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MEMBRANE FLUIDIZING EFFECTS OF THE GENERAL ANESTHETIC METHOXYFLURANE ELICIT AN ACCLIMATION RESPONSE IN *TETRAHYMENA*

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Summary

The general anesthetic methoxyflurane exerts a reversible fluidizing effect upon the membranes of *Tetrahymena pyriformis*. The organism responds by decreasing its fatty acid desaturase activity in much the same fashion observed during its acclimation to high temperature and other environmental factors which increase membrane fluidity.

Tetrahymena pyriformis can react to the membrane fluidizing effects of certain environmental factors, such as temperature changes, by altering the degree of membrane fatty acid unsaturation, thus restoring optimal fluidity [1—6]. To examine further the apparent role of lipid bilayer fluidity per se in triggering this acclimation by the cells, we have tested the response of *Tetrahymena* to methoxyflurane, which, along with other general anesthetics, has been shown to fluidize artificial and natural membranes [8—12].

T. pyriformis Strain NT-1 was grown in enriched medium at 39.5°C [2] and utilized at a density of $2 \cdot 10^5$ cells/ml, as determined with a Coulter counter, Model B. The working concentration of methoxyflurane was determined by observing the physiological effects that various levels of the anesthetic produced in the cells. The standard procedure was to sonicate the desired amount of methoxyflurane in 100 ml inorganic medium [17] and add this to a 100 ml cell suspension of logarithmic phase *Tetrahymena* in a 500 ml Erlenmeyer flask, producing a final density of $1 \cdot 10^5$ cells/ml. The flask was closed with a cotton plug and incubated with shaking at 39.5°C. By removing samples frequently for microscopic examination, the following concentration effects were noted: A final concentration of 1—2.5 mM methoxyflurane produced no discernible effect on cell appearance or growth rate. Methoxyflurane

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at 3.00—4.35 mM caused cells to assume a rounded shape and lose motility despite continued ciliary beating. Cell division was inhibited. A complete recovery followed removal of methoxyflurane by passing a stream of air through the air-space of the culture flask. Methoxyflurane at 4.5—10 mM caused 90% of the cells to lyse and die within 5–10 min.

Unless otherwise specified, all further experiments described herein employed methoxyflurane in the 3.00–4.35 mM concentration range. For initiating such experiments 70 μl of the pure anesthetic was utilized for each final incubation volume of 200 ml. Because of its pronounced volatility, the concentration of methoxyflurane tended to decrease with time, especially since the use of loosely stoppered flasks was necessary in order to maintain aerobic conditions. This normally rapid loss of methoxyflurane could be largely prevented by a slight modification of experimental conditions that involved the insertion of a 1.2 cm diameter test tube 10 cm long containing 70 μl additional undiluted methoxyflurane into the flask 30 min after adding the initial dosage of anesthetic. Gradual diffusion of methoxyflurane from the tube into the air space caused the concentration in the medium to remain within the 3.00—4.35 mM range for at least 4 h of incubation, as monitored by using [1-^{14}C]-methoxyflurane (8.47 μCi/mmol), a generous gift from Dr. Duncan Holaday, University of Miami. Under these conditions, the cells remained viable and could recover completely on removal of the anesthetic.

The effect of methoxyflurane on the physical properties of *Tetrahymena* membranes was studied by freeze-fracture electron microscopy. The degree to which decreasing temperature induces membrane-embedded protein particle aggregation, as detected by freeze-fracture electron microscopy, has been utilized as a quantitative indicator of membrane fluidity [5, 13]. According to that method, the extent of particle aggregation (in the cell's alveolar membrane) is expressed as a particle density index value, the units being % of the maximum possible particle aggregation [5]. It has been proposed that this aggregation of membrane core proteins induced by temperature reduction is due to their exclusion from membrane lipid domains transformed by low temperature from a liquid crystalline to a gel state [14, 15].

After incubating cells with methoxyflurane, as described above, for 5 min or 3 h, 40-ml aliquots of cell suspension were chilled over a 4 min period to 21°C, fixed with 1/3 volume of 4% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.2) and examined by freeze-fracture electron microscopy [5, 13]. The particle density index of control and methoxyflurane-treated cells, both chilled from 39.5 to 21°C, was determined. While the control cells had a particle density index of about 90, as previously observed, values of 35 and 23 were calculated for cells fixed after 5 min and 3 h, respectively, in the presence of methoxyflurane. Thus, the extent of lipid phase separation was significantly reduced in methoxyflurane-treated cells as compared to control cells chilled through the same temperature range, implying a pronounced fluidizing effect of the anesthetic.

Table I describes an experiment in which the composition and radioactivity of phospholipid fatty acids are compared for (A) cells incubated for 7 h in the presence of methoxyflurane; (B) cells incubated for 3 h in the presence of methoxyflurane and then for an additional 4 h after blowing off

TABLE I

FATTY-ACID COMPOSITION AND PATTERN OF RADIOACTIVITY INCORPORATION INTO PHOSPHOLIPID FATTY ACIDS OF CONTROL AND METHOXYFLURANE-TREATED CELLS

A. Cells treated with methoxyflurane as described in the text, but maintained at a relatively steady methoxyflurane concentration for a total of 7 h by adding another 70 μl anesthetic to the indwelling tube after the first 3 h of incubation. 20 μCi [1-^{14}C]acetate (specific activity 50 μCi/mmol) was present during the last 2 h of incubation. B. Cells treated with methoxyflurane for 3 h as described in the text. The anesthetic was then depleted by blowing a stream of filtered air through the air space for 20 min. After a further 1.5 h for recovery, 20 μCi [^{14}C]acetate was added and incubation continued for 2 h. C. Control cells treated with [^{14}C]acetate for 2 h in the absence of methoxyflurane. The absolute radioactivity incorporation into phospholipids, expressed as cpm/μg lipid phosphorous, was: (A) 44 000, (B) 60 000, (C) 51 000. The experiment was repeated several times with minor modifications in design, and the results shown here are representative of all findings.

Fatty acid	% Total radioactivity recovered			Mass % of total fatty acids**		
	A	B	C	A	B	C
14:0*	18.5	13.5	9.8	11.0	9.9	10.7
16:0	32.3	24.7	15.4	19.1	16.0	15.2
16:1	5.3	8.3	8.9	5.2	7.5	9.0
16:2	0.3	2.1	2.7	2.3	3.9	4.4
18:0	1.4	1.0	2.0	3.2	2.7	2.2
18:1	4.8	4.4	3.2	4.2	3.8	3.7
18:2	21.3	23.9	21.0	18.4	17.1	18.3
18:3	5.9	11.6	15.2	25.3	24.1	23.9

*The figure preceding the colon designates the number of carbon atoms in each fatty acid and that following the colon the number of double bonds.
**As determined by gas chromatography [2].

excess methoxyflurane; (C) control cells incubated for 7 h without methoxyflurane. All 3 cultures were treated with [^{14}C]acetate 2 h before harvest.

The results show that methoxyflurane markedly reduced the incorporation of [^{14}C]acetate into the principal polyunsaturated fatty acid (18:3)* and also into 16:1 and 16:2. The reduction was offset by a striking rise in 14:0 and 16:0 radioactivity. Concurrent changes in mass amounts of fatty acids were significantly only in the 16-carbon acids, probably because growth inhibition of the methoxyflurane-treated cells permitted only a limited mass increase during the course of the experiments.

It was surprising that these changes were noted not only in the culture maintained in an inhibitory concentration of methoxyflurane (culture A) but also to a lesser extent in a culture labeled after depletion of methoxyflurane by a stream of air (culture B). Using [1-^{14}C]methoxyflurane, we subsequently found that although blowing air over the cell suspension for 20 min removed almost 80% of the methoxyflurane from the medium, the remaining 20% was lost more slowly and was not completely removed even when the air flow was continued for 45 min (Fig. 1). Most of the retained methoxyflurane was shown to be preferentially associated with the *Tetrahymena* cells, as judged by the increase in bound radioactivity.

Our results with *Tetrahymena* membranes are consistent with other studies indicating the ability of general anesthetics to fluidize artificial lipid bilayers [10, 12], and they extend the phenomenon to membranes of an intact organism. By maintaining the anesthetic concentration relatively constant during the long period of continued metabolic activity, we have demonstrated that *Tetrahymena* compensated for this perturbation by altering its fatty acid

*See Table I legend for explanation of notation for fatty acids.

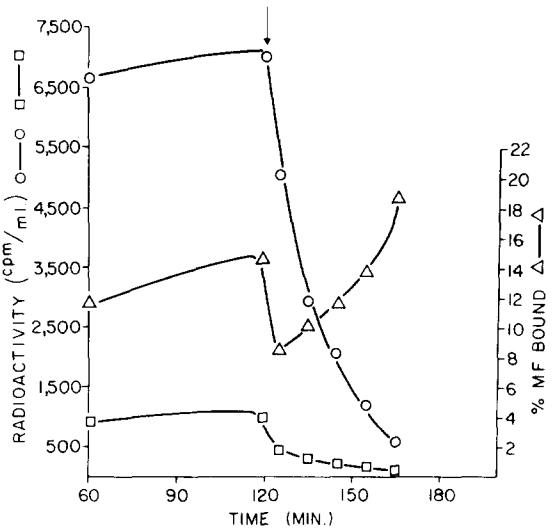

Fig. 1. The recovery of [^{14}C]methoxyflurane in the total cell suspension and in the centrifuged cell pellet at various times, both before and after blowing a stream of air over the surface of the cell suspension. The vertical arrow indicates the time when the stream of air commenced. At each of the sampling times a 4 ml aliquot of cell suspension was removed fro.n the flask. One half (2 ml) was centrifuged in a screw-capped tube for 5 min at 150 x *g* in a clinical centrifuge. The pellet was immediately extracted with 2 ml hexane, and 0.4-ml aliquots of the extract were counted in duplicate. The remaining half was directly extracted in 2 ml hexane and similarly counted to determine counts in the total cell suspension. ○—○, Counts in whole cell suspension; □—□, counts in pellet at corresponding time; △—△, % of counts bound to pellet for equal aliquots of cell suspension at various times.

composition. The alteration was achieved by reducing the extent of enzymatic fatty acid desaturation relative to fatty acid synthesis.

This is basically the same response we have observed when *Tetrahymena* membrane fluidity was raised to an abnormally high level by high temperature [5], or by feeding polyunsaturated fatty acids [1] or methoxy-fatty acids [21]. We consider that the methoxyflurane effect provides important, independent support for the hypothesis that fatty acid desaturase activity is regulated by the physical state of the lipid matrix surrounding these membrane-associated enzymes [5].

By centrifuging aliquots of cell suspension and measuring [^{14}C]methoxyflurane radioactivity at various points during the experiment reported in Fig. 1, the amounts of anesthetic bound to cells could be determined. Assuming that virtually all bound methoxyflurane was associated with cellular membranes, we calculated that at 60, 135, and 165 min methoxyflurane represented 0.05, 0.02 and 0.008% of the membrane lipid volume, respectively. This is slightly lower than the range of occupying volume (0.1—1.0%) for Mullin's rule of anesthesia [18, 19], and within the range of anesthetizing volume occupation within the membrane (0.02%, v/v) as reported by Seeman [7].

These studies raise the interesting question of whether mammalian cells can also reduce their fatty acid desaturation in response to the continued presence of an anesthetic. Certain unexplained phenomena (discussed in ref. 20), such as the higher ethanol level required to induce sleep in habitual alcohol drinkers or the higher concentration of halothane needed to anesthetize alcoholics as compared with normal controls, may stem from prolonged

exposure to the general anesthetic effects of ethanol. Indeed, two recent reports indicate that the long-term ethanol ingestion by mice [22] and rats [23] induces an acclimation in certain membranes to the fluidizing effects of the alcohol. The lipids of these membranes have not been analyzed.

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References

1 Kasai, R., Kitajima, Y., Martin, C.E., Nozawa, Y., Skriver, L. and Thompson, Jr., G.A. (1976) Biochemistry 15, 5228—5233
2 Fukushima, H., Martin, C.E., Iida, H., Kitajima, Y., Thompson, Jr., G.A. and Nozawa, Y. (1976) Biochim. Biophys. Acta 431, 165—179
3 Ferguson, K.A., Davis, M.F., Conner, R.L., Landrey, J.R. and Mallory, F.B. (1975) J. Biol. Chem. 250, 6998—7005
4 Nozawa, Y., Iida, H., Fukushima, H., Ohki, K. and Ohnishi, S. (1974) Biochim. Biophys. Acta 367, 134—147
5 Martin, C.E., Hiramitsu, K., Kitajima, Y., Nozawa, Y., Skriver, L. and Thompson, Jr., G.A. (1976) Biochemistry 15, 5218—5227
6 Conner, R.L. and Stewart, B.Y. (1976) J. Protozool. 23, 193—196
7 Seeman, P. (1972) Pharmacol. Rev. 24, 583—655
8 Metcalfe, J.C., Seeman, P. and Burgen, A.S.V. (1968) Mol. Pharmacol. 4, 87—95
9 Trudell, J.R., Hubbell, W.L. and Cohen, E.N. (1973) Biochim. Biophys. Acta 291, 321—327
10 Trudell, J.R., Payan, D.G., Chin, J.H. and Cohen, E.N. (1975) Proc. Natl. Acad. Sci. U.S. 72, 210—213
11 Vanderkooi, J.M., Landesberg, R., Selick, H. and McDonald, G.G. (1977) Biochim. Biophys. Acta 464, 1—16
12 Miller, W.K. and Pang, K.-Y.Y. (1976) Nature 263, 253—255
13 Kitajima, Y. and Thompson, Jr., G.A. (1977) J. Cell Biol. 72, 744—755
14 Wunderlich, F., Speth, V., Batz, W. and Kleinig, H. (1973) Biochim. Biophys. Acta 298, 39—49
15 Speth, V. and Wunderlich, F. (1972) Biochim. Biophys. Acta 291, 621—628
16 Bligh, E.C. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
17 Hamburger, K. and Zeuthen, E. (1957) Exp. Cell Res. 13, 443—453
18 Mullins, L.J. (1954) Chem. Rev. 54, 289—323
19 Seeman, P. (1975) in Cell Membranes: Biochemistry, Cell Biology and Pathology (Weissman, G. and Claiborne, R., eds.), pp. 239—247, Academic Press, New York
20 Seeman, P. and Roth, S. (1972) Biochim. Biophys. Acta 255, 171—177
21 Kitajima, Y. and Thompson, Jr., G.A. (1977) Biochim. Biophys. Acta, in press
22 Chin, J.H. and Goldstein, D.B. (1977) Science 196, 684—685
23 Curran, M. and Seeman, P. (1977) Science 197, 910—911