chromophore which favors the all-*trans* conformation rather than the 13-*cis* conformation [6]. The formation of the 480 nm pigment may derive from this structural change which is responsible for the increase of interaction between protonated Schiff base and counterion, resulting in the observed blue-shift in the absorption maximum [27]. Hydrogen-bond breaking action of anesthethics was already pointed out by several authors [30,31].

5. Summary and conclusions

The ether-type general anesthetics were used in this work. It was reported that diethylether also induced a second complex form of the retinylidene protein, absorbing maximally at 460 to 490 nm and being called the 460-nm complex [32,33]. At first sight, these facts seem to suggest that the existence of ether type bond in the molecules is important for the formation of bR_{480}. But the ether type bond may not play a special role in the bR_{480} formation, since a similar spectral change is observed in the presence of halothane, a non-ether type anesthetic, as described previously [6,7].

In conclusion, our studies elucidate that volatile anesthetics act on bR in two different manners. In the one, the anesthetics bind on the surface of the membrane and enhance the proton pumping activity. In the other, the anesthetics enter into membranes and interact with protein and lipids in the hydrophobic region. Then, the anesthetics affect largely on the functions and structure of bR, resulting in the decrease of proton pumping efficiency.

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