D_3 at high substrate concentrations (Bjorkhem & Holmberg, 1978). Because the microsomes are believed to be the major physiological site of 25-hydroxylation (Madhok & DeLuca, 1979), inhibition of the in vitro microsomal system would lend support for the latter mechanism. Inhibition was observed with a 100-fold excess of inhibitor (Figure 2) in such an assay. The unique inhibitory properties of 19-OH-10(*S*),19-DHD_3 therefore may result from its selective inhibition of a microsomal vitamin D_3-25-hydroxylase in conjunction with unaffected mitochondrial or exohepatic 25-hydroxylases. However, this will require a more direct investigation before it can be accepted.

Acknowledgments

We thank M. A. Fivizzani, L. E. Reeve, and C. M. Smith for their excellent technical assistance in performing the bioassays.

References

Bhattacharyya, M. H., & DeLuca, H. F. (1974a) *Biochem. Biophys. Res. Commun. 59*, 734.
Bhattacharyya, M. H., & DeLuca, H. F. (1974b) *Arch. Biochem. Biophys. 160*, 58.
Bjorkhem, I., & Holmberg, I. (1978) *J. Biol. Chem. 253*, 842.
Bjorkhem, I., Hansson, R., Holmberg, I., & Wikvall, K. (1979) *Biochem. Biophys. Res. Commun. 90*, 615.
Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
DeLuca, H. F., Paaren, H. E., & Schnoes, H. K. (1979) *Top. Curr. Chem. 83*, 1–65.
Holick, M. F., Kasten-Schraufrogel, P., Tavela, T., & DeLuca, H. F. (1975) *Arch. Biochem. Biophys. 166*, 63.
Holick, S. A., Holick, M. F., Tavela, T. E., Schnoes, H. K., & DeLuca, H. F. (1976) *J. Biol. Chem. 251*, 1025.
Lund, J., & DeLuca, H. F. (1966) *J. Lipid Res. 7*, 739.
Madhok, T. C., & DeLuca, H. F. (1979) *Biochem. J. 184*, 491.
Martin, D. L., & DeLuca, H. F. (1969) *Am. J. Physiol. 216*, 1351.
Mourino, A., & Okamura, W. H. (1978) *J. Org. Chem. 43*, 1653.
Norman, A. W., Hammond, M. L., & Okamura, W. H. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 914.
Onisko, B. L., Schnoes, H. K., & DeLuca, H. F. (1979a) *J. Biol. Chem. 254*, 3493.
Onisko, B. L., Schnoes, H. K., DeLuca, H. F., & Glover, R. S. (1979b) *Biochem. J. 182*, 1.
Paaren, H. E. (1976) Ph.D. Thesis, University of Illinois, Chicago Circle.
Ponchon, G., & DeLuca, H. F. (1969) *J. Clin. Invest. 48*, 1273.
Sulimovici, S., Roginsky, M. S., Duffy, J. L., & Pfeifer, R. F. (1979) *Arch. Biochem. Biophys. 195*, 45.
Tucker, G., III, Grognon, R. E., & Haussler, M. R. (1973) *Arch. Biochem. Biophys. 155*, 47.

Changes in Membrane Potential and Membrane Fluidity in *Tetrahymena pyriformis* in Association with Chemoreception of Hydrophobic Stimuli: Fluorescence Studies†

Hiroshi Tanabe,* Kenzo Kurihara, and Yonosuke Kobatake

ABSTRACT: The fluorescence intensity of rhodamine 6G (Rh6G) and 1,6-diphenyl-1,3,5-hexatriene (DPH) in the presence of *Tetrahymena pyriformis* was measured to monitor changes in the membrane potential and in the gross structure of the surface membrane in response to chemical stimuli. So-called "odorants" for higher vertebrates, which are usually uncharged and hydrophobic compounds, were chosen as chemical stimuli for a model study of the olfactory response. The fluorescence intensity of Rh6G started to increase at the chemotactic thresholds of the stimuli, indicating that negative chemotaxis of *T. pyriformis* to the hydrophobic stimuli is induced by depolarization of the cell. The fluorescence intensity of DPH increased in close association with chemoreception of the hydrophobic stimuli. The increase in the fluorescence intensity was ascribed mainly to uptake of DPH, suggesting that gross structural changes of the surface membrane occur with the reception of hydrophobic stimuli. The membrane fluidity determined by fluorescence polarization of DPH increased in close association with the chemoreception of the hydrophobic stimuli. Inorganic salts such as NaCl, KCl, and $CaCl_2$ did not change the DPH fluorescence intensity or the fluorescence polarization, although these stimuli induced depolarization and negative chemotaxis in *T. pyriformis*.

Living organisms from unicellular organisms to higher vertebrates have an ability to recognize chemical stimuli in external environments. The response of unicellular organisms to chemical stimuli can be seen in chemotaxis. In higher vertebrates, chemical stimuli in the external environment are received at gustatory and olfactory cells. Recently much attention has been paid to the molecular mechanism of chemoreception in the sensory cells, but a detailed mechanism is still unknown. The difficulties encountered in exploring the mechanism come from the limitations of the techniques that can be applied; few techniques besides electrophysiological ones can be applied to intact sensory cells. Furthermore, in the case of olfactory cells, which are terminal swellings of olfactory nerves, intracellular recordings of electrical properties of the cells are extremely difficult because of the small size of the

† From the Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan. *Received November 8, 1979.*

0006-2960/80/0419-5339$01.00/0

cells. It is still unknown how "odorants", which are usually uncharged and hydrophobic compounds, induce changes in the membrane potential of the olfactory cells.

Tetrahymena pyriformis is a ciliated protozoan and is considered to have excitable membranes on the basis of the fact that the related organism *Paramecium* has excitable membranes (Naitoh & Eckert, 1969; Eckert, 1972). *T. pyriformis* exhibits negative chemotaxis to various inorganic salts and hydrophobic compounds (Tanabe et al., 1979). In a previous paper, we showed that chemoreception of *T. pyriformis* to so-called odorants for higher vertebrates has a close correlation to the olfactory response in humans (Ueda & Kobatake, 1977). *T. pyriformis* is a useful model for the chemosensory cells in studying the receptor mechanism at the membrane level, because a large amount of cell suspension is easily available.

In recent years, fluorescence probes have been extensively used to investigate the dynamic properties of biological membranes. Fluorescence probes can be classified as "potential probes" or "structural probes". In a previous paper, we showed that fluorescence changes of rhodamine 6G (Rh6G) well monitor changes in the membrane potential of *T. pyriformis* in response to inorganic salts (Aiuchi et al., 1980). This method is applied in the present study to monitor changes in the membrane potential in response to certain hydrophobic compounds (odorants for higher vertebrates). We also use 1,6-diphenyl-1,3,5-hexatriene (DPH) to monitor gross structural changes of the surface membrane. It is shown that changes in the membrane potential and the membrane fluidity occur in close association with the reception of hydrophobic compounds. The significance of the membrane fluidity changes in the reception of hydrophobic compounds is discussed.

Experimental Procedures

Cell Growth. *T. pyriformis* (strain w) was grown at 22 °C in the medium containing 2% proteose peptone, 1% yeast extract, and 0.6% glucose. The cells taken from 2- or 3-day-old culture were collected by gentle filtration through a filter paper (Whatman No. 3) and washed thoroughly with 1 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl) buffer of pH 7.0 (control solution).

Isolation of Membrane Fraction. The surface membrane fraction (pellicle) was isolated according to the method of Nozawa & Thompson (1971). The fractionated membranes were resuspended in the control solution and used for the experiments.

Fluorescence Measurements. All the fluorescence measurements were made at 22 °C. Fluorescence intensity and polarization were measured with a fluorescence spectrophotometer, Hitachi MPF-2A, equipped with a circulating water bath (Haake).

(*1*) *Measurements of Rh6G Fluorescence.* Rhodamine 6G (Rh6G) dissolved in distilled water was added to a final concentration of 0.7 μM into the cell suspension (1.2×10^4 cells/mL) and equilibrated at 22 °C for 20 min. Into a cuvette, 3 mL of the suspension was pipetted for the fluorescence measurement. A given volume of stimulating solution of varying concentrations was added into the cuvette, and the fluorescence intensity was measured. The excitation wavelength was 520 nm and the fluorescence was detected at 550 nm. Some stimulating chemicals were dissolved in ethanol. The final concentration of ethanol never exceeded 1% in any experiment. The fluorescence intensity was not affected by 1% ethanol itself. The change in the fluorescence intensity was defined as eq 1 where F and F_0 represent the fluorescence intensities in the presence and absence of stimulating chemicals.

$$\Delta F = (F - F_0)/F_0 \quad (1)$$

(*2*) *Fluorescence Labeling with DPH.* The labeling procedure of *T. pyriformis* with DPH was carried out according to Shinitzky & Inbar (1974). Two millimolar 1,6-diphenyl-1,3,5-hexatriene (DPH) in tetrahydrofuran (THF) was diluted 1000-fold with the control solution under vigorous stirring. Stirring was continued for 20 min. One volume of cell suspension (1.5×10^4 cells/mL) in the control solution was mixed with 1 volume of the DPH dispersion and equilibrated at 22 °C for 1 h. *T. pyriformis* in the suspension thus prepared exhibited normal motility for at least 1 day. This indicates that 1 μM DPH and 0.1% THF did not affect their motility. The suspension of unlabeled cells in the same density was used as a reference sample.

The surface membranes (pellicle) were labeled in a manner similar to the case of the intact cells. The equilibration period was 30 min.

(*3*) *Measurements of DPH Fluorescence.* Two milliliters of the cell suspension was pipetted into a cuvette and stirred gently with a magnetic stirrer. After 10 min of stirring, hydrophobic compounds dissolved in ethanol or inorganic salts dissolved in water were added to the cuvette. The final concentration of ethanol was <1%. Control experiments indicated that the presence of 1% ethanol had no significant effect on the DPH fluorescence. After 15 min, $I_\parallel$ and $I_\perp$ were measured, where $I_\parallel$ and $I_\perp$ are the fluorescence intensities detected through a polarizer oriented parallel and perpendicular to the direction of polarization of the exciting beam.

DPH was excited at 360 nm and the fluorescence was detected at 430 nm through a 390-nm cutoff filter. Because of the reversible self-bleaching of DPH, the DPH-labeled systems were exposed to the exciting beam for <10 s.

Fluorescence intensity and polarization were obtained by measurements of $I_\parallel$ and $I_\perp$. The contribution of scattering light and the intrinsic fluorescence of *T. pyriformis* was corrected according to Shinitsky et al. (1971) by measurements of the fluorescence intensity of the unlabeled sample. The degree of fluorescence polarization, P, and the total fluorescence intensity, F, were defined as the equations

$$P = \frac{(I_\parallel - I_\parallel^s) - (I_\perp - I_\perp^s)}{(I_\parallel - I_\parallel^s) + (I_\perp - I_\perp^s)} \quad (2)$$

$$F = (I_\parallel - I_\parallel^s) + 2(I_\perp - I_\perp^s) \quad (3)$$

where $I_\parallel^s$ and $I_\perp^s$ stand for the parallel and perpendicular components of the fluorescence intensity at 430 nm for the unlabeled sample. The change in the fluorescence intensity of DPH was also defined as eq 1.

For the membrane fraction, the suspension of isolated surface membranes at a protein concentration of 67 μg of protein/mL was measured after 15 min of incubation with and without stimulating chemicals. In this case, no correction for the scattering light was made, because its contribution was <2% of the total fluorescence intensity.

Excited-State Lifetime of DPH in the Liposomal Suspension. The excited-state lifetime of DPH in the liposomal suspension was measured at 22 °C by a pulse technique with a time amplitude converter (TAC). The excitation wavelength was 337 nm obtained with a nitrogen gas laser, and the emission was detected at 430 nm.

Liposomes were prepared as follows: the total lipids were extracted from *T. pyriformis* according to the method of Bligh & Dyer (1959). The lipids obtained were dissolved in chlo-

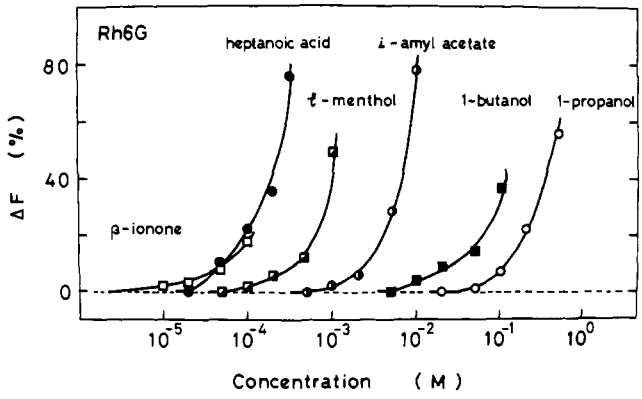

FIGURE 1: Changes in fluorescence intensity (ΔF) of Rh6G as a function of concentration of stimuli. 0.7 μM Rh6G was added to a *T. pyriformis* cell suspension of 1.2 × 10^4 cells/mL at 22 °C.

roform and stored at −20 °C in nitrogen gas until use. The solvent was evaporated under vacuum. The dispersions of the lipids were made in the control solution by agitation of a flask with a Vortex mixer. The dispersed lipids were sonicated for 10 min, and the liposomal suspension was centrifuged at 20000*g* for 30 min. The supernatant was used for the experiments. The final concentrations of liposomes and DPH were 0.5 mg of lipid/mL and 1 μM, respectively.

Binding Studies. Two samples of *T. pyriformis* cell suspension (7.5 × 10^3 cells/mL) were prepared containing 1 μM DPH. Isoamyl acetate dissolved in ethanol was added to one sample to the final concentration of 5 mM, and the ethanol of the same volume was added to another. After 10 min of standing, samples were centrifuged at 8000*g* for 10 min. One milliliter of each supernatant was pipetted into a cuvette in which 1 mL of THF was placed, and the fluorescence intensity was measured. The fluorescence intensity of a 1:1 mixture of 1 μM DPH solution and THF was also measured as reference.

Reagents. The following chemicals were obtained from the indicated sources and used without further purification: rhodamine 6G [commercial name, Rhodamine 6GO, 3,6-bis(ethylamino)-2,7-dimethyl-9-(2′-carbethoxyphenyl)-xanthenyl chloride] (Chroma Gesellschaft Schmid & Co.); 1,6-diphenyl-1,3,5-hexatriene (Tokyo Chemical Industry, Ltd.); 1-propanol, 1-butanol, *l*-menthol, skatole, and coumarin (Wako Pure Chemical Industries, Ltd.); isoamyl acetate, 1-heptanoic acid, and β-ionone (Nakarai Chemical, Ltd.).

Results

Changes in Fluorescence Intensity of Rh6G. Changes in the membrane potential of *T. pyriformis* in response to certain hydrophobic compounds (odorants for higher vertebrates) were monitored by measuring fluorescence intensity of Rh6G. An addition of the hydrophobic compounds to Rh6G–*T. pyriformis* cell suspension increased the fluorescence intensity. As shown in the previous paper (Aiuchi et al., 1980), an increase in the fluorescence intensity of Rh6G implies that depolarization occurs in response to the stimuli. Figure 1 represents changes in the fluorescence intensity, ΔF, as a function of the concentration of chemical stimuli. The fluorescence intensity starts to increase at certain concentrations for respective compounds, which are hereafter refered to as C_F^{Rh6G}. In Figure 2 (closed circles), the threshold concentrations of chemotaxis (C_{taxis}) in response to the hydrophobic compounds, which are taken from the previous paper (Ueda & Kobatake, 1977), are plotted against C_F^{Rh6G}. The figure shows that there is a good correlation between the two, indicating that changes in the membrane potential occur in close association with the

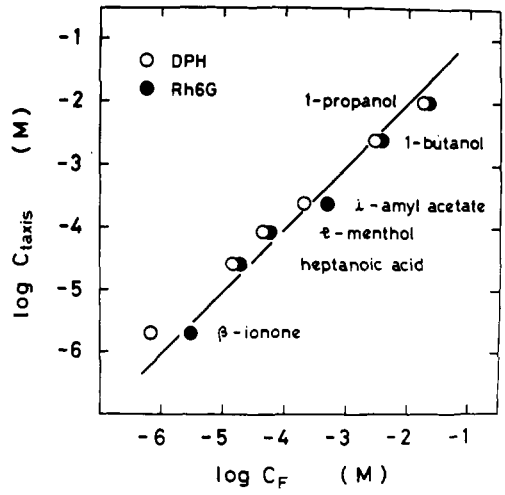

FIGURE 2: Relationship between thresholds in fluorescence change, C_F, and those in chemotaxis, C_{taxis}. (●) Rh6G; (○) DPH.

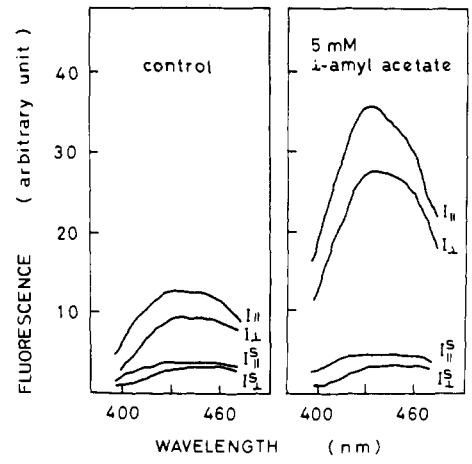

FIGURE 3: The effect of 5 mM isoamyl acetate on the fluorescence spectra of 1 μM DPH bound to *T. pyriformis*. I^S in the figure represents the cell background (see text). The excitation wavelength was 360 nm, and the fluorescence was detected through a 390-nm cutoff filter.

chemoreception of the hydrophobic compounds.

Fluorescence Spectra and Locus of DPH. The DPH fluorescence was measured to obtain information of the gross structural change in the surface membrane of *T. pyriformis* in response to the chemical stimuli. Figure 3 illustrates the fluorescence spectra of DPH bound to *T. pyriformis* in the absence (A) and presence (B) of 5 mM isoamyl acetate. The excitation wavelength was 360 nm, and DPH bound to the cell has an emission maximum at 430 nm. I^S in Figure 3 represents the fluorescence spectra of unlabeled cell suspension measured under the same condition. The correction for the scattering light and the intrinsic fluorescence of *T. pyriformis* was made as described under Experimental Procedures.

Incubation of the cells with an aqueous dispersion of DPH is accompanied by a sharp increase in fluorescence intensity, which levels off after ~1 h at 22 °C as shown in Figure 4. Simultaneously, the degree of fluorescence polarization (P) was determined. Figure 4 also shows that the value of P remains constant throughout the labeling process. The P value obtained with intact cells in the absence of chemical stimuli (control in Table I) was 0.242 ± 0.010, which is close to the value determined with the surface membranes of *T. pyriformis* (0.246 ± 0.008).

Changes in Fluorescence Intensity of DPH. Figure 5 shows a plot of changes in the fluorescence intensity (ΔF) of DPH

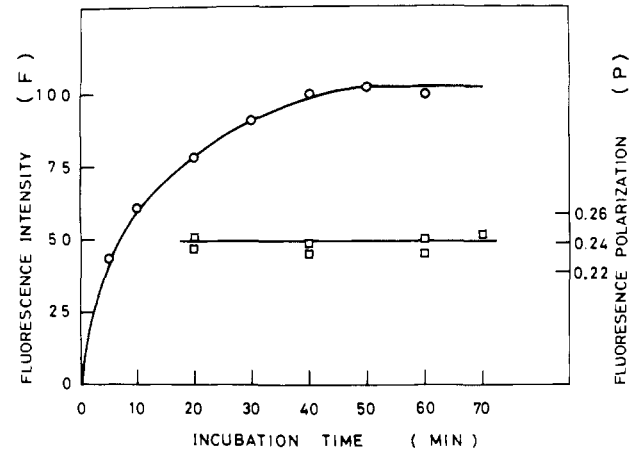

FIGURE 4: The increase in fluorescence intensity (F) with time of 7.5 $\times 10^3$ cells/mL incubated with 1 μM DPH dispersion in the control solution at 22 °C. The degree of the fluorescence polarization (P) at different times of incubation was also included.

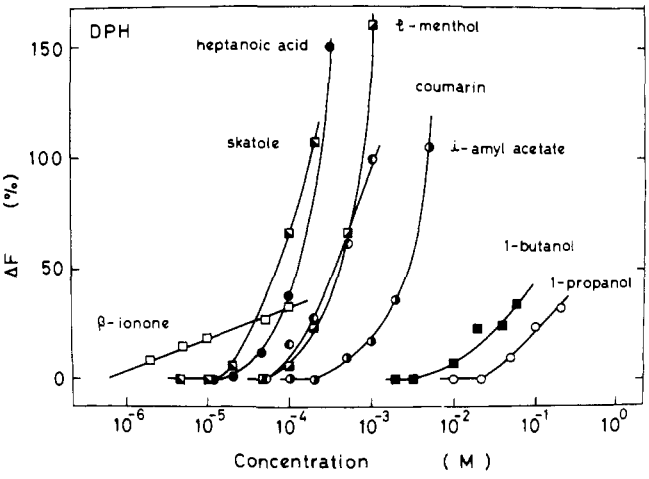

FIGURE 5: Changes in fluorescence intensity (ΔF) of DPH bound to the cells as a function of chemical stimuli. The fluorescence intensity was measured at 430 nm through a 390-nm cutoff filter. The contribution of the cell background was substracted as described under Experimental Procedures.

bound to the cells as a function of the concentration of hydrophobic compounds. The fluorescence intensity starts to increase at certain concentrations for respective chemicals, which is referred to as C_F^{DPH}. Figure 2 (open circles) shows a good correlation between C_{taxis} and C_F^{DPH}, indicating that the fluorescence intensity of DPH starts to increase in close association with the chemoreception of hydrophobic compounds. Contrary to the case of the hydrophobic compounds, inorganic salts such as NaCl, KCl, and $CaCl_2$ did not change the DPH fluorescence.

The fluoresence intensity of the DPH dispersion in the absence of the cells was a few percent of that in the presence of the cells. Therefore, most of the fluorescence intensity from the latter suspension comes from DPH bound to *T. pyriformis* cells. The above results showing that the presence of the hydrophobic compounds increased the DPH fluorescence intensity suggest that the application of the hydrophobic stimuli to *T. pyriformis* brought about an increase of the dye bound to the cells.

Binding Studies. The measurement of DPH bound to the cells indicated that the dye bound to the cells was ~12% of the dye added. The addition of 5 mM isoamyl acetate increased the amount of the dye bound to ~35%. These results indicate that an increase in the fluorescence intensity of DPH

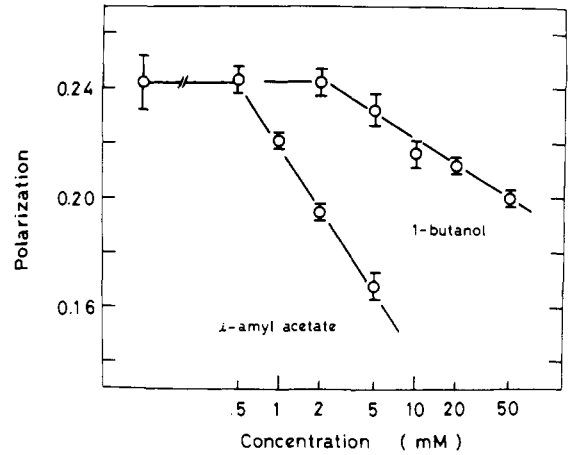

FIGURE 6: Dependence of the fluorescence polarization on concentration of isoamyl acetate or 1-butanol. The bars represent the range of experimental deviation.

Table I: Fluorescence Polarization Measured in the Presence of Hydrophobic Compounds

chemical	concn (M)[a]	polarization[b]
control		0.242 ± 0.010
1-propanol	2×10^{-1}	0.210 ± 0.001
1-butanol	3×10^{-2}	0.205 ± 0.005
isoamyl acetate	2×10^{-3}	0.195 ± 0.005
l-menthol	5×10^{-4}	0.166 ± 0.001
1-heptanoic acid	2×10^{-4}	0.198 ± 0.005
β-ionone	5×10^{-4}	0.220 ± 0.005

[a] The concentrations are 10 times the chemotactic threshold concentrations for respective chemicals except for β-ionone. In the case of β-ionone, the concentration is 250 times the chemotactic threshold. [b] Each value is the average of two or three determinations. The value of the control is the average of all determinations.

brought about the addition of hydrophobic compounds was mainly attributed to an increase in the amount of the dye bound to the cells.

Changes in Fluorescence Polarization of DPH. Figure 6 shows a plot of the P value against concentration of isoamyl acetate and 1-butanol. The degree of fluorescence polarization decreases linearly with concentration of the chemical applied. It should be noted that the concentrations at which the fluorescence polarization starts to decrease are practically identical with respective C_{taxis} or C_F^{DPH}.

As shown in Figure 6, the P value is a function of the concentration of chemical stimuli. Table I compares the P value in the presence of chemical stimuli of 10 times the chemotactic threshold concentrations. In the case of β-ionone, the P value at 250 times the threshold is listed, since β-ionone has only a small effect on the P value. All the hydrophobic compounds examined decreased the fluorescence polarization, but the degree of its change varies among the chemicals. On the other hand, inorganic salts such as NaCl, KCl, and $CaCl_2$ did not change the fluorescence polarization, although these inorganic salts induced changes in the membrane potential and negative chemotaxis in *T. pyriformis*.

The comparison of the fluorescence spectra of DPH in various solvents suggested that the locus of solubilization of DPH is the hydrocarbon region of the membrane (Shinitzky et al., 1971; Shinitzky & Inbar, 1974; Cogan et al., 1973). This was also supported by the following results. A decrease in the P value in response to hydrophobic compounds was observed in the DPH–liposome suspension, similar to the DPH–*T. pyriformis* cell suspension, although the degree of

the decrease of the *P* value in the former suspension was less than that of the latter. For estimation of the effect of the hydrophobic stimuli on the excited-state lifetime of DPH, it was measured in the liposomal suspension in the absence and presence of 5 mM isoamyl acetate. The lifetime of DPH in the liposomal suspension was 8.3 ns, and the addition of 5 mM isoamyl acetate did not change it. These results suggest that the decrease in the fluorescence polarization in response to the reception of hydrophobic compounds is caused by an increase in the fluidity of the surface membrane.

The present results showed that the addition of hydrophobic compounds to the DPH–cell suspension increased the fluorescence intensity of DPH and decreased the *P* value. These changes induced by the addition of the hydrophobic compounds were fully reversible; the fluorescence intensity and the *P* value returned to the original value after the hydrophobic compounds added were washed out and the cells were resuspended in the control solution. Furthermore, it was confirmed that no protein was released from the cell by the addition of hydrophobic compounds.

Discussion

Rh6G fluoresces in an aqueous solution, and its fluorescence is quenched by the adsorption of the dye onto the membrane. Depolarization of cells leads to a release of the dye into an external medium, which in turn increases the fluorescence intensity. The fluorescence change of Rh6G reflects a change in the membrane potential. In the previous paper (Aiuchi et al., 1980), we showed that negative chemotaxis of *T. pyriformis* to various inorganic salts is induced by depolarization of the cell. The present results indicate that negative chemotaxis of *T. pyriformis* against the hydrophobic compounds is also induced by depolarization. In these studies, more than 20 chemicals including inorganic salts and hydrophobic compounds depolarized *T. pyriformis* cells, and all these chemicals induced negative chemotaxis. On the basis of the studies on *Paramecium* (Naitoh & Eckert, 1969), depolarization of the cell opens the voltage-sensitive Ca channel and induces a transient "Ca response". In fact, we have found that the elimination of Ca^{2+} from the external medium by an addition of [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) suppressed the chemotactic responses in *T. pyriformis* to inorganic salts and hydrophobic compounds without affecting its motility.

Eukaryotic cells have a number of hydrophobic intracellular locations, and hence DPH may bind to these locations. In general, the membrane fluidity varies among membranes of different organelles in the same species of cells. For example, the microsomal membranes of *T. pyriformis* are much more fluid than the surface membranes (Shimonaka et al., 1978). Hence the *P* value must change during the labeling process of DPH if DPH molecules penetrate into *T. pyriformis* cell and bind to intracellular locations. However, the *P* value was unchanged during the labeling process where the labeling reached a steady level in about 1 h. Furthermore, the *P* value obtained with intact cells in the present study was practically identical with that obtained with the fractionated surface membranes. These results suggest that DPH is bound to the surface membrane of *T. pyriformis* under the experimental condition employed in the present study.

The application of the hydrophobic compounds to the *T. pyriformis* cell suspension increased the fluorescence intensity of DPH. The binding studies indicated that the increase in the fluorescence intensity was mainly due to uptake of DPH by *T. pyriformis* in association with chemoreception. It is unlikely that the uptake of DPH by the cell is ascribed to changes in the membrane potential since inorganic salts did not increase the fluorescence intensity and DPH molecule has no electric charge. Probably, the gross structural change of the surface membrane induced by the reception of hydrophobic stimuli leads to the uptake of DPH. An increase in the fluorescence intensity of uncharged lipophilic agent was also observed when colicin was added to the suspension of *Escherichia coli* labeled with *N*-phenylnaphthylamine (NPN) (Nieva-Gomez et al., 1976). This increase in the fluorescence intensity was also explained as a result of an increase of the dye bound to the cell.

In the liposomal system, hydrophobic compounds did not affect the fluorescence lifetime of DPH. This result suggests that the change in the fluorescence polarization of DPH reflects that in the membrane fluidity. The reception of hydrophobic stimuli in *T. pyriformis* led to an increase in the fluidity of the surface membrane, while that of inorganic salts led to no change in the fluidity. Since the concentrations at which the membrane fluidity starts to increase were practically identical with respective thresholds of the chemotactic responses, the increase in the membrane fluidity seems to be essentially important for the initial process of chemotaxis.

Although both inorganic salts and hydrophobic compounds depolarized *T. pyriformis*, inorganic salts did not change the membrane fluidity. In previous studies (Aiuchi et al., 1980), we showed that changes in the surface potential of the cell contribute significantly to those in the membrane potential in response to inorganic salts. Inorganic salts seem to interact directly with the hydrophilic region of the membrane surface and induce the change in the surface potential. Recently, we have monitored the change in the surface potential of *T. pyriformis* in response to the hydrophobic compounds by use of 8-anilino-1-naphthalenesulfonate (Ans) and observed that the hydrophobic compounds also change the surface potential of the cell in close association with their chemoreception (H. Tanabe, K. Kurihara, and Y. Kobatake, unpublished data). The hydrophobic compounds used are electrically neutral, and hence their simple adsorption itself on the surface membrane of the cell does not induce changes in the surface potential. Probably, changes in the surface potential in response to the hydrophobic compounds are brought about by a conformational change of the surface membrane.

Koyama & Kurihara (1972) measured an increase in the surface pressure of the lipid monolayer induced by an adsorption of various odorants. Plots of log C_F^{DPH} against log C_{sp}, where C_{sp} is the concentration giving the surface pressure increase of 1 dyn/cm in the monolayer, give a straight line. One plausible explanation on the linear relationship is as follows: odorants are adsorbed on the hydrophobic region of the lipid layer of the surface membrane of *T. pyriformis* and increase the surface pressure of the membrane, which in turn leads to the gross structural change of the membrane. This increase in the surface pressure also leads to an increase in the membrane fluidity. Such structural changes of the membrane will lead to changes in the surface potential of the membrane. Further study will be needed to confirm the above explanation.

Acknowledgments

We express our thanks to Drs. T. Takemura, N. Suzuki, and Y. Yamada, The Research Institute of Applied Electricity, Hokkaido University, for measuring the fluorescence lifetime.

References

Aiuchi, T., Tanabe, H., Kurihara, K., & Kobatake, Y. (1980) *Biochim. Biophys. Acta 628*, 355.

Bligh, E. C., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911.
Cogan, U., Shinitzky, M., Weber, G., & Nishida, T. (1973) *Biochemistry 12*, 321.
Eckert, R. (1972) *Science 176*, 473.
Koyama, N., & Kurihara, K. (1972) *Nature (London) 236*, 402.
Naitoh, Y., & Eckert, R. (1969) *Science 164*, 963.
Nieva-Gomez, D., Konisky, J., & Gennis, R. B. (1976) *Biochemistry 15*, 2747.
Nozawa, Y., & Thompson, G. A., Jr. (1971) *J. Cell Biol. 49*, 722.
Shimonaka, H., Fukushima, H., Kawai, K., Nagao, S., Okano, Y., & Nozawa, Y. (1978) *Experientia 34*, 586.
Shinitzky, M., & Inbar, M. (1974) *J. Mol. Biol. 85*, 603.
Shinitzky, M., Dianoux, A. C., Gitler, C., & Weber, G. (1971) *Biochemistry 10*, 2106.
Tanabe, H., Kurihara, K., & Kobatake, Y. (1979) *Biochim. Biophys. Acta 553*, 369.
Ueda, T., & Kobatake, Y. (1977) *J. Membr. Biol. 34*, 351.

Kinetics of Binding of [^{3}H]Acetylcholine and [^{3}H]Carbamoylcholine to *Torpedo* Postsynaptic Membranes: Slow Conformational Transitions of the Cholinergic Receptor†

Norman D. Boyd and Jonathan B. Cohen*

ABSTRACT: The kinetics of binding of [^{3}H]acetylcholine (AcCh) and [^{3}H]carbamoylcholine (Carb) to membrane-bound nicotinic receptor from *Torpedo* electric tissue have been measured on the second time scale by rapid mixing and ultrafiltration. The concentration dependence of the association kinetics of agonist binding and the kinetics of ligand dissociation and receptor reisomerization following the removal of agonist are analyzed in terms of a model in which the observed binding is by a single population of receptors that exists in the absence of agonist in two interconvertible conformations, one binding agonist weakly (R_1) and the other binding with high affinity (R_2). A computer simulation has been used to determine values of rate and equilibrium constants characterizing the ligand interactions with the two conformations and for the conformational equilibrium in the presence and absence of agonist. At 4 °C, R_1/R_2 = 4.5, and the half-time for isomerization for low to high affinity of unliganded receptor is equal to 200 s, while for receptors occupied by either AcCh or Carb the half-time is reduced to ~4 s. For AcCh the apparent dissociation constants of the low- and high-affinity conformations are 800 nM and 2 nM, respectively (K_{eq} = 8 nM), and for Carb the values are 30 μM and 25 nM (K_{eq} = 100 nM). The dissociation rate constant of [^{3}H]AcCh from R_2 is equal to 0.04 s^{-1}. The results are further discussed in terms of alternate less satisfactory reaction models and are compared with the receptor conformational equilibria deduced by the use of other kinetic techniques.

Permeability control by nicotinic cholinergic receptors is characterized by two kinetically distinct processes: the primary permeability response (channel opening) that occurs within a fraction of a millisecond of the release of acetylcholine (AcCh)[1] from nerve and the slow decrease of that permeability response (desensitization) that occurs when a constant concentration of AcCh or other agonist is maintained for seconds or longer. Electrophysiological data concerning both channel opening and desensitization have been interpreted in terms of models where it is postulated that the nicotinic receptor (the AcCh binding protein and its associated channel) exists in a limited number of conformations differing both in their ligand binding properties and in the functional state of the ion channel [for reviews, see Gage (1976), Magazanik & Vyskocil (1976), and Colquhoun (1979)]. Thus, an "open channel" conformation is transiently stabilized when AcCh or another agonist is bound by the receptor, while prolonged exposure to AcCh permits the formation of a thermodynamically preferred "desensitized" conformation.

To provide direct evidence for these postulated receptor conformations, it is necessary to characterize under identical conditions the conformational equilibria defined by ligand binding and the functional state of the ion channel. Nicotinic postsynaptic membranes isolated from *Torpedo* electric tissue provide a unique preparation for such studies [for a review, see Heidmann & Changeux (1978)]. The cholinergic receptor in those membranes remains functional since cholinergic agonists cause increased cation permeabilities (Popot et al., 1976) and exposure to agonists for minutes or longer results in desensitization (Sugiyama et al., 1976; Bernhardt & Neumann, 1978). The use of rapid-mixing and quenched-flow techniques permits quantitative characterization of agonist dose–response relations for the *Torpedo* vesicles as well as a definition of the kinetics of channel activation and desensitization (Neubig & Cohen, 1980).

Analysis of the kinetics of binding of cholinergic ligands has begun to provide a definition of the conformational equilibria of the cholinergic receptor. The effects of cholinergic ligands on the kinetics of binding of radiolabeled α-neurotoxins (Weber et al., 1975; Weiland et al., 1977; Barrantes, 1978; Quast et al., 1978; Weiland & Taylor, 1979) provided evidence for

† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received February 4, 1980.* This research was supported by U.S. Public Health Service Grant NS 12408 and by a grant from the Sloan Foundation. N.D.B. was supported by a Muscular Dystrophy Association Postdoctoral Fellowship and by Postdoctoral Training Grant NS 07009 from the U.S. Public Health Service; J.B.C. was supported by Career Development Award NS 00155 from the National Institutes of Health.

[1] Abbreviations used: AcCh, acetylcholine; α-BgTx, α-bungarotoxin; Carb, carbamoylcholine; DFP, diisopropyl phosphorofluoridate; TPS, *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0, and 0.02% NaN_3).

0006-2960/80/0419-5344$01.00/0