Biochimica et Biophysica Acta, 1062 (1991) 7–12

ADONIS 000527369100089P

BBAMEM 75126

Liposomes having high sensitivity to odorants

Shuichi Enomoto, Makoto Kashiwayanagi and Kenzo Kurihara

Faculty of Pharmaceutical Sciences, Hokkaido University Sapporo (Japan)

(Received 19 September 1990)

Key words: Liposome; Odorant; Membrane potential; Membrane fluidity

The conditions to increase the sensitivities of liposomes to odorants were examined. The results obtained are as follows. (1) The minimum concentration of amyl acetate to induce the membrane potential changes (threshold) in phosphatidylcholine (PC) liposomes was about 10^{-4} M and addition of 10 or 20% phospatidylserine (PS) lowered the threshold to about 10^{-9} M, which was lower than the thresholds for amyl acetate in the turtle and frog olfactory systems. (2) Similar to amyl acetate, addition of PS to PC greatly lowered the threshold for β-ionone. On the other hand, addition of PS to PC in certain ratio increased the threshold for citral, suggesting that addition of PS to PC does not always increase the responses to all odorants. (3) The membrane fluidity change of the liposomes in response to odorants occurred at similar concentration regio.: where the membrane potential changes occurred. The presence of $CaCl_2$ in external solution much greatly increased both the magnitude of the membrane potential changes and the membrane fluidity changes of the PC-PS liposomes in response to amyl acetate than the presence of NaCl and $MgCl_2$. These results suggest that the membrane fluidity change is related to generation of the membrane potential change. (4) It was estimated that adsorption of less than a few molecules of amyl acetate on single liposome elicits detectable changes in the membrane potential and the membrane fluidity.

Introduction

Recently there has been an increased demand for chemical sensors in various fields. In living systems, the olfactory cells are the most typical chemical sensor. They sense multifarious substances with extremely high sensitivity and discriminate such multifarious substances. Elucidation of the receptor mechanisms in olfactory systems is valuable for the development of a new type of chemical sensors.

It has been postulated that olfactory cells have specific receptor proteins for odorants. A number of binding proteins for certain odorants have been isolated [1–3], but no protein was verified to be a true receptor protein. On the other hand, various odorants were found to activate adenylate cyclase in frog olfactory cilia [4,5], which suggested that there exist specific receptor proteins coupled to the GTP-binding protein. It was, however, reported that the same odorants that activate the olfactory adenylate cyclase activate adenylate cyclase in frog melanocytes, which are of course unrelated to olfactory cells, at similar concentrations [6]. Hence activation of adenylate cyclase by odorants does not always support existence of specific receptor proteins [7]. It was proposed that both mechanisms via cyclic AMP [4,8] and not via cyclic AMP [9–11] are involved in olfactory transduction.

The responses to odorants are seen not only in olfactory systems but also in nonolfactory systems such as the turtle trigeminal nerve [12], the *Helix* ganglion [13], the fly taste nerve [14], the frog taste cells [15] and the neuroblastoma cells [16,17]. These nonolfactory systems do not seem to provide specific receptor proteins for odorants, which suggests that specific receptor proteins are not required for odor reception in these systems. In previous papers [18–20], we showed that liposomes made of azolectin are depolarized by various odorants. The sensitivity of the liposomes to odorants was, however, much less than that in olfactory systems. In the present paper, we show that liposomes having certain lipid composition respond to certain odorants with high sensitivity comparable to that of olfactory systems.

Materials and Methods

Materials

The sources for the reagents used are as follows: egg phosphatidylcholine (PC), Nippon Oil and Fats Co.

Abbreviations: PC, egg phosphatidylcholine ; PS, bovine brain phosphatidylserine; diS-C_3(5), 3,3′-dipropylthiocarbocyanine iodide; CA, cholesteryl anthracene-9-carboxylate.

Correspondence: K. Kurihara, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan 060.

(Tokyo); bovine brain phosphatidylserine (PS) and valinomycin, Sigma Chemical Co. (St. Louis); 3,3′,-dipropylthiocarbocyanine iodide (diS-C_3(5)), Nihon Sensitive Dye Laboratories Co. (Okayama); cholesteryl anthracene-9-carboxylate (CA), Molecular Probes (Junction City). Odorants used were of the best grade available and dissolved in ethanol.

Preparation of liposomes

Unilamellar liposomes were prepared as described previously [18]. 200 mM mannitol solution containing 0.1 mM NaCl was used as an internal solution for the liposomes throughout the present study. 100 mM NaCl, 2 mM $CaCl_2$ or 2 mM $MgCl_2$ solution was used as an external solution. Difference in osmotic pressure between the internal and external water phases was compensated by adding mannitol of proper concentration to external solutions. Liposomes used in the experiments for Fig. 2 were prepared in a solution containing 99 mM KCl and 1 mM NaCl. The phospholipid concentration was determined by measuring phosphorus content [21].

Measurement of membrane potential changes

Changes in the membrane potential in response to odorants were monitored by measuring changes in the fluorescence intensity of diS-C_3(5) as described previously [18]. The change in the fluorescence intensity (ΔF) is defined as $\Delta F(\%) = 100(F - F_0)/F_0$ where F and F_0 represent the fluorescence intensity in the presence and absence of odorants, respectively. The temperature was maintained at 30°C by circulating water through the cuvette holder of the photometer. It is noted that addition of any odorant to the dye solution in the absence of liposomes did not affect the fluorescence of the dye.

It was confirmed in a previous paper [18] that the fluorescence changes of diS-C_3(5) in response to odorants in neuroblastoma cells-the dye suspension are closely related to the membrane potential changes of the cells measured with a microelectrode.

Measurement of fluorescence polarization

The polarization of the CA fluorescence in the liposomes was measured by a method similar to that employed for 1,6-diphenyl-1,3,5-hexatriene [18]. The polarization of the CA fluorescence in liposomes was measured as follows: 5 μl of tetrahydrofuran solution of CA was added to 2 ml of liposome suspensions. Tetrahydrofuran itself of the concentration used here did not bring about any effect both on the fluorescence intensity of diS-C_3(5)-liposomes suspension and the fluorescence polarization of CA-liposome suspensions. After the dye-liposome suspensions were stirred for 20 min at 30°C, the polarization was measured. The final concentrations of phospholipids and the dye were 50 and 0.63 μM, respectively. The temperature was maintained at 30°C by circulating water through the cuvette holder of the photometer. 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 average P value ± S.E. of PC and PC-PS liposomes in the absence of an odorant was 0.055 ± 0.001 and there was no essential difference between PC and PC-PS liposomes. The emission of CA was excited at 386 nm and monitored at 464 nm.

Measurement of size distribution of liposomes

The size distributions of liposomes composed of PC and PS (PS/PC = 0.2) were determined by quasi-elastic light scattering analysis, performed utilizing a Nicomp Model 370 Laser Particle Sizer with a 5 mW Helium-Neon Laser at an exciting wavelength of 632.8 nm [22]. Quasi-elastic light scattering, also referred to as dynamic light scattering or photon correlation spectroscopy, employed digital autocorrelation to analyze the fluctuations in scattering light intensity generated by the diffusion of vesicles in solution. The measured diffusion coefficient was used to obtain the average hydrodynamic radius and hence the mean diameter of the vesicles.

The size distributions of liposomes were also determined by scanning electron microscope. The liposomes were fixed according to the method employed by Ishii et al. [23]. The fixed liposomes were viewed in the scanning electron microscope (S-430, Hitachi).

Results and Discussion

Fig. 1 shows the responses of liposomes containing PC and PS in different ratios to various odorants. Here 100 mM NaCl solution is used as an external solution. The sensitivity of liposomes to odorants varies with species of odorants. The minimum concentration of amyl acetate to induce the response (referred to as threshold) in the PC liposomes is about 10^{-4} M. Addition of 10 or 20% PS lowers the threshold to about 10^{-9} M and increases the magnitude of the response. Similar to amyl acetate, addition of PS to PC greatly lowers the threshold for β-ionone and increases the magnitude of the response. On the other hand, the effects of addition of PS on the response to citral are not simple; the order of the magnitude of the response to citral at equimolar is PS/PC = 0.1 > PC > PS/PC = 0.2 > PS/PC = 0.05. Thus addition of PS to PC does not always increase the responses to all odorants.

In order to confirm that the fluorescence change of diS-C_3(5) reflects the membrane potential change of the present system, KCl concentration in external medium for the liposome (PS/PC = 0.2) was changed in the presence of $5 \cdot 10^{-11}$ M valinomycin and the fluorescence change was measured. Fig. 2A shows that ΔF

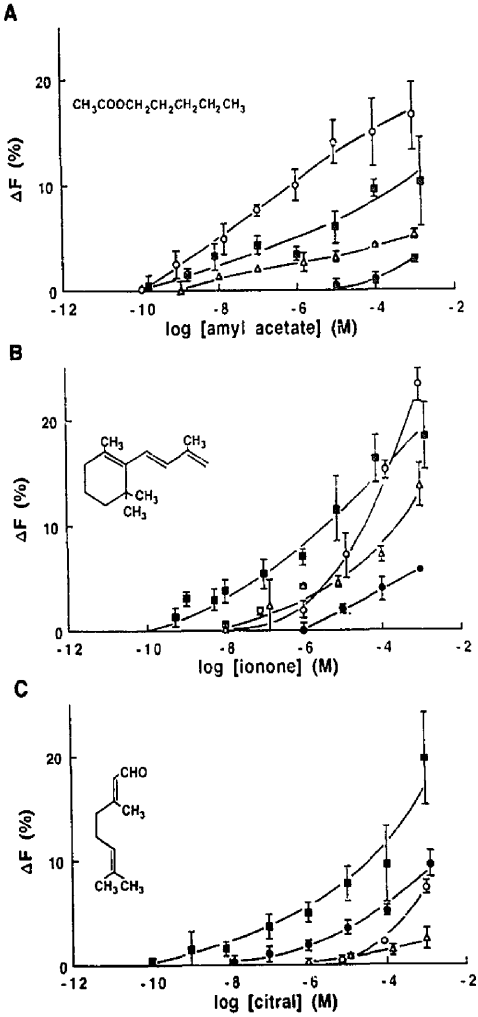

Fig. 1. Changes in the fluorescence intensity (ΔF) of diS-C_3(5) added to suspension of liposomes composed of PC and PS in different ratios as a function of concentration of amyl acetate (A), β-ionone (B) and citral (C). Each point is the mean value ± S.E. of data obtained from at least four preparations. ●, PC; △, PS/PC = 0.05; ■, PS/PC = 0.1; ○, PS/PC = 0.2.

value is linearly changed with logarithmic concentration of KCl, suggesting that the fluorescence change well reflects the membrane potential change. Fig. 2B shows the fluorescence change of the dye-liposomes in the presence of valinomycin in response to amyl acetate of varying concentrations when a solution containing 10 mM KCl and 90 mM NaCl is used as an external solution. The concentration-response curve shown in Fig. 2B (solid line) is essentially similar to that shown in Fig. 1A (dotted line), although the magnitude of the response under this conditions is a little lower than that in Fig. 1A at equimolar concentration.

Fig. 3A and B show effects of $CaCl_2$ on the responses of the liposomes to odorants. The responses of the PC liposomes to amyl acetate or citral in the presence of 2 mM $CaCl_2$ are a little larger or smaller than respective responses in the presence of 100 mM NaCl. The responses of the PC-PS liposomes (PS/PC = 0.2) to both odorants are remarkably enhanced by the presence of $CaCl_2$. In Fig. 4, the magnitude of the response of the PC-PS liposomes to amyl acetate is plotted as a function of $CaCl_2$ concentration. The response is increased with an increase of $CaCl_2$ and reaches a saturation level at about 2 mM. Fig. 3C and D shows the effect of $MgCl_2$ on the responses of the liposomes. The responses of the PC liposomes to amyl acetate in the

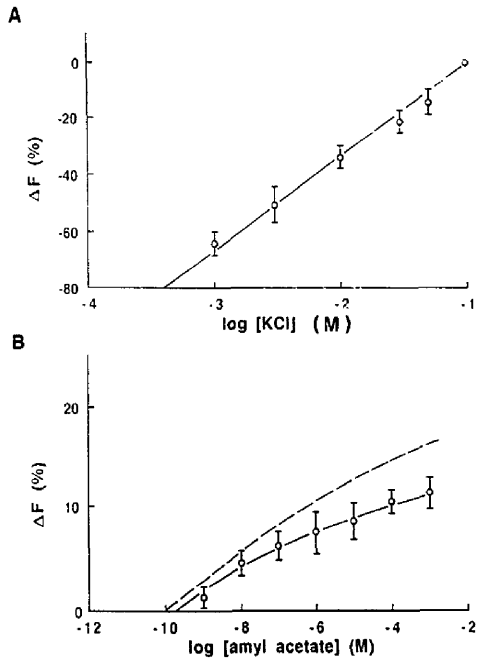

Fig. 2. Changes in the fluorescence intensity (ΔF) of diS-C_3(5) added to suspension of liposomes (PS/PC = 0.2) containing $5 \cdot 10^{-11}$ M valinomycin as a function of KCl concentration in external medium where total salt concentration of KCl and NaCl was kept constant (100 mM) (A) and as a function of concentration of amyl acetate when a solution containing 10 mM KCl and 90 mM NaCl is used as an external solution (B). The liposomes were prepared in a solution containing 99 mM KCl and 1 mM NaCl. In (A), the ΔF value is defined as $\Delta F(\%) = 100(F - F_0)/F_0$ where F and F_0 represent the fluorescence intensity in solutions containing various concentrations of KCl and a solution containing 99 mM KCl and 1 mM NaCl (the same solution as the internal solution), respectively. In (B), ΔF is defined as described in Materials and Methods. Dotted line shows the curve shown in Fig. 1A. Each point is the mean value ± S.E. of data obtained from four preparations.

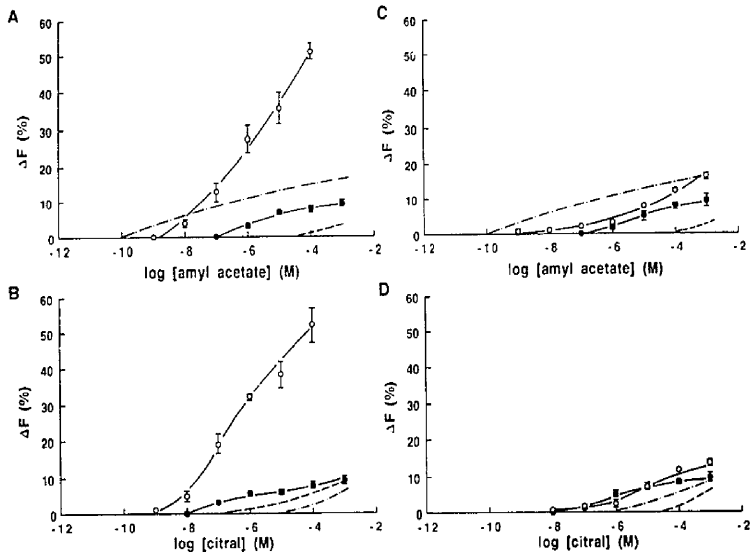

Fig. 3. Effects of 2 mM $CaCl_2$ and 2 mM $MgCl_2$ on the response of liposomes to odorants. Changes in the fluorescence intensity (ΔF) of diS-C_3(5) added to suspension of the PC and the PC-PS (PS/PC = 0.2) liposomes are plotted as a function of concentration of amyl acetate (A, C) and citral (B, D). A solution containing 2 mM $CaCl_2$ and 194 mM mannitol (A, B) and that containing 2 mM $MgCl_2$ and 194 mM mannitol (C, D) were used as external solutions. Each point is the mean value ± S.E. of data obtained from at least four preparations. Data in the presence of 100 mM NaCl represented by dashed line (- - - - -, PC; - · - · -, PS/PC = 0.2) are taken from Fig. 1. ●, PC; ○, PC-PS.

presence of $MgCl_2$ are a little larger than those in the presence of NaCl, while those of the PC-PS liposomes are decreased by the presence of $MgCl_2$. The responses of both liposomes to citral are a little increased by the presence of $MgCl_2$. Thus the results shown in Fig. 3 demonstrate that among NaCl, $CaCl_2$ and $MgCl_2$, only $CaCl_2$ remarkably enhances the response of the PC-PS liposomes to the odorants.

The membrane fluidity changes in response to amyl acetate were examined by measuring fluorescence polarization of CA in the liposomes. Fig. 5 represents the membrane fluidity changes of the liposomes as a function of concentration of amyl acetate. The membrane fluidity of the PC and the PC-PS liposomes in the presence of 100 mM NaCl (Fig. 5A) starts to be changed around 10^{-5} and 10^{-8} M, respectively. Thus the concentration region of amyl acetate where the membrane fluidity changes of the PC-PS liposomes occur is close to the region where the membrane potential changes occur, although the membrane potential changes of the PC liposomes occur at a little lower concentration of amyl acetate than the membrane potential changes (see Fig. 1A). In the presence of 2 mM $CaCl_2$ (Fig. 5B) and 2 mM $MgCl_2$ (Fig. 5C), there is also close relation between the membrane fluidity and the membrane potential changes (see Fig. 3A and C). The membrane fluidity changes in the presence of $CaCl_2$ are much larger than those in the presence of NaCl or $MgCl_2$ (note that the scale in the ordinate for Fig. 5B is larger than that for Fig. 5A or 5C). As shown in Fig. 5, the membrane potential changes in the presence of $CaCl_2$ are much larger than those in the presence of NaCl or $MgCl_2$. These correlation between the membrane fluidity and the membrane potential changes suggests that the membrane fluidity changes are related to generation of the membrane potential changes. Ohnishi and Ito [24] examined the effects of ions on PS-PC membranes using PC spin labels and demonstrated that Ca ion

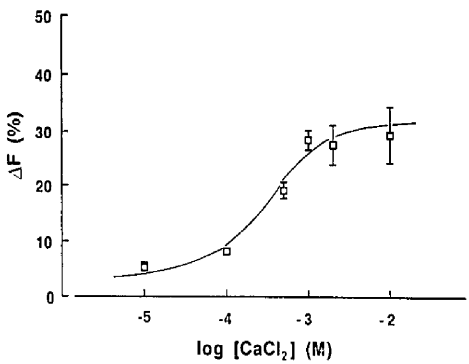

Fig. 4. Changes in the fluorescence intensity (ΔF) of diS-C_3(5) added to suspension of the PC-PS (PS/PC = 0.2) liposomes in response to 10^{-6} M amyl acetate as a function of $CaCl_2$ concentrations. Each point is the mean value ± S.E. of data obtained from at least four preparations. The osmotic pressure of $CaCl_2$ solution was kept constant by addition of mannitol.

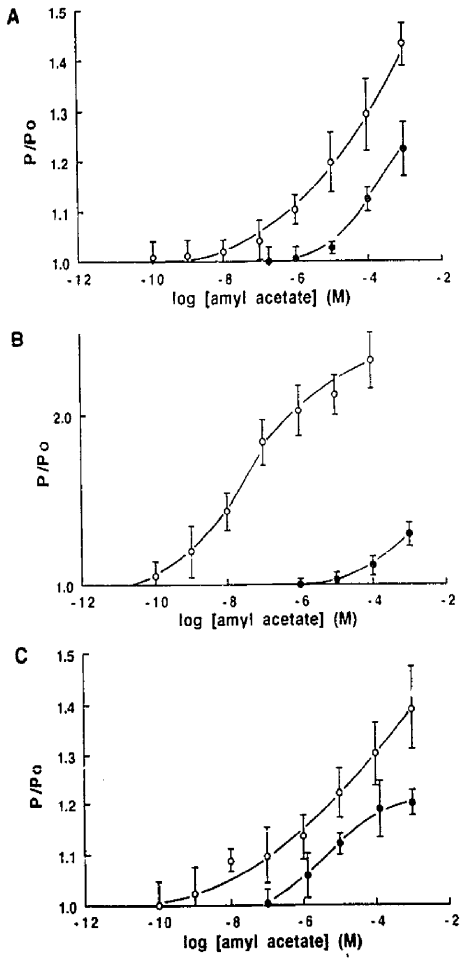

Fig. 5. Changes in relative polarization value (P/P_o) of the CA fluorescence in the presence of the PC (●) and the PC-PS (PS/PS = 0.2) (○) liposomes as a function of concentrations of amyl acetate. P_o and P represent the polarization in the absence and presence of amyl acetate, respectively. 100 mM NaCl solution (A), 2 mM $CaCl_2$ solution (B) and 2 mM $MgCl_2$ solution (C) were used as external solutions. Mannitol was added to external solution to adjust osmolarity constant. Each point in the figure represents the mean value ± S.E. of data obtained from at least four preparations.

induces phase separation of PS-PC bilayer membranes into a solid phase of PS aggregates bridged by Ca chelation and a fluid phase of PC molecules, whereas Mg ion is ineffective. Comparison of the data reported by Ohnishi and Ito [24] with the present results indicates that the effects of Ca and Mg ions on the phase separation and the clustering in PS-PC membranes resemble those of these ions on the membrane potential changes in response to odorants. Hence the phase separation and the clustering of the membranes seem to be related to enhancement of the sensitivity of the membranes to odorants, although exact mechanism of the enhancement of the sensitivity to odorants by addition of PS to PC is still unknown.

The olfactory thresholds for amyl acetate were determined to be 10^{-4} M for the frog [16] and 10^{-7} M for the turtle [25], whose olfactory sensitivity is comparable to that of the dog [26]. Hence the threshold of the PC-PS liposomes (PS/PC = 0.2) to amyl acetate is comparable to or lower than the olfactory thresholds in these animals.

The average diameter of the PC-PS liposomes (PS/PC = 0.2) was determined to be 65 ± 0.14 (mean ± S.E., n = 35,000) nm by light scattering and 56 ± 0.46 (mean ± S.E., n = 1900) nm by scanning electron microscope. It was estimated from the data reported by Huang and Mason [27] that single liposome having diameter of 65 nm is composed of 25000–34000 molecules of phospholipids. The concentration of the lipids used in the present study was 50 μM and hence numbers of the liposomes in a cuvette containing 2 ml were calculated to be $1.7\text{–}2.3 \cdot 10^{12}$, although this was approximate estimation. The minimum concentrations of amyl acetate to induce changes in the membrane potential or the membrane fluidity in the PC-PS liposomes are between 10^{-9} and 10^{-8} M. The numbers of amyl acetate molecules in 2 ml are between $1.2 \cdot 10^{12}$ and $1.2 \cdot 10^{13}$. Hence this calculation suggests that adsorption of less than a few molecules of amyl acetate on single liposome elicits detectable changes in the membrane potential and the membrane fluidity.

In the previous studies [18,19], we demonstrated that the specificity of liposomes to odorants is greatly dependent on the lipid composition and proposed that quality of odor is discriminated by postulating that composition of lipids and proteins of each olfactory cell membrane is different from cell to cell. This idea was supported by the present results; e.g., the PC-PS liposomes (PS/PC = 0.2) respond sensitively to amyl acetate and less sensitively to citral, while the PC liposomes respond sensitively to citral and less sensitively to amyl acetate as shown in Fig. 1. Detail analysis of the adsorption sites for various odorants in lipid membranes was carried out using various fluorescence dyes which monitor the membrane fluidity changes in different regions of the membrane and it was shown that different odorants are adsorbed on different regions in the membranes having complex lipid composition while different odorants are adsorbed on similar region in the membranes having simple lipid composition [28]. These results suggested that odorants are adsorbed on hydrophobic spaces between lipids.

PS has a negative charge, but incorporation of negative lipids such as phosphatidic acid (PA) or cardiolipin (CL) into PC liposomes did not bring about appreciable enhancement of the response to amyl acetate: the response of PA/PC = 0.2 liposomes to amyl acetate was

only slightly larger than that of PC liposomes at equimolar concentration of amyl acetate and that of CL/PC = 0.1 liposomes was smaller than that of PC liposomes. Hence the negative charge itself of PS does not contribute to enhancement of the response to amyl acetate. As shown above, there is close relation between the membrane potential changes and the membrane fluidity changes in response to odorants. Hence, it seems that PC-PS liposomes exhibit high sensitivity to amyl acetate because structure changes of liposomes are easily induced by amyl acetate. Easiness of structural changes of liposomes in response to odorants depends on various factors such as species of odorants, lipid composition, or ionic environment, and hence the sensitivity of the membrane potential changes of liposomes to odorants also seems to depend on these factors.

In general, the membrane potential is composed of two phase boundary potentials at both sides of the membrane and diffusion potential of ions within the membrane [29,30]. In the membrane system having high permeability for ions, where ions within the membrane phase easily reach an equilibrium state, changes in the phase boundary potential are canceled out by changes in the diffusion potential within the membrane [31,32], and hence the changes in the phase boundary potential do not lead to the membrane potential changes under the equilibrium conditions. In the membrane system having low ionic permeability, the ion distribution within the membrane does not easily change in response to changes in the phase boundary potential; the diffusion potential to cancel the changes in the phase boundary potential is not easily produced [31,32]. Hence the change in the phase boundary potential leads to the membrane potential change. This type of the membrane potential is produced only in the nonequilibrium state [33]. This mechanism is applicable to the lipid membranes. In fact, the membrane potential changes of the liposomes or the planar lipid bilayer induced by hydrophobic substances such as odorants [18,20] or bitter substances [34] occurred when there is no ion gradient across the membranes, indicating that the membrane potential changes in response to the hydrophobic substances stem from the phase boundary potential. The magnitude of the fluorescence change of the liposomes containing valinomycin in response to amyl acetate is a little smaller than that containing no valinomycin (Fig. 2B). This seems to be because valinomycin increases permeability of potassium ion.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and grants from the Chemical Materials Research and Development Foundation.

References

1 Pevsner, J., Trifiletti, R.R., Strittmatter, S.M. and Snyder, S.H.P. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 3050–3054.
2 Pevsner, J., Sklar, P.B. and Snyder, S.H. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 4942–4946.
3 Price, S. (1978) Chem. Senses Flavor 3, 51–56.
4 Pace, U., Hanski, E., Salmon, Y. and Lancet, D. (1985) Nature 316, 255–258.
5 Sklar, P.B., Anholt, R.R.H. and Snyder, S.H. (1986) J. Biol. Chem. 261, 15538–15543.
6 Lerner, M.R., Reagan, J., Gyorgyi, T. and Roby, A. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 261–264.
7 Dionne, V.E. (1988) Trends Neurosci. 11, 188–189.
8 Nakamura, T. and Gold, H. (1987) Nature 325, 442–444.
9 Bruch, R.C. and Teeter, J.H. (1989) in Chemical Senses I (Brand, J.G., Teeter, J.H., Cagan R.H. and Kare, M.R., eds.), pp. 283–298, Marcel Dekker, Inc., New York.
10 Labarca, P., Simon, S.A. and Anholt, R.R.H. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 944–947.
11 McClintock, T.S., Schutte, K. and Ache, B.W. (1989) Chemical Senses 14, 817–827.
12 Tucker, D. (1971) in Handbook of Sensory Physiology (Beidler, L.M., Ed.) pp. 151–181, Vol. IV-1, Springer-Verlag, Berlin.
13 Arvanitaki, A., Takeuchi, H. and Chalazonitis, N. (1967) Olfaction and Taste II, 573–598.
14 Dethier, V.G. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 2189–2192.
15 Kashiwagura, T., Kamo, N., Kurihara, K. and Kobatake, Y. (1977) Comp. Biochem. Physiol. 56C, 105–108.
16 Kashiwayanagi, M. and Kurihara, K. (1984) Brain Res. 293, 251–258.
17 Kashiwayanagi, M. and Kurihara, K. (1985) Brain Res. 359, 97–103.
18 Nomura, T. and Kurihara, K. (1987a) Biochemistry 26, 6135–6140.
19 Nomura, T. and Kurihara, K. (1987b) Biochemistry 26, 6141–6145.
20 Nomura, T. and Kurihara, K. (1989) Biochim. Biophys. Acta 1005, 260–264.
21 Allen, R.J.L. (1940) Biochem.J. 34, 858–865.
22 Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Biochim. Biophys. Acta 858, 161–168.
23 Ishii, F., Takamura, A. and Noro, S. (1982) Membrane 7, 307–308.
24 Ohnishi, S. and Ito, T. (1974) Biochemistry 13, 881–887.
25 Shoji, T., Kashiwayanagi, M. and Kurihara, K. (1991) Comp. Biochem. Physiol., in press.
26 Tonosaki, K. and Tucker, D. (1982) Behav. Neur. Biol. 35, 187–199.
27 Huang, C. and Mason, J.T. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 308–310.
28 Kashiwayanagi, M., Suenaga, A., Enomoto, S. and Kurihara, K. (1990) Biophys. J. 58, 887–895.
29 Teorell, T. (1935) Proc. Soc. Exp. Biol. 33, 282–285.
30 Meyer, K.H. and Sievers, J.F. (1936) Helv. Chim. Acta 19, 649–664.
31 Miyake, M. and Kurihara K. (1983) Biochim. Biophys. Acta 762, 256–264.
32 Kurihara, K., Yoshii, K. and Kashiwayanagi, M. (1986) Comp. Biochem. Physiol. 85A, 1–22.
33 Kamo, N. and Kobatake, Y. (1974) J. Colloid Interface Sci., 46, 85–93.
34 Kumazawa, T., Nomura, T. and Kurihara, K. (1988) Biochemistry, 27, 1239–1244.