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EFFECTS OF ANESTHETICS, DIBUCAINE AND METHOXYFLURANE ON THE ATPASE ACTIVITY AND PHYSICAL STATE OF *TETRAHYMENA* SURFACE MEMBRANES

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Abstract—The effect was studied of a local anesthetic, dibucaine, and an inhalation anesthetic, methoxyflurane, on the ATPase activity and the membrane fluidity in the surface membrane, pellicle, of *Tetrahymena pyriformis* NT-I. Both anesthetics were observed to inhibit ATPase activity and to lower its transition temperature (28° → 21°). It was also found that these anesthetics exerted fluidizing and depression of the phase transition temperature and that the disordering effect by methoxyflurane was much greater than that by dibucaine.

These results have demonstrated that the anesthetic-induced alterations in the physical property of the pellicular membrane lead to inhibition of ATPase activity bound to the membrane, thus providing evidence suggesting the relationship between the function and physical state of the biological membrane.

A large body of evidence has shown that various membrane functions are closely associated with the physical state of membrane lipids [1, 2]. Therefore, alterations in membrane fluidity may affect biological activities taking part in the membrane, For a better understanding of such close interrelationship between dynamic structure and functions of the biological membrane, we have chosen an eukaryotic protozoan, *Tetrahymena pyriformis*, as a suitable model membrane system and conducted a series of experiments [3, 4]. There are several advantages in using this cell; bacteria-like rapid growth, well-defined organelles, ease of isolating membrane fractions [5, 6], feasible modification of membrane lipids [7–9] etc. In the previous studies, we have shown that the activities of adenylate cyclase and desaturase are dependent upon the physical state of the surface membrane (pellicle) and endoplasmic reticulum (microsomes) of *T. pyriformis* NT-I, respectively [10–13]. In the present study, effects of altered membrane fluidity caused by anesthetics upon adenosine triphosphatase (ATPase) in the pellicular membrane were examined. Results are presented which show that the increased (superoptimal) fluidity as inferred by 1,6-diphenyl-1,3,5-hexatriene (DPH) leads to the decrease in the activity and changes in Arrhenius Kinetics of the surface membrane-bound ATPase.

MATERIALS AND METHODS

Isolation of the pellicular membrane. The surface membrane pellicle was isolated essentially according to Nozawa and Thompson's procedure as described previously [5]. Cells were harvested from the culture of *T. pyriformis* NT-I which reached to the mid-log phase, resuspended in 0.2 M potassium phosphate buffer (pH 7.2), and homogenized by hand in a tightly fitted glass homogenizer. The homogenate was centrifuged at 117 *g* for 5 min, the resulting pellet, consisting of mostly pellicles, being subject to 1.0 M and 1.72 M discontinuous sucrose density gradient centrifugation at 4,050 *g* for 5 min. The purified pellicular mambrane fraction was obtained from the interface of the two sucrose gradient layers, and washed in 25 mM Tris–HCl buffer, pH 7.4.

Fluorescence polarization measurement. The pellicular membrane suspension was incubated with dibucaine (3.2×10^{-3} M) or methoxyflurane (8.6×10^{-3} M) at 35° for 10 min. The incubation mixture was made up to 2 ml. Methoxyflurane was added to the membrane suspension by injecting its pure solution without dilution. For monitoring membrane physical properties, a fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) was used. This compound was dissolved in tetrahydrofuran at a concentration of 10^{-3} M. The labeling procedure of membranes was carried out essentially according to Shinitzky and Inbar's method [14]. Immediately prior to use, it was diluted 1:1000 in 25 mM Tris–HCl buffer by vigorous mixing on a cyclomixer. One volume of this DPH solution was mixed with 1 vol. of the pellicular membrane suspension, and the reaction solution was incubated at 35° for 20 min [15]. Incubation with methoxyflurane was carried out in the tightly stoppered tube to avoid loss of the anesthetic by evaporation. Measurements of the degree of fluorescence polarization (P) and intensity (I) were performed at desired temperatures with an Elscint MV-I microviscometer (Haifa, Israel),

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

$I_{\parallel}$, $I_{\perp}$: the fluorescence intensities polarized parallel and perpendicular to the direction of polarization of the excitation beam, respectively.

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Assay of adenosine triphosphatase activity. After pre-incubation at a specified temperature for 2 min, the reaction was initiated by the addition of 0.25 ml of 20 mM ATP to 0.75 ml of pellicular membrane suspension in Tris–HCl buffer containing 75 mM KCl, 50 mM sucrose, 1 mM $MgCl_2$ and 0.5 mM EDTA. After the reaction was stopped by the addition of 0.25 ml perchloric acid (70%), the produced inorganic phosphate was measured using a modification of the method of Allen [16]. Incubations for methoxyflurane-treated pellicles were carried out in the capped tubes to prevent loss of the anesthetic. ATPase activity is expressed as μmoles P_i/mg protein/hr. The protein content was measured by the method of Lowry *et al.* [17].

RESULTS AND DISCUSSION

Alterations in ATPase activity of the pellicular membrane

Effects of dibucaine or methoxyflurane on the ATPase activity of the pellicular membrane are presented in Fig. 1. The untreated, control membrane shows a discontinuity around 28°, the activation energies above and below this transition point being 8.7 and 16.0 kcal/mole, respectively. Treatment of the membrane with local anesthetic, dibucaine or inhalation anesthetic, methoxyflurane is shown to inhibit and lower the transition temperature of the ATPase activity. Table 1 shows the percent activities of ATPase of both anesthetic-treated membranes, indicating marked inhibitory effects on the ATPase activity. Especially, a drastic depression of its activity was observed with the dibucaine-treated membranes incubated at 15°. Dibucaine appears less effective in inhibition of its activity than methoxyflurane. However, it might be possible that such a trend of the inhibitory effect would be changed in the presence of the drugs at a different concentration.

Table 1. Inhibition of ATPase activity by dibucaine and methoxyflurane

	Percentage of ATPase activity				
	39°	31°	27°	21°	15°
Control	100	100	100	100	100
Dibucaine-treated	77.3	66.1	70.9	71.0	47.0
Methoxyflurane-treated	60.2	57.5	52.3	64.5	56.0

The results of several studies have demonstrated that anesthetics, either general or local agents, bring about inhibiting effect on ATPase activities in various membranes. For example, the inhalation anesthetic, halothane, inhibits myofibrillar ATPase [18], and the local anesthetic, tetracaine, causes a decrease in activity of the enzyme of bovine brain cortex [19] and human red cell membranes [20].

Furthermore, it is of interest to note that the transition temperatures of ATPase activity of dibucaine- and methoxyflurane-treated membranes were shifted from 28° for the control membrane to lower temperatures around 21°. The energies of activation above and below the transition temperature (21°) are 11.9 and 29.3 kcal/mole respectively for the dibucaine-treated membrane, while the corresponding values for methoxyflurane-treated membrane are 10.1 and 22.0 kcal/mole. Therefore, there is no significant change in the energy of activation in either membrane. Such a marked lowering induced by anesthetics of transition temperature of ATPase activity would suggest that the physical state of the pellicular membrane where ATPase activity is associated must be altered by insertion of lipophilic anesthetic molecules into the membrane lipid bilayer.

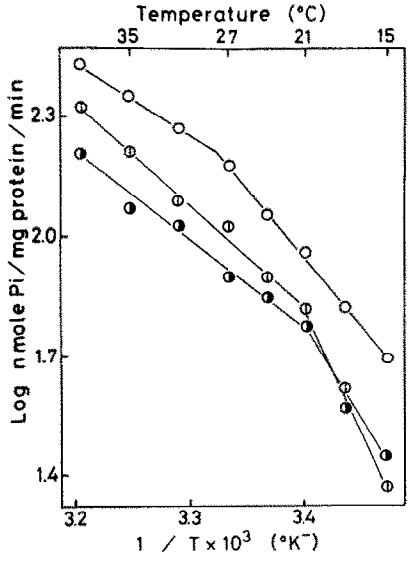

Fig. 1. Arrhenius plot of ATPase activity of the dibucaine- or methoxyflurane-treated pellicular membrane of *Tetrahymena pyriformis* NT-1.
○:control; ○:3.2 × 10^{-3} M dibucaine; ◑:8.6 × 10^{-3} M methocyflurane.

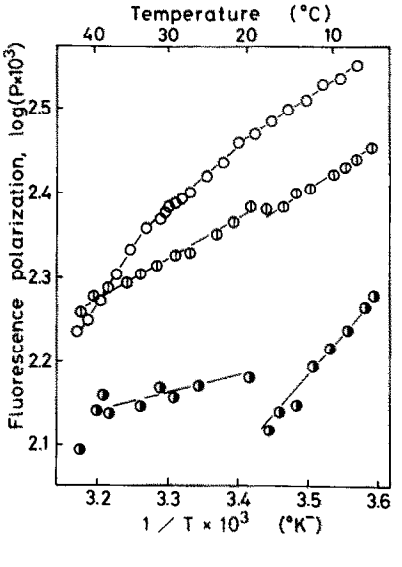

Fig. 2. Fluorescence polarization plotted as a function of temperature of the dibucaine- or methoxyflurane-treated pellicular membrane of *Tetrahymena pyriformis* NT-1.
○:control; ○:3.2 × 10^{-3} M dibucaine; ◑:8.6 × 10^{-3} M methoxyflurane.

Alterations in the physical state of the anesthetic-treated membranes

The results presented as above, demonstrating inhibition and depression of transition temperature of ATPase, prompted us to examine the effects of anesthetics on the physical property of the pellicular membrane. For this purpose we employed a fluorescence polarization technique using 1,6-diphenyl-1,3,6-hexatriene as a fluorescent probe. Figure 2 shows an Arrhenius plot depicting the relationship between the temperature and the degree of fluorescence polarization of the pellicular membranes which are untreated, dibucaine- and methoxyflurane-treated. There is a sharp break around 32° and a mild bend around 20° for the control, untreated membranes. The transition at 32° was also observed by our earlier work with freeze-fracture electron microscopy which has proved useful for detecting the thermotrophic phase transition of the biological membrane [3, 21, 22]. The membrane-intercalated particles of the outer alveolar membrane are homogeneously distributed at the growth temperature, 39.5°, but they begin to aggregate at 32° and this results in the formation of round areas devoid of membrane particles. This might imply that the initiation temperature of the membrane particle aggregation closely corelates with the onset point of phase separation in the membrane lipid bilayer.

In the anesthetic-treated membranes, no distinct breaks due to phase transition are observed at 32°, suggesting that the transition point was shifted to the lower temperature, around 20°, at which the breaks or jumps are seen. This indicates that both anesthetics bring about the phase transition of the membrane lipids at a new temperature below that at which this transition normally occurs. Especially, methoxyflurane exerts an abrupt change in activation energy immediately above and below 20°. Taking into consideration that, as depicted in Fig. 1, the transition point of ATPase activity was also depressed to 21° by anesthetic, the activity of this ATPase appears greatly dependent upon the physical state of membrane lipids. Recently, the anesthetic-induced shift of the thermotropic phase transition point to lower temperatures was observed with artificial lipid membranes by several investigators [23–25], but little has been reported about such depression of phase transition temperature in the biological membrane. Therefore, this *Tetrahymena* pellicular membrane is a typical example to show the lowering effect by anesthetics of transition temperature.

In addition to depression of thermotropic transition, both anesthetics have a disordering or fluidizing effect of the pellicular membrane, as observed in other membranes [26–28]. Figure 2 shows the increased fluidity induced by dibucaine or methoxyflurane. Methoxyflurane exerts a drastic enhancement of the membrane fluidity within all temperature ranges tested, while dibucaine has the smaller degree of disordering effect, assuming that both anesthetics would have no direct effect on the lifetime or rotation of DPH in the membrane. Moreover, since the concentration of the drugs is thought to affect the membrane dynamic structure, a confirmative conclusion should be drawn after further detailed experiments using different concentrations of the anesthetics. Some recent studies have demonstrated that different fluorescence probes may show different microviscosities even in a given membrane [29, 30], giving a warning that one should be careful to interpret data obtained using a single probe. Such differences in the potency of fluidizing of the membrane would be in part due to differences in the chemical structure. It can be speculated that a small molecule methoxyflurane is highly hydrophobic and penetrates into the hydrocarbon core of the membrane bilayer, while the nonpolar moiety of dibucaine (19Å long) is inserted in parallel with the fatty acyl chains, most likely to the depth of the seventh or eighth methylene group. Therefore, it may be possible that both anesthetics affect the physical property of the membrane in different manners. Rosenberg *et al.* [31] has demonstrated by spin-labeling study that halothane has a biphasic action, low concentrations: ordering and high concentrations: disordering (fluidizing). Our present condition for treatment of the membrane with methoxyflurane is similar to the high concentration condition and therefore produces a disordering effect. Furthermore, Rosenburg *et al.* have drawn the conclusion that the volatile anesthetic, halothane, has its action primarily on the lipid region of membranes, and that on the other hand, lidocaine, a local anesthetic intereacts primarily with non-lipid and charged radicals in membranes [31]. Thus, the results present in Fig. 2, which shows different effects between the volatile anesthetic, methoxyflurane, and the local anesthetic, dibucaine, on the physical properties of the pellicular membrane, would be explicable according to their proposed thesis.

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