Comparative studies on the cellular response to chemical loading in unicellular eucaryote, *Euglena gracilis*, strains Z and SMZ

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Euglena gracilis, a single cell organism with plant and animal characteristics is useful tool in studying cellular response to various chemical stresses. *E. gracilis* strains Z and achlorophyllous mutant SMZ were used in this study. In elucidation of the mechanism of the cellular response to the chemical loading in *E. gracilis*, we hypothesized that G-protein and phosphatidylinositol biphosphate mediated signal transduction pathway participates in the cellular response of *E. gracilis*. Then, changes of their morphology, deflagellation and the intracellular calcium level by tributyltin chloride (TBTCl) were compared concerning several calcium agonists and antagonists. By 50μM of TBTCl loading on Z strain, a lost of cell motility, morphological change, deflagellation and increase of intracellular calcium level were observed, however, halt of cell motility without distinctive morphological change and a behavior of intracellular calcium level were observed in SMZ strain. Interestingly, the response to a concentration jump of inositol 1, 4, 5-trisphosphate (10 nM) loading in Z and SMZ strain was not the same, suggesting some difference in inositol 1, 4, 5-trisphosphate mediated signal transduction system.

Abbreviations used in this paper : TBTCl, tributyltin chloride ; IP_3, inositol 1, 4, 5-trisphosphate ;

Introduction

Calcium ions have been known to implicate in the cell function like a signal transduction and regulation of cell shape in eukaryote[1]. In addition, a Ca^{2+}-mobilized second messenger, inositol 1, 4, 5-trisphosphate (IP_3) has been studied in a variety of organisms in the signal transduction pathway[2].

The unicellular phytoplankton, *Euglena gracilis*, is highly sensitive to various chemical stresses. *E. gracilis* changes their cell shape responding to stresses, and this response is reversible under favorable conditions (Fig. 1). In response to certain extracellular stimuli as from chemical stresses, *E. gracilis* excise their flagella, change their morphology from spindle form to cyst form via teardrop form, and lost its maneuverability[3]. Though it has been well demonstrated that Ca^{2+} ions are participated in the movement and locomotion of *E. gracilis*[4] and that regulation of cell shape of *E. gracilis* in a clock-controlled rhythm[5, 6], the detailed mechanism of morphological change in response to stresses has been obscure. Using this *E. gracilis* as a model organism, we have been studied the effect of some trace minerals, such as calcium and magnesium, on the tributyltin chloride (TBTCl)-intoxicated cell morphology and motility[7, 8, 9]. An endocrine disruptor in marine environment, TBTCl, has been known to play as ionophore and ATPase inhibitor in animal cells[10]. However, the study on the cellular response to chemical stress in *E. gracilis* has not been investigated.

In the course of our present study, we set up the hypothesis that IP_3 may trigger off the rapid cellular response

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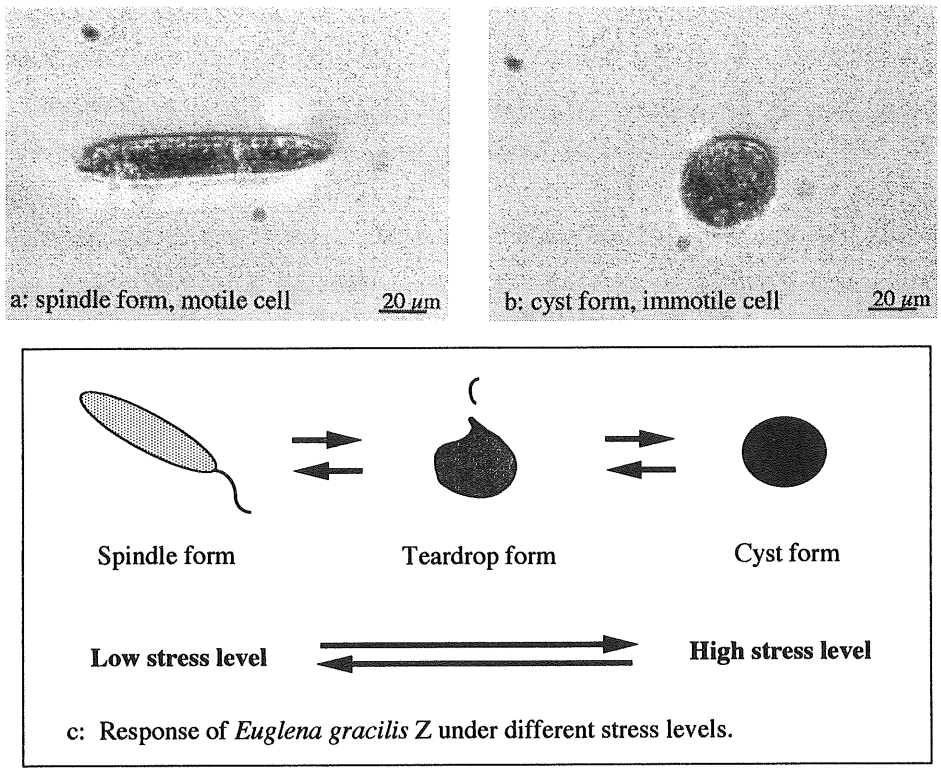


Fig. 1 Change of cell morphology of *Euglena gracilis*.
Cell images of *Euglena gracilis*. a; spindle form cell (motile cell) and b; cyst form cell (immotile cell). Images were taken with Olympus video microscope (type IMT-2) equipped with CCD camera (CoolSNAP, Nippon Roper K,K., Japan). Bar represents 20 μm. The fig. c shows the cell response to stress in *E. gracilis*.

owing to the alteration of intracellular calcium in *E. gracilis* (Fig. 2). Quarmby L. M. *et al* reported that inositol phospholipid trigger flagella excision in the unicellular green alga, *Chamydomonas reinhardtii*[11]. Moreover, deflagellation by low pH shock in *Chamydomonas reinhardtii*, has revealed involvement of phosphatidyl inositol metabolism concerning G-protein playing an important role[12]. Besides Matsuda W. *et al* reported that IP_3 induces Ca^{2+} release from *Euglena* microsomes[13]. Therefore, we have tried to verify our hypothesis to investigate changes of morphology, deflagellation and intracellular calcium level by using several calcium agonists and antagonists.

Materials and Methods

Culture conditions

Euglena gracilis, strains Z and SMZ, were used in all experiments. Cells were grown in Koren-Hutner liquid medium (pH 5.5) at 28℃ for 3 days[14]. Cultures were exposed to a 12 h light and 12 h dark cycle with a light at 2,800 lx. The cells used for these experiments were at the early stationary phase.

Experimental design

Tri-*n*-butyltin chloride (TBTCl) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). A phospholipase C inhibitor, neomycin, was purchased from Sigma (Tokyo, Japan) and dissolved in Tris-HCl buffer (pH 6.5). A G-protein activator, mastoparan, calcium ionophore, A23187, a Ca^{2+}-channel blocker, verapamil and caged-IP_3 were purchased from Calbiochem®, Co., California, U.S.A. The caged-IP_3 was used for producing a concentration jump of inositol 1, 4, 5-trisphosphate by the irradiation with a light of about 360 nm. All other chemicals were of analytical grade.

Evaluation of cell morphology and deflagellation

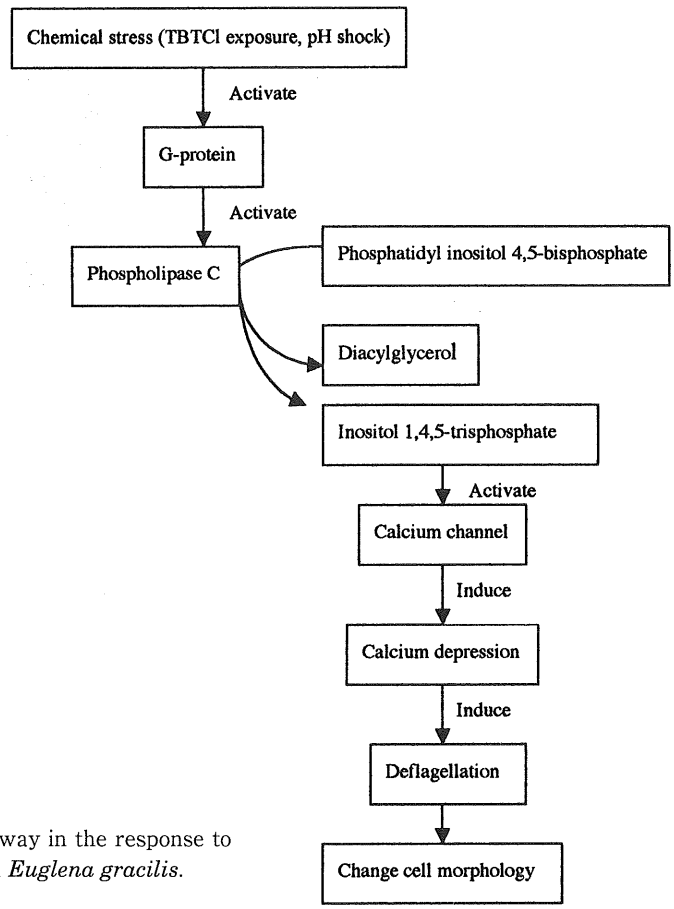


Fig. 2 Hypothesized signal pathway in the response to chemical stresses on *Euglena gracilis*.

E. gracilis cells (*ca.* 10^3 cells/ml) were exposed to each chemical for 5 min by gently mixing. After exposure of chemicals, *E. gracilis* cells in a cyst form (or spherical form) were examined microscopically by an inverted microscope (type IMT-2, Olympus Opticals, Co., Ltd., Japan) equipped with a video image analyzer (ARGUS-100, Hamamatsu Photonics, Co., Japan).

Analysis intracellular calcium concentration

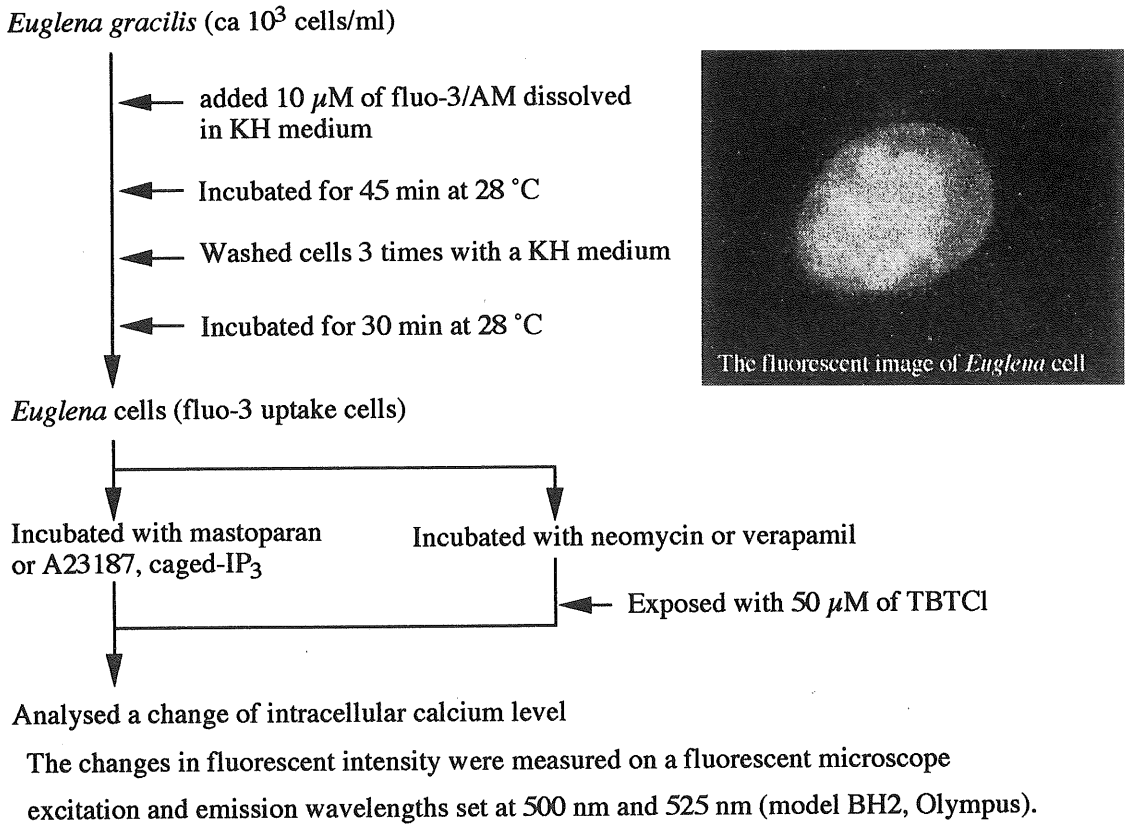
The level of free calcium ion in the cytoplasm was investigated using a calcium indicator, fluo-3/AM (Dojindo Molecular Technologies, Inc., Japan). *Euglena* cells (*ca.* 10^3 cells/ml) were loaded with 10 μM of fluo-3/AM for 45 min at 28℃ under the darkness. Then cells were washed with Koren-Hutner medium and incubated in the same buffer for a further 30 min at 28℃ to allow time for cytoplasmic deesterification of the fluo-3/AM dye. For an observation of fluorescent signal quality, a fluorescence microscope (BH-2, Olympus Optical, Co., Ltd., Japan) equipped with CCD camera was used. The excitation light is supplied by a xenon lamp, and the excitation and emission wavelengths were selected by 500 nm and 525 nm. The resultant image data was analyzed by OPTIMAS software (Media Cybernetics, L. P., M. D., U.S.A.).

Statistical analysis

To determine whether there were significant differences in the level of intracellular free calcium ion after TBTCl exposure or after the use of inhibitors, a statistical analysis was carried out by using ANOVA test.

Results and Discussion

To compare the response to chemical stress in *E. gracilis* strains Z and SMZ, morphological changes and deflagellation were observed. Fig. 3 shows *E. gracilis* cell images after 50 μM of TBTCl intoxication (a, Z strain and b,

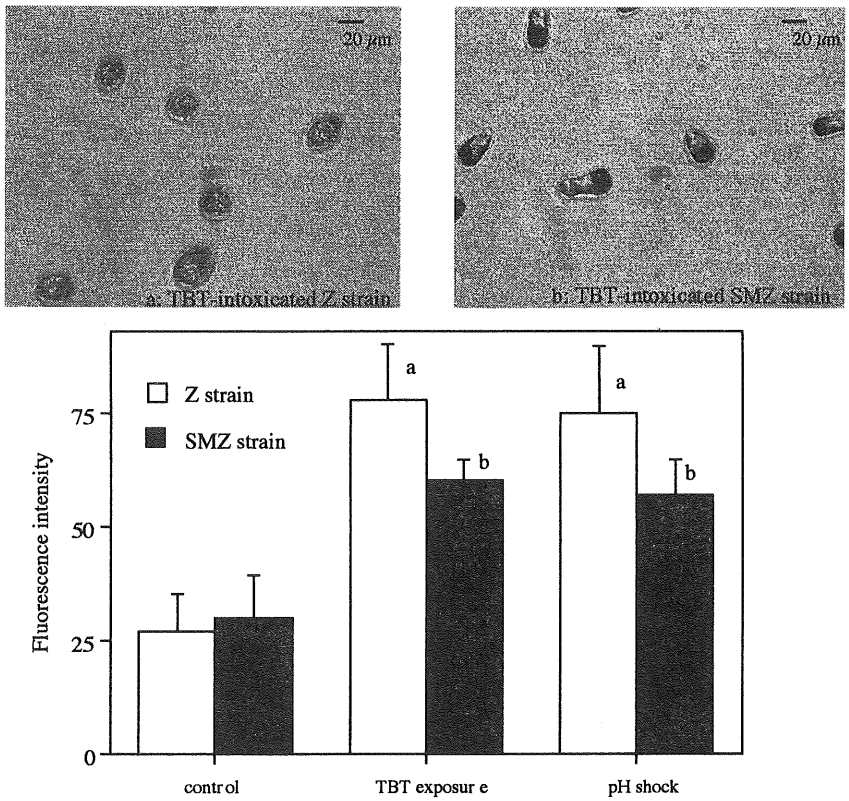


Scheme 1. Outline of experimental design.
Procedure for the determination of the intracellular calcium level. The image is the fluorescent image of fluo-3 dyed *Euglena* cell taken after 50 μM TBTCl intoxication.

SMZ strain) and changes of intracellular calcium level (c). Different morphological change of *E. gracilis* and intracellular calcium level took place by TBTCl exposure and pH shock. In Z strain, the cellular response to TBTCl exposure was a change of cell shape from spindle to completely spherical, cyst form (a). As for SMZ strain, they did not change their cell shape from spindle to cyst form, and they changed their shape not to spherical, to distorted form by TBTCl exposure (b). The change to a distorted form was also observed in SMZ strain by pH shock (data not shown). To be clear the dynamic change of intracellular calcium mobilization by chemical stress in *E. gracilis*, TBTCl exposure and pH shock, the change of intracellular calcium concentration level were observed (Fig. 3, c). With the change of cell morphology, the intracellular calcium level increased in cyst form compared with that of the control, spindle form. Interestingly, the intracellular calcium level in distorted cell shape was lower than that of the cyst form.

As described in the introduction, we hypothesized that the cellular response in *E. gracilis* may be due to the inositol trisphosphate mediated signal transduction pathway. Therefore, the morphological change and the intracellular calcium levels were investigated in treatments of G-protein activator, mastoparan and phospholipase C inhibitor, neomycin[6]. As shown in Fig. 4, the intracellular calcium level increased by the treatment of mastoparan with the change of cell morphology from spindle to cyst or distorted cell shape (Table 1). On the other hand, the response to TBTCl exposure was inhibited by pretreatment of neomycin. Neomycin treatment only did not cause the change in both intracellular calcium level and cell shape (data not shown). These results obtained from mastoparan and neomycin treatment suggest participation of IP_3 mediated signal transduction pathway as the cellular response to the stress in *E. gracilis*.

As a further investigation, to be clear the participation of IP_3 in the cellular response to stresses in *E. gracilis*,



c: Effect of TBT exposure and pH shock on intracellular calcium level

Fig. 3 The effect of TBTCl exposure and pH shock on the cell morphology and the intracellular calcium. Cell images of TBTCl-intoxicated *Euglena gracilis* strains Z (image : a) and SMZ (image : b). c : Effect of TBTCl exposure and pH shock on intracellular calcium level. The fluorescence intensity represents the data subtracted the back ground fluorescence. Data presented as the mean ± S.D. (n=10). Bar represents 20 μm.

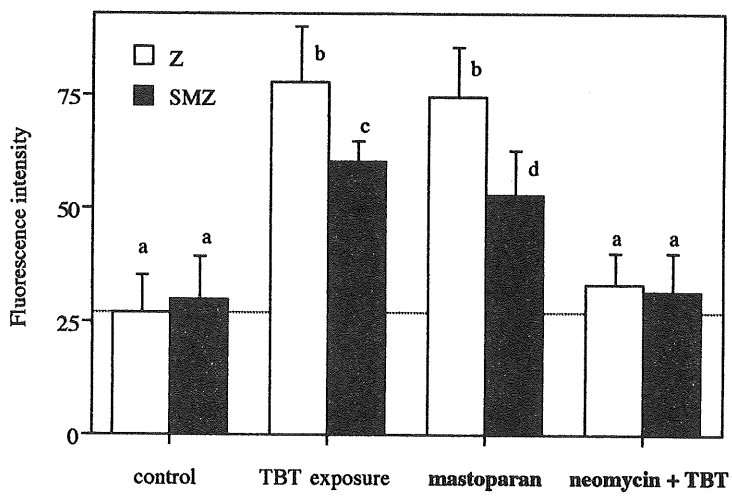


Fig. 4 Changes of intracellular Ca^{2+} levels by treatments of mastoparan and neomycin.
An intracellular calcium level was assessed by measuring fluorescence using the calcium indicator, fluo-3. The fluorescence intensity represents the data subtracted the back ground fluorescence. Data presented as the mean ± S.D. (n=10).

the change of intracellular calcium level was observed in a concentration jump of IP_3, the addition of calcium ionophore, A23187 and calcium-channel blocker, verapamil (Fig. 5). The same morphological change and alteration of calcium level as TBTCl exposure were observed by the addition of IP_3 (Table 2). On the other hand, the cell shape of SMZ strain changed to spherical, cyst form by the increase of intracellular calcium owing to the addition of A23187. Moreover, the increase of intracellular calcium level observed in TBTCl exposure was blocked by the pre-treatment of verapamil. These results suggested that an intracellular calcium regulate system in SMZ strain is somewhat different from Z strain.

We have demonstrated that calcium agonist and antagonist used in this study cause the morphological change and the alteration of intracellular calcium level in *E. gracilis*. The results obtained from our present study, strongly suggest that TBTCl stress is mediated through G-protein/IP_3 mediated signal transduction system, and the data

Table 1. Morphological changes of *Euglena* cell by mastoparan and neomycin treatment.

	Z strain	SMZ strain
Normal cell	Spindle	Spindle
pH shock	Cyst	Distorted
TBT exposure (50μM)	Cyst	Distorted
Mastoparan (10μM)	Cyst	Distorted
Neomycin (50μM)+TBT	Spindle	Spindle

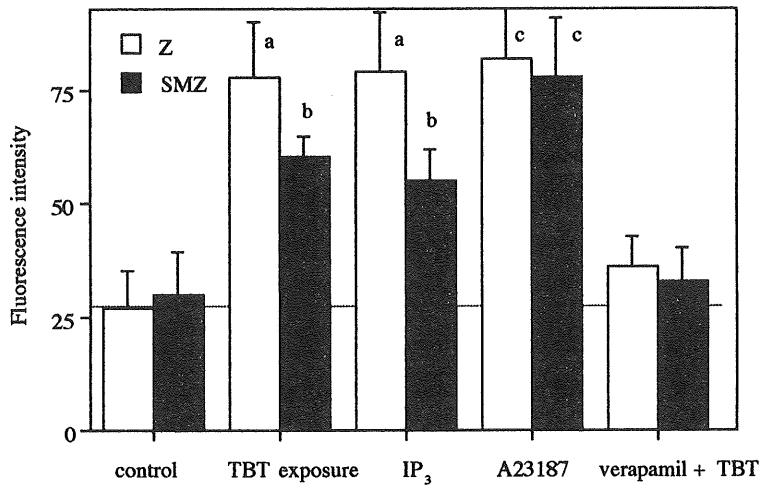


Fig. 5 The effect of IP_3, A23187 and verapamil loading on the intracellular calcium level.
An intracellular calcium level was assessed by measuring fluorescence using the calcium indicator, fluo-3. The fluorescence intensity represents the data subtracted the back ground fluorescence. Data presented as the mean ± S.D. (n=10).

Table 2. Morphological change of *Euglena* cell IP_3, A23187 and pretreatment of verapamil.

	Z strain	SMZ strain
Normal cell	Spindle	Spindle
TBT exposure (50μM)	Cyst	Distorted
IP_3 (10μM)	Cyst	Distorted
Ca ionophore (50μM)	Cyst	Cyst
Verapamil (10μM)+TBTCI	Spindle	Spindle

were principally the same as those of Matuda, W *et al*'s report[13] with *in vitro* system and with Cheshire, J. L. using *Chlamydomonas reinhardtii*. It is likely that these agents are achieving IP_3 through the activation and inactivation of the series of our hypothesized signal transduction pathway, but it is unclear whether or not they are acting the same point. It remains to be confirmed the accumulations of intracellular IP_3 directly using radioisotope technique. It might be a potentially useful tool to probe how the stress cause the physiologically response. Although the difference in the signal transduction pathway in *Euglena gracilis* Z and SMZ has not been fully revealed yet, calcium plays an important role in both strains.

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