Membrane fluidity changes of liposomes in response to various odorants

Complexity of membrane composition and variety of adsorption sites for odorants

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ABSTRACT Three kinds of liposomes prepared from phosphatidylcholine (PC), azolectin, and azolectin-containing membrane proteins of the canine erythrocytes were used as models for olfactory cells. To explore properties of the adsorption sites of odorants, membrane fluidity changes in response to various odorants were measured with various fluorescence dyes which monitor the fluidity at different depths and different regions of the membranes. (*a*) Application of various odorants changed the membrane fluidity of azolectin liposomes. The patterns of membrane fluidity changes in response to odorants having a similar odor were similar to each other and those in response to odorants having different odors were different from each other. These results suggested that odorants having a similar odor are adsorbed on a similar site and odorants having different odors are adsorbed on different sites. (*b*) Such variation of the pattern was not seen in liposomes of a simple composition (PC liposome). (*c*) In the proteoliposomes whose composition was more complex than that of azolectin liposomes, the patterns of membrane fluidity changes varied among odorants having a similar odor. It was concluded that liposomes of complex membrane composition have the variety of adsorption sites for odorants.

INTRODUCTION

Olfactory systems respond sensitively to various volatile chemicals and discriminate structures of multifarious chemicals. Although olfactory systems have such interesting characteristics, fundamental mechanisms of olfactory reception are still unknown. An idea that olfactory reception occurs via specific receptor proteins for odorants has been suggested by a number of investigators. The results that lectin concanavalin A (1) and protein modified regents (2) preferentially blocked rat olfactory responses to some odorants supported the above idea, although those are indirect evidence. Attempts to isolate receptor proteins have been made by many investigators (3–6). For example, Fesenko et al. isolated binding proteins for decanal and camphor from rat olfactory epithelium and demonstrated that antibodies to these proteins blocked olfactory responses to these odorants (6). Pevsner et al. isolated a binding protein for isobutylmethoxypyrazine (IBMP) from the bovine olfactory mucosa (5). Later they showed that the IBMP-binding protein was water soluble and existed in mucus covering olfactory epithelium and also in the tears (7). Thus few proteins have been verified to be a true receptor protein for odorants.

Olfactory cells respond to multifarious odorants. The question arises regarding whether the olfactory cells provide specific receptor proteins for multifarious odorants. It is known that odorants induce the response not only in olfactory systems but also in nonolfactory systems which must have no specific receptor proteins for odorants. Tucker reported that all the odorants that stimulated the turtle olfactory system also induced sensitively the response in the turtle trigeminal nerve (8). In a previous paper (9), we reported that the mouse neuroblastoma cell (N-18 clone) is depolarized by various odorants and there is a good correlation between the order of the threshold concentrations for various odorants in the N-18 cell and that in the frog olfactory responses. On the other hand, Pace et al. found that odorants increased the adenylate cyclase activity of the frog olfactory cilia and suggested that receptor proteins for odorants coupled with G-protein exist in the ciliary membrane (10). This finding does not, however, support existence of the odorant receptor proteins because the same odorants that activate the frog olfactory adenylate cyclase at similar concentrations increased the adenylate cyclase activity of the frog melanophore which is unrelated to olfactory cells and must have no specific receptor proteins for odorants (11).

Recently, we reported that various odorants also depolarized various liposomes and there is a good correlation between the order of the threshold concentrations for various odorants in azolectin liposomes and that in the frog and the porcine olfactory responses (12). The present study purposes to explore characteristics of odor receptor sites using liposomes as a model for an olfactory cell. Liposomes of simple composition such as phosphatidylcholine (PC) liposomes and those of complex composition such as azolectin liposomes and azolectin liposomes containing membrane proteins of the canine erythrocyte

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(proteoliposome) are prepared for the experiments. We measure the membrane fluidity changes of liposomes in response to various odorants with six fluorescence dyes which monitor the membrane fluidity at different depths and in different regions. The results obtained indicate that various odorants change the membrane fluidity of liposomes monitored with various dyes and that odorants having different odors induce different patterns of the membrane fluidity changes of liposomes of complex composition, suggesting that the membrane of complex composition has the variety of adsorption sites for odorants.

MATERIALS AND METHODS

Preparation of azolectin and PC liposomes

Liposomes were prepared essentially as described in the previous paper (12). Azolectin was washed three times with ice-cold acetone and dissolved in chloroform. The dried lipid film in the flask was dispersed in the 200-mM mannitol solution containing 0.1 mM NaCl. The lipid suspension was sonicated at 15°C for 1 h in a bath-type sonicator (W-375; Heat Systems-Ultrasonic Inc., New York). The sonicated suspension was centrifuged at 100,000 × *g* for 60 min at 4°C. The supernatant was used in the present study. The phospholipid content was determined by measuring the phosphorus content (13).

Preparation of proteoliposome

Gosts of canine erythrocyte were prepared by the modified method described by Kasahara and Hinkle (14). Flesh canine blood in the ACD solution (8.5 mM sodium citrate, 4.2 mM citric acid, 12.2 mM glucose) was centrifuged at 2,000 × *g* for 15 min and washed three times by the 0.9% NaCl solution. The precipitate was suspended in the deionized water and centrifuged at 10,000 × *g* for 20 min and washed seven times by the deionized water. The washed ghosts were incubated with the 200-mM mannitol solution containing 0.5% Triton X-100 and 0.1 mM NaCl at 4°C. After centrifugation at 45,000 × *g* for 60 min, the supernatant was treated with Bio-Beads SM-2 overnight (6 g wet weight of beads/20 ml) to remove Triton X-100 from proteins and stored at −80°C. Protein was measured by the method of Lowry et al. (15).

The Triton-extract (0.05 mg) was added to the sonicated azolectin liposomes (7.5 mg) prepared as above. The mixture was quickly frozen in liquid nitrogen and thawed at room temperature for 5 min. This procedure was repeated four times and the mixture was sonicated for 20–30 s at 20°C in the bath-type sonicator.

Liposome labeling with various fluorescence dyes

The procedure of labeling with various fluorescence dyes was described in previous papers (16, 17). After the liposomes were added to the 5 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethane sulfonic acid (Hepes)-NaOH buffer (pH 7.3) containing 93 mM NaCl and 7 mM KCl, the fluorescence dyes were added to the liposome suspension. The final concentration of phospholipids was 50 μM. The mixture was stirred for 20 min at 30°C and then the polarization was measured. Concentrations of the probes were 0.63 μM for cholesteryl anthracene-9-carboxylate (CA), *n*-(9-anthroyloxy) stearic acids (2-AS, 7-AS and 12-AS), and 16-(9-anthroyloxy) palmitic acid (16-AP), and 1.25 μM for 12-(9-anthroyloxy) oleic acid (12-AO).

Measurement of fluorescence polarization

The fluorescence polarization of dyes in the membrane was measured in fluorescence spectrophotometer (650-10S; Hitachi Ltd., Tokyo) as described in the previous paper (16). All experiments were carried out at 30°C. The degree of polarization was calculated as $P = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$. Here, $I_\parallel$ and $I_\perp$ stand for the fluorescence intensity parallel to and that perpendicular to the plane of polarization of the excitation beam, respectively. The background intensity was subtracted from the fluorescence intensity measured.

Solutions

Stock dye solutions were as follows. 0.25 mM CA dissolved in tetrahydrofuran: 0.25 mM 2-AS, 7-AS, 12-AS, 16-AP, and 1 mM 12-AO dissolved in ethanol.

Chemicals

CA, 2-AS, 7-AS, 12-AS, 16-AP, and 12-AO were purchased from Molecular Probes Inc. (Junction City, Eugene, Oregon). 3, 3'-dipropylthiocarbocyanine iodide (diS-C_3[5]) was purchased from Nihon Sensitive Dye Laboratories Co. (Okayama, Japan). Egg PC was purchased from Nihon Seika Co. (Osaka, Japan). Azolectin, *n*-amyl acetate, cyclohexanone, ethyl acetate, heptanol, hexanol, menthone, nonanol, octanol, and 2-octanol were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Citral was purchased from Kanto Chemical Corporation (Tokyo). β-Ionone was purchased from Nakarai Chemicals Ltd. (Kyoto, Japan). *l*-Menthol, *d*-menthol, muscone, and oxalide were kindly supplied from Takasago International Corporation (Tokyo). Odorants used were of the best grade available.

Regions in the membrane monitored with various fluorescence dyes

Various fluorescence dyes used in the present study monitor the membrane fluidity at different depths and in different regions (Fig. 1). Probes with saturated fatty acid (2-AS, 7-AS, 12-AS, and 16-AP) monitor the membrane fluidity at the gel-like region of the membrane and a probe with unsaturated fatty acid (12-AO) monitors the membrane fluidity at the fluid-like region (18). CA monitors the membrane fluidity at the region where cholesterol exists.

RESULTS

1. Membrane fluidity changes in azolectin liposome

Dose-dependent fluidity changes in response to β-ionone

Fig. 2 shows the membrane fluidity changes of azolectin liposome monitored with various fluorescence dyes when β-ionone of varying concentrations is used as a stimulant.

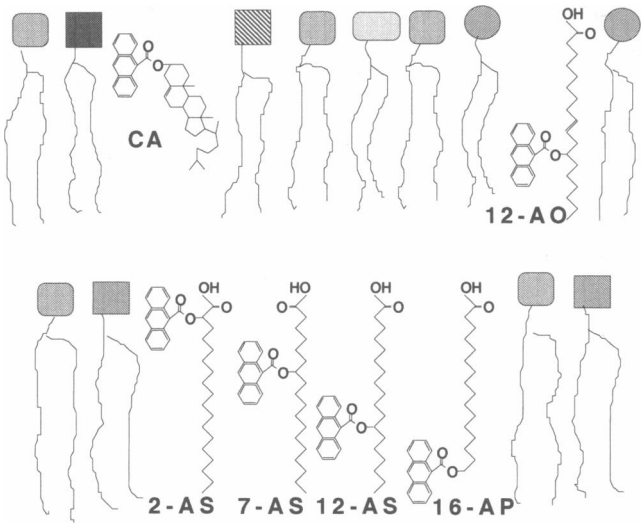

FIGURE 1 Schematic diagram illustrating distribution of various fluorescence dyes in the membrane.

Application of β-ionone leads to a dose-dependent increase in the degree of fluorescence polarization of all fluorescence dyes used, indicating that β-ionone decreases the membrane fluidity of all portions in the membrane.

Patterns of membrane fluidity changes in response to various odorants

Various species of odorants change the membrane fluidity of the azolectin liposome measured with various probes. Fig. 3 plots the P/P_0 values of six dyes in response to various odorants. P/P_0 represents relative value of the polarization degree of the fluorescence where P_0 and P indicate the degree of the polarization in the odorant-free and odorant solutions, respectively. The concentrations of odorants used are those to depolarize sufficiently the membrane potential of the azolectin liposome.

Odorants used in the present study are divided into five groups according to the patterns of the P/P_0 values in response to odorants. β-Ionone, citral, muscone, and oxalide significantly increase the P/P_0 values monitored with CA, 2-AS, 7-AS, and 16-AP (Figs. 3, *a* and *b*), indicating that these odorants decrease the fluidity of the portions in the membrane monitored with these probes. While β-ionone and citral significantly increase the P/P_0 values monitored with 12-AS, muscone and oxalide practically unchanged these values. Correlation coefficients of the P/P_0 values are 0.99 (β-ionone and citral, which have floral odor and an enone structure) (19) and 0.98 (muscone and oxalide, which have musky odor) (Table 1).

Dependence of the patterns of the P/P_0 values in

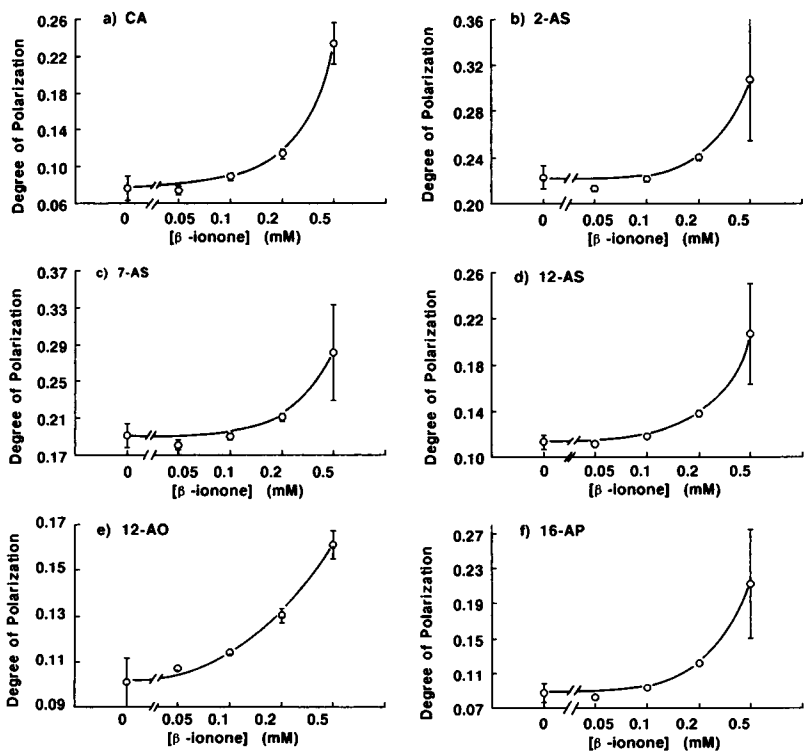

FIGURE 2 The degree of fluorescence polarization monitored with various fluorescence dyes as a function of concentration of β-ionone. Each point is the mean value ± SD obtained from at least three preparations.

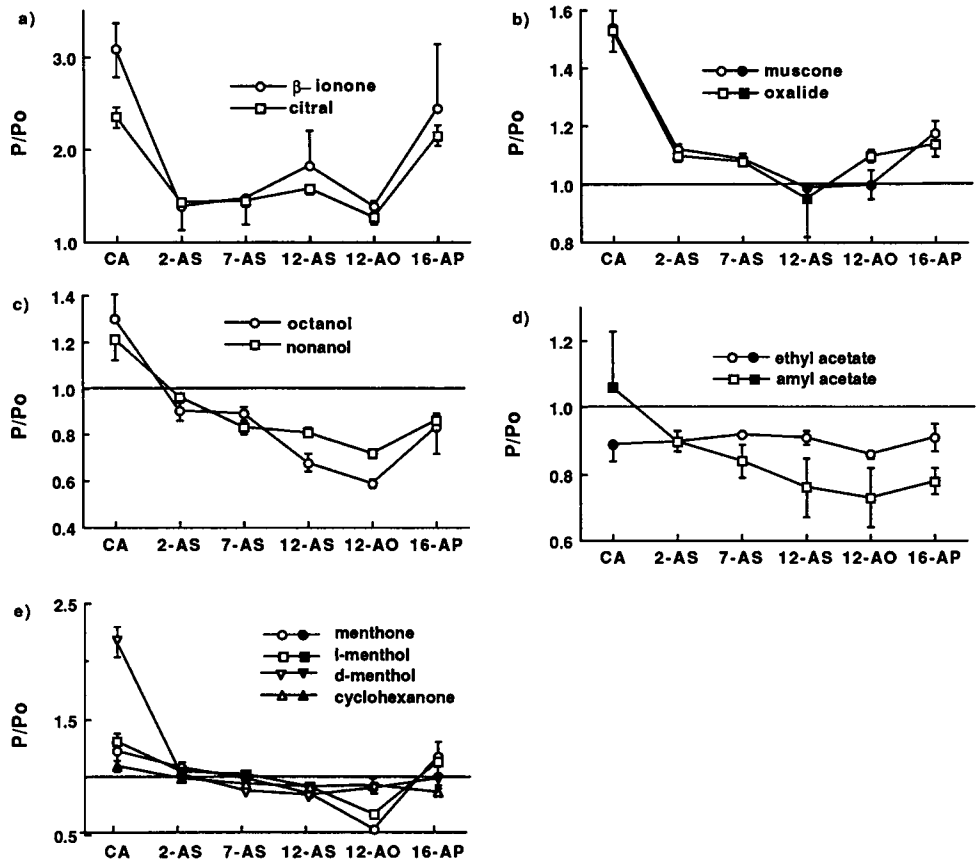

FIGURE 3 Changes in membrane fluidity of azolectin liposome monitored with various fluorescence dyes in response to various odorants. (*a*) 0.5 mM β-ionone and 1 mM citral, (*b*) 0.1 mM muscone and 0.1 mM oxalide, (*c*) 5 mM octanol and 0.5 mM nonanol, (*d*) 200 mM ethyl acetate and 20 mM amyl acetate, and (*e*) 5 mM menthone, 5 mM *l*-menthol, 5 mM *d*-menthol, and 10 mM cyclohexanone were used as stimulants. Odorants shown in Fig. 3 change the fluorescence intensity of membrane potential sensitive dye, diS-$C_3(5)$, from 7 to 32%. Changes in the fluorescence intensity were measured as described in the previous paper (12). Each point is the mean ± SD obtained from at least five preparations. Open circle, open square, and open triangle; $p < 0.05$ vs. control.

response to odorants on odorant concentrations was examined. Correlation coefficients for 0.1:0.2 mM, 0.1:0.5 mM, and 0.2:0.5 mM β-ionone were 0.95, 0.90, and 0.98, respectively, indicating the patterns of the membrane fluidity changes in response to β-ionone are independent of its concentration.

Octanol, nonanol, ethyl acetate, and amyl acetate significantly decrease the P/P_0 values monitored with 2-AS, 7-AS, 12-AS, 12-AO, and 16-AP (Figs. 3, *c* and *d*). While octanol and nonanol significantly increase the P/P_0 values monitored with CA, ethyl acetate and amyl acetate practically unchange these values. Correlation coefficients of the P/P_0 values are 0.99 (octanol and nonanol, which have floral odor and a hydroxy group) and 0.81 (ethyl acetate and amyl acetate, which have floral odor and an ester structure). Menthone, *l*-menthol, *d*-menthol, and cyclohexanone, which have a minty odor, also change the membrane fluidity. Patterns of the changes in the P/P_0 values do not apply those described above (Fig. 3 *e*). These results indicate that certain odorants having a similar odor induce a similar pattern of fluidity changes. While musky odorants (muscone and oxalide) increase the P/P_0 values monitored with 2-AS, 7-AS, and 16-AP, floral odorants (alcohol; octanol and nonanol) decrease those values and the correlation coefficients of the P/P_0 values between musky and floral odorants (alcohol) are 0.31 or less, indicating that odorants having different odors induce different patterns.

2. Membrane fluidity changes in PC liposome

Various odorants also change the membrane fluidity of PC liposome (Fig. 4). Patterns of membrane fluidity

TABLE 1 **Correlation coefficients of P/P_0 values of azolectin liposome in response to two different odorants***

	ION	CIT	MUS	OXA	OCT	NON	ETH	AMY	MEN	*l*-M	*d*-M
CIT	0.99										
MUS	0.89	0.88									
OXA	0.87	0.84	0.98								
OCT	−0.12	−0.13	0.30	0.25							
NON	−0.11	−0.12	0.31	0.26	0.99						
ETH	−0.86	−0.84	−0.63	−0.67	0.54	0.52					
AMY	−0.51	−0.53	−0.11	−0.14	0.90	0.90	0.81				
MEN	−0.14	−0.17	0.25	0.21	0.94	0.96	0.49	0.86			
l-M	0.45	0.49	0.70	0.58	0.73	0.73	0.02	0.40	0.62		
d-M	0.65	0.60	0.89	0.89	0.60	0.61	−0.31	0.28	0.58	0.71	
CYC	−0.27	−0.33	0.15	0.17	0.88	0.89	0.52	0.90	0.90	0.39	0.55

*Odorants used were 0.5 mM β-ionone (ION), 1 mM citral (CIT), 0.1 mM muscone (MUS), 0.1 mM oxalide (OXA), 5 mM octanol (OCT), 0.5 mM nonanol (NON), 200 mM ethyl acetate (ETH), 20 mM amyl acetate (AMY), 5 mM menthone (MEN), 5 mM *l*-menthol (*l*-M), 5 mM *d*-menthol (*d*-M), and 10 mM cyclohexanone (CYC).

changes in response to odorants except for β-ionone and citral are similar to each other; the odorants increase or unchange the membrane fluidity monitored with 12-AO, 12-AS, and 16-AP and decrease the membrane fluidity monitored with CA. Most correlation coefficients between these odorants are 0.41 or more (Table 2), indicating that patterns of the membrane fluidity changes in response to those odorants are similar to each other.

3. Membrane fluidity changes in proteoliposome

To examine the effects of odorants on the membrane fluidity of a membrane of more complex composition, we measured membrane fluidity changes of proteoliposome (Fig. 5). In the proteoliposome, patterns of membrane fluidity changes vary among odorants having a similar odor in the human sense. For example, nonanol, hexanol, heptanol, and 2-octanol, which have floral odor and hydroxy group, decrease the P/P_0 values monitored with 2-AS, 7-AS, 12-AS, 12-AO, and 16-AP. Octanol, which also has a floral odor and hydroxy group, increases the P/P_0 values monitored with 2-AS, 7-AS, and 16-AP. Correlation coefficients of the P/P_0 values between octanol and other alcohol odorants with floral odor are negative values (Table 3). In proteoliposomes, patterns of membrane fluidity changes in response to *l*- and *d*-menthol, which have a minty odor, are similar to each other and are distinct from that in response to menthone, which also has a minty odor. While menthone decreases the P/P_0 values monitored with 7-AS, 12-AS, 12-AO, and 16-AP, *l*- and *d*-menthol increase the P/P_0 values monitored with 16-AP. Correlation coefficient of the P/P_0 values between *l*- and *d*-menthol is 0.98, whereas that between menthone and *l*- or *d*-menthol is below 0.70.

4. Difference in correlation coefficient among different liposomes

To visualize difference in patterns of membrane fluidity changes of three liposomes, distribution of the correlation coefficient between different combinations of odorants in liposomes is summarized in Fig. 6. The order of the magnitude of the frequency above 0.6 of the correlation coefficient is PC liposome > azolectin liposome > proteoliposome. On the other hand, the order of the frequency magnitude below 0.2 of the correlation coefficient is proteoliposome > azolectin liposome > PC liposome. These results indicate that diversity of patterns of membrane fluidity changes increases with an increase in complexity of the membrane composition.

DISCUSSION

The order of the degree of fluorescence polarization of saturated fatty acid probes in the odorant-free solution was 2-AS > 7-AS > 12-AS > 16-AP (Fig. 2). These results coincide with the report that the fluidity at the deep region of the membrane is larger than that near the membrane surface of dipalmitoyl phosphatidylcholine liposomes (20) and human erythrocytes (21). The degree of fluorescence polarization of 12-AO which monitors the membrane fluidity at fluid-like region is smaller than that of 12-AS, which monitors the fluidity at gel-like region (18). Thus, various fluorescence dyes used in the present study monitor changes in membrane fluidity at various depths and various regions in the liposome membranes. It is noted that these fluorescence dyes also monitored the membrane fluidity changes of the N-18 cell (16) and cells

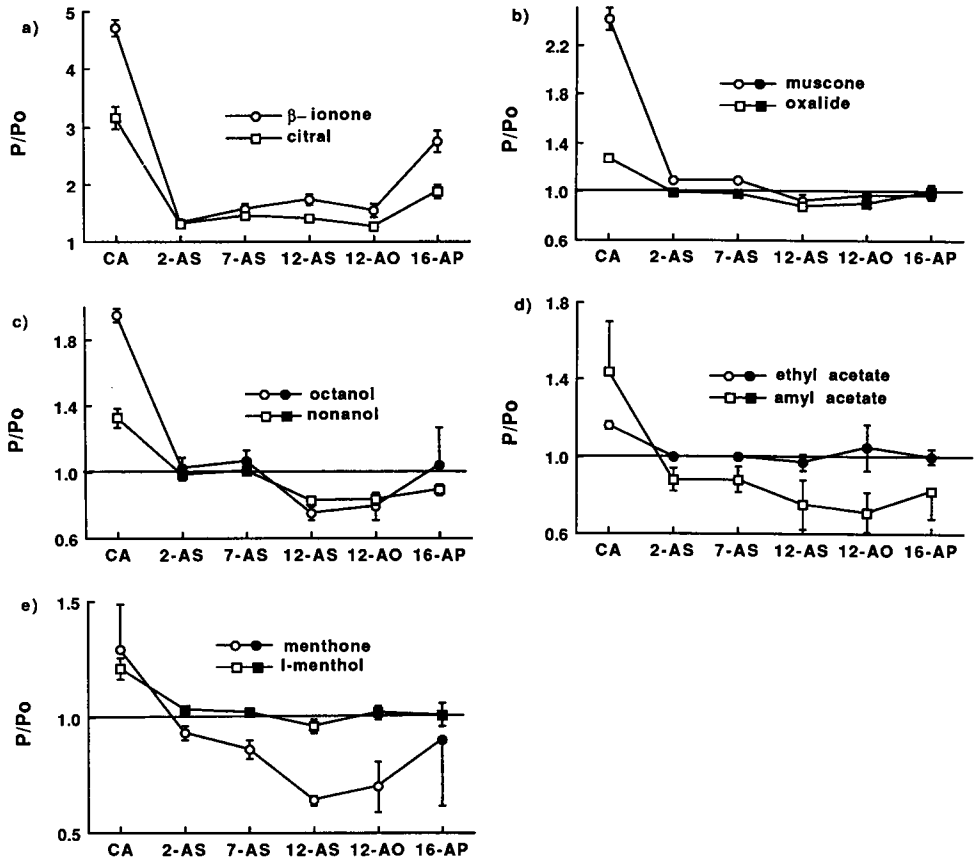

FIGURE 4 Changes in membrane fluidity of PC liposome monitored with various fluorescence dyes in response to various odorants. (*a*) 0.5 mM β-ionone and 1 mM citral, (*b*) 0.1 mM muscone and 0.1 mM oxalide, (*c*) 5 mM octanol and 0.5 mM nonanol, (*d*) 200 mM ethyl acetate and 20 mM amyl acetate, and (*e*) 5 mM menthone and 5 mM *l*-menthol were used as stimulants. Each point is the mean ± SD obtained from at least five preparations. Open circle, open square, and open triangle; $p < 0.05$ vs. control.

from porcine olfactory mucosa (17) in response to various odorants.

In the present study, three types of liposomes were used as a model for an olfactory cell. The responses of liposomes to odorants were reported to be similar to those of olfactory cells in the following points. (*a*) The azolectin liposome responded to many species of odorants. (*b*) Concentrations of various odorants to depolarize the azolectin liposome closely correlated with those to induce the frog and porcine olfactory responses (12). (*c*) Changes

TABLE 2 Correlation coefficients of P/P_0 values of PC liposome in response to two different odorants*

	ION	CIT	MUS	OXA	OCT	NON	ETH	AMY	MEN
CIT	1.00								
MUS	0.86	0.88							
OXA	0.41	0.44	0.81						
OCT	0.78	0.80	0.96	0.87					
NON	0.45	0.47	0.80	0.98	0.91				
ETH	0.83	0.83	0.94	0.72	0.87	0.68			
AMY	0.32	0.34	0.70	0.95	0.82	0.96	0.59		
MEN	0.15	0.16	0.51	0.85	0.71	0.89	0.43	0.94	
l-M	0.86	0.88	0.98	0.77	0.95	0.77	0.96	0.64	0.50

*Odorants used were 0.5 mM β-ionone (ION), 1 mM citral (CIT), 0.1 mM muscone (MUS), 0.1 mM oxalide (OXA), 5 mM octanol (OCT), 0.5 mM nonanol (NON), 200 mM ethyl acetate (ETH), 20 mM amyl acetate (AMY), 5 mM menthone (MEN), and 5 mM *l*-menthol (*l*-M).

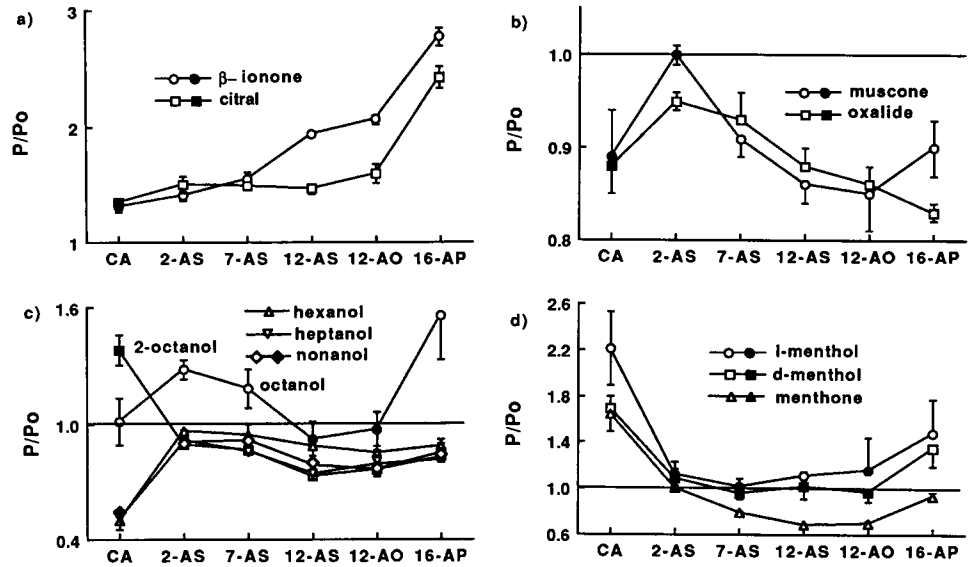

FIGURE 5 Changes in membrane fluidity of proteoliposome monitored with various fluorescence dyes in response to various odorants. (*a*) 0.5 mM β-ionone and 1 mM citral, (*b*) 0.1 mM muscone and 0.1 mM oxalide, (*c*) 5 mM octanol, 5 mM 2-octanol, 10 mM heptanol, 10 mM hexanol, and 0.5 mM nonanol, and (*d*) 5 mM *l*-menthol, 5 mM *d*-menthol, and 5 mM menthone were used as stimulants. Each point is the mean ± SD obtained from at least five preparations. Open circle, open square, open triangle, open rhombus; $p < 0.05$ vs. control.

in lipid composition of liposomes led to great changes in specificity of the responses to odorants (22). (*d*) The membrane potential change of azolectin liposome in response to odorants did not appear in the absence of salts and appeared in the presence of low concentrations of salts (23). Such ion dependence of odor responses of azolectin liposome closely resembled that of the carp olfactory responses (24). Thus liposomes are a good model for olfactory cells.

In previous papers (16, 22), we proposed the following hypothesis about receptor sites for odorants. In general, biological membranes are composed of various species of lipids and proteins. Combinations of various species of lipids and proteins will provide various sites whose properties such as size, shape, or affinity to chemicals are different, and an odorant is assumed to be adsorbed at the site where the odorant can fit. This hypothesis well explains that olfactory systems respond to multifarious odorants.

If odorants are adsorbed in the site in the membrane described above, a membrane having simple composition may not have the variety of receptor sites for odorants. In fact, variation of the pattern of membrane fluidity changes in response to different odorants was not seen in liposomes

TABLE 3 Correlation coefficients of P/P_0 values of proteoliposome in response to two different odorants*

	ION	CIT	MUS	OXA	HEX	HEP	OCT	2-O	NON	MEN	*l*-M
CIT	0.97										
MUS	−0.86	−0.78									
OXA	−0.95	−0.92	0.95								
HEX	−0.52	−0.54	0.75	0.76							
HEP	−0.69	−0.68	0.88	0.88	0.96						
OCT	0.70	0.83	−0.32	−0.57	−0.22	−0.31					
2-O	−0.57	−0.46	0.39	0.37	−0.29	−0.03	−0.28				
NON	−0.69	−0.68	0.87	0.88	0.97	0.99	−0.30	−0.04			
MEN	−0.34	−0.21	0.21	0.12	−0.48	−0.25	−0.07	−0.95	−0.26		
l-M	0.49	0.55	−0.62	−0.70	−0.97	−0.89	0.35	0.40	−0.90	0.62	
d-M	0.43	0.52	−0.49	−0.61	−0.89	−0.80	0.41	0.48	−0.80	0.70	0.98

*Odorants used were 0.5 mM β-ionone (ION), 1 mM citral (CIT), 0.1 mM muscone (MUS), 0.1 mM oxalide (OXA), 10 mM hexanol (HEX), 10 mM heptanol (HEP), 5 mM octanol (OCT), 5 mM 2-octanol (2-O), 0.5 mM nonanol (NON), 5 mM menthone (MEN), 5 mM *l*-menthol (*l*-M), and 5 mM *d*-menthol (*d*-M).

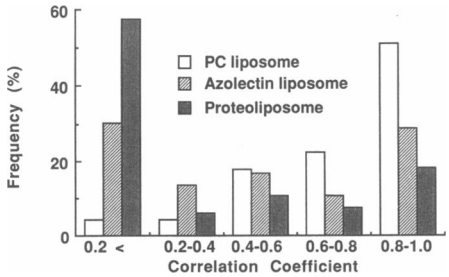

FIGURE 6 Distribution histogram of correlation coefficients of the P/P_0 values between two different odorants. The data of correlation coefficients of azolectin liposome, PC liposome, and proteoliposome were taken from Tables 1, 2, and 3, respectively.

having a simple composition such as PC liposomes. Correlation coefficients between most odorants were 0.41 or more, suggesting that most odorants adsorb at a similar site in the membrane having simple composition.

In azolectin liposomes, patterns of fluidity changes in response to enone-floral odorants (β-ionone and citral), musky odorants (muscone and oxalide), alcohol-floral odorants (octanol and nonanol), and ester-floral odorants (ethyl acetate and amyl acetate) were different from each other. Azolectin is composed of various phospholipids such as PC, phosphatidylethanolamine, and phosphatidylinositol (25) and hence various types of receptor sites for odorants seem to be formed by combination of various species of lipids.

In the proteoliposome whose composition is more complex than that of azolectin liposome, 61% of correlation coefficients of the P/P_0 values between different odorants were below 0.2, indicating that the variety of patterns of membrane fluidity changes increases with an increase in complexity of the membrane composition. These results support an idea that odorants are adsorbed on the variety of sites formed by different combinations of lipids and membrane proteins in olfactory cells.

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