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FLUORESCENCE CHANGES OF RHODAMINE 6G ASSOCIATED WITH CHEMOTACTIC RESPONSES IN *TETRAHYMENA PYRIFORMIS*

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Summary

1. The chemotactic responses in *Tetrahymena* to various inorganic salts and octanol were examined quantitatively. *Tetrahymena* exhibited negative chemotaxis to the salts and octanol above respective threshold concentrations. The order of the thresholds of salts was $NH_4Cl \simeq NaCl > LiCl > KCl > CaCl_2 \simeq MgCl_2 > LaCl_3$.

2. Fluorescence intensity of rhodamine 6G added to *Tetrahymena* suspension increased with increasing concentration of stimuli above respective thresholds. The fluorescence changes were closely correlated with the chemotactic responses for all the chemical stimuli examined.

3. Measurements of the fluorescence intensity of the supernatant of dye-*Tetrahymena* suspension showed that the dye was taken up by *Tetrahymena* and the addition of salts led to a release of the dye into an external medium.

4. The adsorption of dye on the liposome also led to the quenching of its fluorescence. Addition of salts to the dye liposome suspension brought about an increase of the fluorescence. The order of the effectiveness was monovalent < divalent < trivalent. The fluorescence intensity changed linearly with $\log[K]_{out}$ in the presence of valinomycin. It was concluded that the changes in fluorescence of rhodamine 6G reflect both changes in the surface potential and those in the intramembrane diffusion potential, which in turn reflect changes in the total membrane potential across the membrane. We suggest that the surface potential as well as the diffusion potential contributes to the changes in the membrane potential in *Tetrahymena* associated with the chemotactic responses.

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

Introduction

The chemical stimulation in the sensory cells of higher vertebrates is induced by adsorption of chemical stimuli on the receptor membrane [1]. The adsorption induces change in the membrane potential in sensory cells, which leads to generation of impulses in the sensory nerves. Much interest has been paid to the mechanism by which the adsorption of chemical stimuli induces the receptor potentials [1—5], but a detailed molecular mechanism is still unknown. The difficulties encountered in exploring the mechanism come from the limitations of the technique to be employed: few techniques besides an electrophysiological one can be applied to the intact sensory cells.

Many lower organisms exhibit chemotactic response to various chemical stimuli and provide a suitable system for investigation of the molecular mechanism in chemoreception. Some protozoa especially have excitable membranes [6,7] and hence are most desirable as a model for chemoreceptor system in higher vertebrates. The ciliated protozoan *Tetrahymena* exhibits tactic responses to various chemical stimuli. In the previous studies, we examined the chemotactic responses of *Tetrahymena* to so called 'bitter substances' [8] and 'odorants' [9] and found that the order of the threshold concentrations of various stimuli was quite similar to that in the sensory organs of the higher vertebrates. In addition, the chemotactic response in *Tetrahymena* is considered to be induced by changes in the membrane potentials inferred from the studies on *Paramecium* [6,7,10] which also belongs to ciliated protozoa. Thus *Tetrahymena* is a suitable model for the investigation of the chemoreceptive mechanism in higher vertebrates.

In the present study, the chemotactic responses in *Tetrahymena* to various inorganic salts and octanol, which is a typical organic chemoeffector, are examined quantitatively. We have used rhodamine 6G as a fluorescence probe to monitor a change in the membrane potential in *Tetrahymena* and have found that the fluorescence change of the dye is closely correlated with the chemotactic response. The mechanism of the fluorescence change of rhodamine 6G has been investigated with use of liposomes. Based on the results obtained, the mechanism of the potential change induced by various chemical stimuli in *Tetrahymena* is discussed.

Materials and Methods

Measurements of chemotactic responses

Tetrahymena pyriformis (strain w) was grown at 22°C in an enriched proteose peptone medium. The cells taken from 3 day culture were used for the experiments. They were collected by gentle filtration through a filter paper (Whatman No. 3) and washed thoroughly with control solution (1 mM Tris-HCl buffer, pH 7.0).

Chemotactic response was evaluated according to the following equation by measuring the density of the cells in stimulating solution (n_1) and that in control solution (n_2). All measurements were performed at 22°C.

$$R = (n_1 - n_2)/(n_1 + n_2)$$

The experimental details are described in a previous paper [11].

Preparation of liposomes

The lipids were obtained from *Tetrahymena* and *Escherichia coli*. *Tetrahymena* taken from 3 day culture was used for the lipid extraction. *E. coli* (strain B, IAM-1268) was grown at 37°C in the medium containing 1% polypeptone, 0.1% yeast extracts, 0.1% glucose and 0.8% NaCl. The cells in logarithmic phase were harvested. The total lipids were extracted from the organisms according to the method of Bligh and Dyer [12]. The lipids obtained were dissolved in chloroform and stored at −20°C in nitrogen gas until use.

Liposomes were prepared as follows: the solvent was evaporated under vacuum and dispersions of the lipids were made in the control solution, or a solution containing 99 mM NaCl, 1 mM KCl and 10 mM Tris-HCl buffer, pH 7.0, by agitating of a flask with a Vortex mixer. The dispersed lipids were sonicated for 60 min and the suspension obtained was centrifuged at $10^5 \times g$ for 20 min. The supernatant was used for the experiments.

Fluorescence measurements

The fluorescence dye, rhodamine 6G (commercial name Rhodamine 6G0; 3,6-bis(ethylamino)-2,7-dimethyl-9-(2′-carbethoxyphenyl)-xanthenyl chloride), was purchased from Chroma-Gesellshaft Schmid and Co. Fluorescence was measured with a Hitachi MPF-2A at 22°C (an excitation wavelength of 520 nm and an emission wavelength of 550 nm).

Tetrahymena. The organisms, washed thoroughly with the control solution, were resuspended in the control solution ($1.2 \cdot 10^5$ cells/ml). A given volume of rhodamine 6G solution (final concentration of 0.7 μM) was added to the suspension and this suspension was allowed to stand for 20 min at 22°C. 3 ml of the suspension were pipetted into a cuvette for fluorescence measurements. A given volume of salt solution of varying concentrations was added into one cuvette and the same volume of water was added into another cuvette. For the subsequent discussion, the change in the fluorescence intensity, Δf, is defined as

$$\Delta f = (f - f_0)/f_0 \times 100\ (\%)$$

where f and f_0 stand for the fluorescence intensities in dye-*Tetrahymena* suspension in the presence and in the absence of chemical stimuli, respectively.

Liposomes. 3 ml of liposomal suspension (0.05 mg lipid per ml of control solution) were pipetted into a cuvette and a given volume of the dye solution (final concentration of 0.5 μM) was added. After addition of salt solution of varying concentrations, the fluorescence was measured.

Fluorescence of liposomal suspension in the presence of valinomycin was measured as follows. Liposomes prepared in a solution containing 99 mM NaCl, 1 mM KCl and 10 mM Tris-HCl buffer, pH 7.0, were used. External KCl concentration was varied under the condition where the total concentration of [NaCl + KCl] in 10 mM Tris-HCl buffer solution was kept at 100 mM. The final concentration of liposomes, rhodamine 6G and valinomycin were 0.02 mg lipids/ml 2 μM and 0.07 μM, respectively. The change in fluorescence intensity, Δf, is defined as is similar to the cases of the dye-*Tetrahymena* suspension.

Results

Chemotactic responses

The solid lines in Fig. 1 represent the chemotactic responses of *Tetrahymena* to inorganic salts. The organisms exhibit negative chemotaxis to all the salts examined above respective threshold concentrations (C_{taxis}). The magnitude of the response increases linearly with logarithmic concentration of chemical stimuli. The threshold concentrations of monovalent cations vary in the order: $NH_4Cl \simeq NaCl > LiCl > KCl$. The thresholds of salts of polyvalent cations are lowered with an increase of valence of cations. No significant difference is found between the threshold of $CaCl_2$ and $MgCl_2$. *Tetrahymena* exhibited negative chemotaxis to chemical stimuli besides salts. The dotted line in Fig. 1 represents the chemotactic response to octanol. The threshold concentration of octanol is 10^{-4} M.

Fluorescence changes in rhodamine 6G-Tetrahymena suspension

Rhodamine 6G, a fluorescence dye, was added to *Tetrahymena* suspension and changes in the fluorescence intensity in response to various inorganic salts and octanol were measured. The addition of rhodamine 6G did not bring about any effect on the mobility of the organism under the experimental conditions employed here. Rhodamine 6G in *Tetrahymena* suspension exhibits emission maximum at 548 nm and the addition of the chemical stimuli brought about an increase in the fluorescence intensity without the shift of the emission maximum. The addition of inorganic salts or octanol to the dye solution did not cause any change in the fluorescence. Hence it is evident that the change in the fluorescence reflects a change in a certain state of *Tetrahymena* in response to the chemical stimuli. Fig. 2 represents changes in fluorescence intensity as a function of concentration of inorganic salts (solid lines) and octanol (dotted line). The figure shows that the fluorescence intensity starts to change above certain concentration for respective chemical stimuli, which hereafter is referred to as the threshold of fluorescence changes ($C_{fluor.}$). In Fig. 3, $C_{fluor.}$ is plotted against C_{taxis} determined in Fig. 1. Good correlation is found between both thresholds. Note that the correlation holds with octanol as well as salts.

Inferred from the studies on *Paramecium* [7], the chemotactic response in

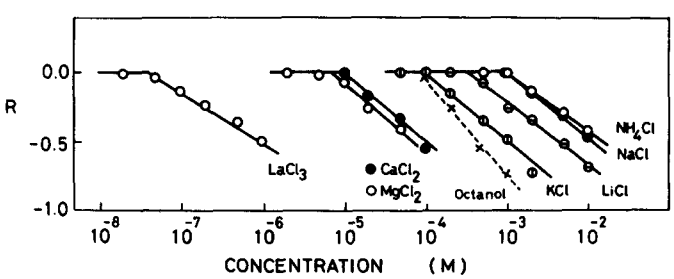

Fig. 1. The magnitude of chemotactic responses of *Tetrahymena* to various inorganic salts and octanol. Data shown here besides NH_4Cl, LiCl and octanol were cited from a previous paper [11]. Negative sign of *R* represents negative taxis.

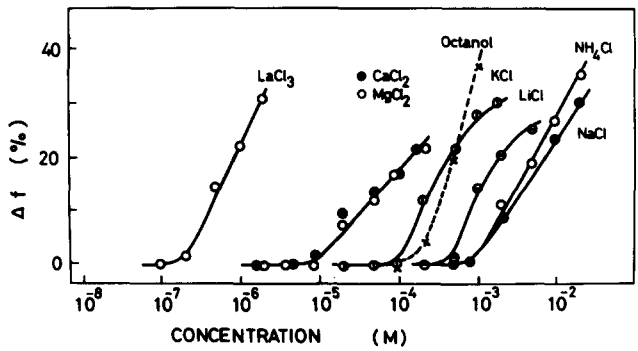

Fig. 2. Fluorescence change (Δf) of 0.7 μM rhodamine 6G in *Tetrahymena* suspension ($1.2 \cdot 10^5$ cells/ml) as a function of concentration of chemicals.

Tetrahymena is considered to be induced by a change in the membrane potential of cells. The correlation shown in Fig. 3 suggests that the fluorescence change of rhodamine 6G monitors the membrane potential of *Tetrahymena*. Elucidation of the mechanism by which the fluorescence of the dye reflects the membrane potential would aid the interpretation of the mechanism of the potential changes in *Tetrahymena* in response to chemical stimuli. In the following, we examine the mechanism of the fluorescence change with use of *Tetrahymena* and liposomes as a simple model system.

Release of the dye from Tetrahymena

In order to examine whether or not rhodamine 6G is released from *Tetrahymena* cells into an external medium in response to addition of chemical stimuli, the dye-*Tetrahymena* suspension in the presence of salts of varying concentration was centrifuged and the fluorescence of the supernatant was measured. Fig. 4 shows fluorescence change of the supernatant as a function of

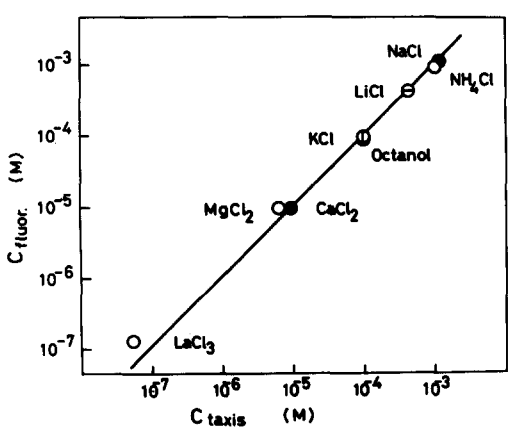

Fig. 3. Relation between the threshold concentrations in fluorescence ($C_{fluor.}$) and those in chemotaxis (C_{taxis}). The line in the figure has a unit slope.

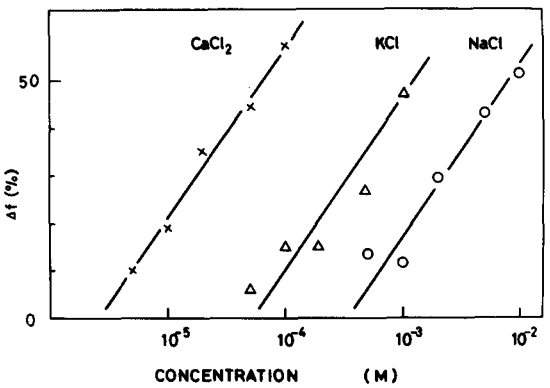

Fig. 4. Fluorescence change (Δf) of the supernatant obtained by the centrifugation of 0.7 μM rhodamine 6G-*Tetrahymena* suspension in the presence of varying concentrations of salt.

concentration of NaCl, KCl and $CaCl_2$ added to the suspension. As seen from the figure, the fluorescence in the supernatant increases with increase of salt concentration. Hence it is evident that the dye is released from *Tetrahymena* cells into the external medium in response to addition of salts.

The effectiveness of salts to induce changes in the fluorescence of the supernatant was in the order: $NaCl < KCl < CaCl_2$, which is coincident with the order in the case of the dye-*Tetrahymena* suspension. The concentrations of salts where the fluorescence change is induced were a little lower than respective $C_{fluor.}$ obtained with the dye-*Tetrahymena* suspension.

Fluorescence change in rhodamine 6G-liposome suspension

Rhodamine 6G in aqueous solution exhibits fluorescence spectrum having an

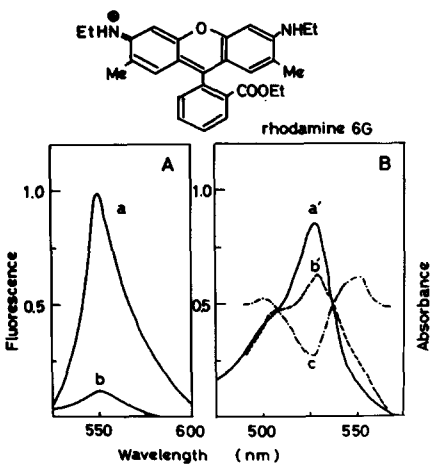

Fig. 5. Effect of liposomes on the fluorescence and absorption spectra of rhodamine 6G. (A) Curves a and b; fluorescence spectra of 10 μM rhodamine 6G without and with liposomes (0.09 mg lipid/ml). (B) Curves a′ and b′; absorption spectra of 10 μM rhodamine 6G without and with liposomes (0.09 mg lipid/ml). Curve c; difference spectrum of 10 μM rhodamine 6G between a′ and b′. The liposomes were made of the total lipids from *Tetrahymena*.

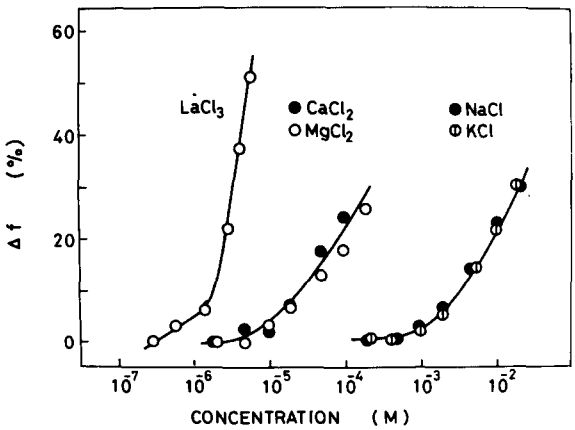

Fig. 6. Fluorescence change (Δf) of 0.5 μM rhodamine in the liposomal suspension (0.05 mg lipid/ml) as a function of salt concentrations. The liposomes were made of the total lipids extracted from *Tetrahymena*.

emission maximum at 478 nm. Yaginuma et al. [13] reported that the fluorescence was decreased when negatively charged chemicals such as sodium dodecyl sulfate, tetraphenylboron and dextran sulfate were added to aqueous rhodamine solution. Addition of proteins such as albumin, lysozyme or trypsin to aqueous dye solution did not change the fluorescence. The fluorescence increased in polar organic solvents such as alcohol or acetone. The dye exhibited practically no fluorescence in non-polar solvents such as toluene or pentane.

When the liposomes made of the total lipids from *Tetrahymena* were added to aqueous dye solution, the fluorescence was greatly quenched, as shown by curves a and b in Fig. 5A. In Fig. 5B, the absorption spectra in the absence (a′) and presence (b′) of the liposomes are illustrated. Curve c represents the difference spectrum between a′ and b′. The figure indicates that the addition of the liposomes leads to a decrease of the absorbance at 522 nm and an increase of that at 548 nm. The fluorescence change at 548 nm is proportional to the increase in absorption at 522 nm and to the decrease in that at 548 nm.

Fig. 6 represents the fluorescence changes in the liposomes suspension as a function of concentration of various inorganic salts. Above certain concentrations for respective salts, the fluorescence increases with increasing salt concentration. The order of the effectiveness of cations to induce the fluorescence change is trivalent > divalent > monovalent. These results resemble those observed in the dye-*Tetrahymena* suspension, although no difference in the effectiveness among salts of monovalent cations was found in the liposomal suspensions. As discussed later, the above results, that the effectiveness of cations increased with increasing valency of cations, suggest that the fluorescence of rhodamine 6G reflects changes in the surface potential of the membrane.

The fluorescence of rhodamine 6G also reflects changes in the membrane potential induced by permeation of ions across the membrane as shown below. The liposomes loaded with 1 mM KCl were prepared and KCl concentration in

the external medium was varied in the presence of valinomycin under the condition where the total ionic strength in the medium was kept constant. In this experiment, the liposomes made of the lipids from *E. coli* were used, since the changes in the fluorescence of rhodamine 6G in the liposomal suspension were larger than those in the suspension of liposomes made of the lipids from *Tetrahymena*. The fluorescence of rhodamine 6G added to the liposomal suspension was increased linearly with logarithmic concentration of KCl in the external medium, while no change was observed in the absence of valinomycin. The membrane potential, $\Delta\psi$, induced by permeation of K^+ is represented by Eqn. 1.

$$\Delta\psi = \frac{RT}{F} \ln \frac{[K]_{out}}{[K]_{in}} \quad (1)$$

where $[K]_{in}$ and $[K]_{out}$ represent K^+ concentration in the internal and external medium, respectively and other symbols have their usual meanings. The linear increase of the fluorescence with ln $[K]_{out}$ satisfied Eqn. 1.

Discussion

Rhodamine 6G in an aqueous solution fluoresces and is quenched in non-polar solvents. Addition of liposomes to aqueous dye solution led to quenching. These results suggest that a change in the amount of the dye bound to the hydrophobic region of the surface membrane in *Tetrahymena* is a cause for the fluorescence change. In fact, it has been shown in Fig. 4 that rhodamine 6G is taken up by *Tetrahymena* and the addition of salts leads to the release of dye into an external medium.

An addition of salts to rhodamine 6G-liposome suspension brought about an increase of the fluorescence. The order of the effectiveness of cations was monovalent < divalent < trivalent and no variation was found within the groups of cations of the same valence. Similar results were obtained with 8-anilino-1-naphthalenesulfonate (ANS)-liposome suspension [14] and 9-amino-acridine (9-AA)-chloroplast thylakoid membrane [15]. Searle et al. [15] calculated the relative concentration of the dye at the membrane surface with various addition of monovalent, divalent and trivalent cations to the negatively charged membrane with the use of Gouy-Chapman theory and obtained results similar to those observed in 9-AA-thylakoid membrane. Rhodamine 6G bears a positive charge and the above mechanism seems to be applicable. That is, an increase of the salt concentration, which leads to a decrease in the surface potential, brings about a release of rhodamine 6G bound to the liposome into bulk solution, which in turn results in an increase in the fluorescence.

The fluorescence of rhodamine 6G-liposome suspension increases with ln $[K]_{out}$ in the presence of valinomycin, which indicated that the fluorescence also monitors changes in the membrane potential induced by permeation of the ion across the membrane.

According to the theory of Teorell-Meyer-Sievers [16,17], the membrane potential, $\Delta\psi$, is represented by an algebraic sum of two phase boundary potentials at the membrane-solution interface (which is considered to be approx. equal to the surface potentials) and the intramembrane diffusion

potential, $\Delta\psi_d$. For the consideration of the membrane potentials of the vesicles, the phase boundary potentials are represented by $\Delta\psi_i$ and $\Delta\psi_o$ where the suffixes stand for the inside and outside of the vesicles.

$$\Delta\psi = \Delta\psi_i + \Delta\psi_d + \Delta\psi_o \quad (2)$$

In the case of liposome or *Tetrahymena*, $\Delta\psi_i$ can be considered to be constant under the experimental conditions. Therefore, the total membrane potential, $\Delta\psi$, is a function of $\Delta\psi_d$ and $\Delta\psi_o$. The theoretical treatment described in a separate paper indicated that the dye bound to the membrane is released into a bulk solution when either of $\Delta\psi_d$ or $\Delta\psi_o$ is changed in the direction of 'depolarization' (the inside potential of the vesicles changes in the positive direction); fluorescence of rhodamine 6G reflects change in the total membrane potential no matter how either $\Delta\psi_d$ or $\Delta\psi_o$ is changed. This leads to conclusion that the chemotactic response in *Tetrahymena* is induced by a change in the membrane potential (depolarization) since the fluorescence of rhodamine 6G increased closely associating with the chemotactic response in all the cases examined. The order of the effectiveness of cations to induce the membrane potential change in *Tetrahymena* was also monovalent < divalent < trivalent although there was variation in the group of monovalent cations. Furthermore, practically no difference was found in the effectiveness between $CaCl_2$ and $MgCl_2$. These results suggest that the surface potential as well as the intramembrane diffusion potential greatly contributes to the total membrane potential in *Tetrahymena* especially when polyvalent cations are applied to the organisms. A similar mechanism was proposed for the membrane potential change in *Physarum polycephalum* [18] and for the generation of taste receptor potentials in higher vertebrates [3,4].

The fluorescence changes of rhodamine 6G-*Tetrahymena* suspension seem to reflect the resting potential changes. The resting potential changes in direction of depolarization will induce a transient 'Ca response', which is essential for the generation of chemotaxis. In fact we have found that the elimination of Ca^{2+} from the external medium by addition of EGTA suppressed the chemotactic response in *Tetrahymena* without affecting its motility.

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