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Article

New effect of alkali metal ions on the orientation of cortical microtubule in *Spirogyra* cell

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Effect of alkali metal ions on the orientation of cortical MTs (microtubules) was investigated using *Spirogyra* cells. With $GdCl_3$, inhibitor of mechanosensing receptor, alkali metal ion affected the orientation of cortical MTs in a characteristic manner. Na^+ and Rb^+ induced the ratio of cells having transverse MTs to increase, whereas K^+ and Cs^+ had almost no effects. Na^+ and Rb^+ had the same effect, while K^+ and Cs^+ had a different effect, that was already reported in the movement of water molecules treated with far infrared radiating ceramic and is the first report in living things. In addition, other interesting phenomena of MT orientation related to trehalose is reported.

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Key words: alkali metal ion, cortical microtubule, $GdCl_3$, trehalose

Introduction

Alkali metal ions, especially Na^+ and K^+ are abundant in nature including the biosphere. Recently, it was shown that Na^+ and Rb^+ had the same effect, while K^+ and Cs^+ had a different effect on the movement of water molecules[1]. When water is treated with far-infrared radiating ceramic, its movement together with Na^+ or Rb^+ decreases, whereas that of K^+ or Cs^+ increases. Ceramic is a material made from granite consisting of silicate, that exists in the natural physical environment together with water and alkaline metal ions, these being all related to the phenomenon detailed above. In addition, ceramic has effects on living organisms, such as the promotion of plant growth and resistance to high temperatures[2)3]. These all imply that the same effects by Na^+ and Rb^+, as well as K^+ and Cs^+, could occur in plant physiology. Moreover, it is assumed that such a phenomenon has not been reported.

Therefore, a demonstration of this phenomenon was attempted using *Spirogyra* algae, where the orientation of cortical microtubules (MTs) is affected by various factors including alkali metal ions[4),5]. Cortical MTs in plant cells exist in parallel beneath the plasma membrane and are believed to regulate cell morphology[6]. In the *Spirogyra* cell, MT orientation is perpendicular to the elongated orientation of the cell[7,8]. After the destruction of MTs with an anti-MT chemical, the orientation of reorganized MTs is affected by certain factors[9-12].

Alkali metal ions alone are known to affect MT orientation in a similar manner[4,5]. Hence, the effect of a combination of alkali metal ions and a selected chemical compound affecting plasma membrane was investigated. We hypothesized that both Na^+ and Rb^+ affect MT orientation similarly, while both K^+ and Cs^+ affects MT orientation in a different manner. Test results are reported herein, together with other interesting phenomena detected regarding MT orientation. Thus, effects of trehalose, a naturally occurring sugar, on MT orientation, were also assessed.

Materials and methods

Culture conditions

Specimens of *Spirogyra fluviatilis* Hilse were collected in a pond in Joyo, Kyoto, Japan and cultured in Closterium medium[13] in a photoperiod of 16 h (light) – 8 h (dark) at 23℃.

Treatment of Spirogyra with chemicals[7,8]

Artificial pond water (APW (0.1 mM each of KCl, NaCl, $CaCl_2$, and 5 mM HEPES-Tris [pH7.5])) was used as the experimental medium. Cell treatment with amiprophos-methyl (APM) was performed as reported previously[4]. The dissolution of APM in DMSO was carried to a concentration of 5 mg ml^{-1}. Cells were treated with 3 µg ml^{-1} APM for 1 h or 24 h to depolymerize cortical MTs. The removal of APM followed, by washing cells quickly three times with distilled water. Cortical MTs were

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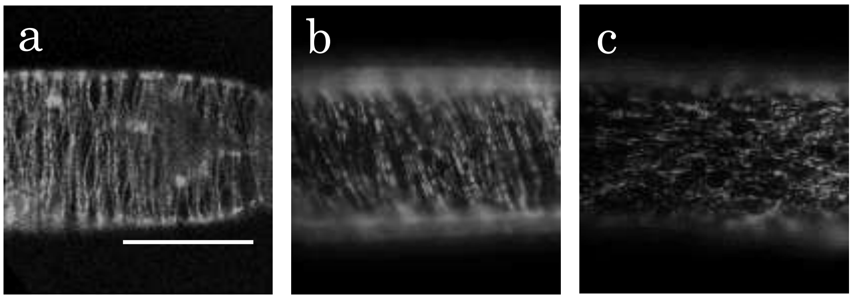

Fig. 1 Photographs of cortical MTs of *Spirogyra* Cell. a transverse MTs (80-90°of the cell axis) ;b oblique MTs (10-80°of the cell axis) ;c longitudinal MTs (0-10°of the cell axis) Bar=30μm.

reorganized in APW, or in an alkali metal ion or other solution, where solutes were dissolved in APW for 1.5 h. Materials dissolved in this manner included 50 mM NaCl, 50 mM KCl, 50 mM RbCl, or 50 mM CsCl solution with or without 20 μM $GdCl_3$ (a mechanosensitive channel blocker)[15], 100μM sodium orthovanadate (inhibitor of ATPase) or 1 mM EGTA. Moreover, 0.3 M mannitol and other sugars (sorbitol, glucose, arabinose, sucrose, maltose, lactose and trehalose) in APW, respectively, were also used.

Immunostaining and observation of MTs [7,8]

Cells were fixed with 3.7% glutaraldehyde in 50 mM sodium-phosphate buffer (pH 7.0) for 30 min. Next, they were cut with a double-blade razor in a fixative. After washing three times with 50 mM sodium-phosphate buffer (pH 7.0) they were treated with a detergent solution (1% Nonidet P-40, 0.4 M mannitol in 50 mM sodium-phosphate buffer [pH 7.0]) for 3 h. Upon subsequent removal of the detergent solution, cells were labeled overnight with a 1:100 dilution of a monoclonal anti-α tubulin antibody raised against chicken α-

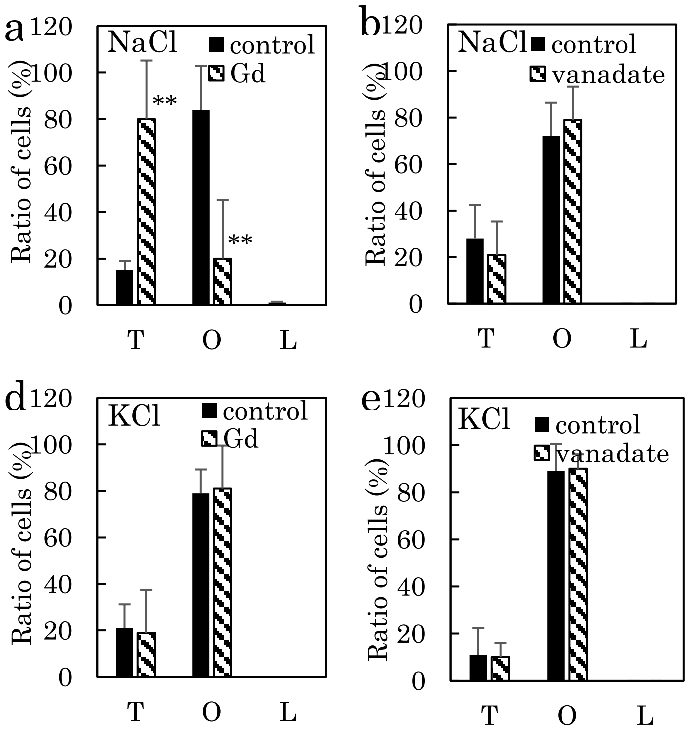

Fig. 2 Orientation of cortical MTs reorganized in 50 mM NaCl (a,b,c) or KCl (d,e,f) solution after treatment with APM for 1h. a,d with 10μM $GdCl_3$; b,e with 100μM sodium ortho vanadate; c,with 1mM EGTA. Control indicates orientation of cortical MTs without $GdCl_3$ (a,d), vanadate (b,e) or EGTA (c). T, O, L indicate transverse, oblique and longitudinal MT, respectively. Experiments were repeated five times. 20 cells were counted in each experiment. *P<0.05, **P<0.01 (t-test).

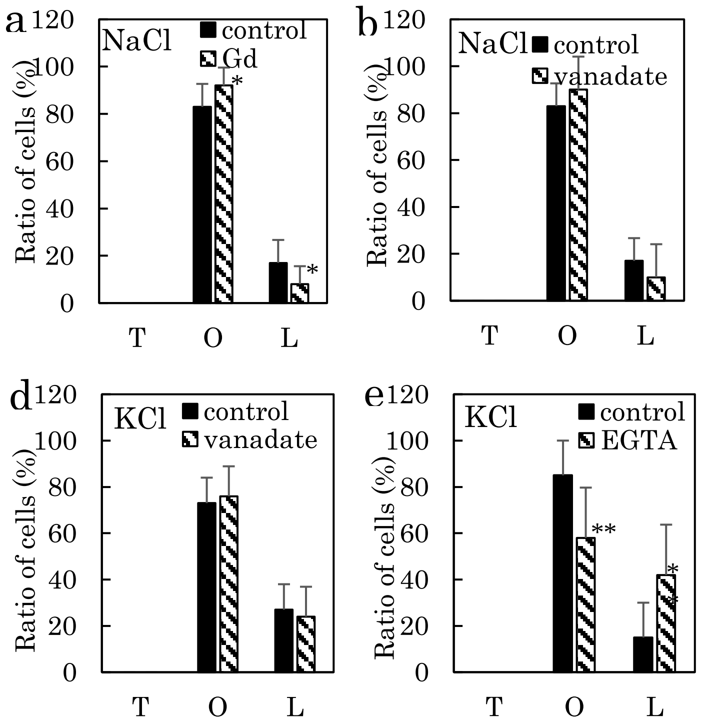

Fig. 3 Orientation of cortical MTs reorganized in 50 mM NaCl (a,b,c) or KCl (d,e) solution after treatment with APM for 24h. a with 10μM $GdCl_3$; b,d with 100μM sodium orthovanadate; c,e with 1mM EGTA. Control indicates orientation of cortical MTs without $GdCl_3$ (a), vanadate (b,d) or EGTA (c,e).T, O, L indicate transverse, oblique and longitudinal MT, respectively. Experiments were repeated five times. 20 cells were counted in each experiment. *P<0.05, **P<0.01 (t-test).

	NaCl(1h)	KCl(1h)	NaCl(24h)	KCl(24h)
$GdCl_3$	T	±	T	/
vanadate	±	±	±	±
EGTA	L	/	L	L

Table 1 Effect of $GdCl_3$ sodium orthovanadate and EGTA on MT orientation. T indicates increase of ratio of cells having transverse MTs or decrease of that of longitudinal MTs. L indicates increase of ratio of cells having longitudinal MTs or decrease of that of transverse MTs. ± indicates no change. / indicates that cells were damaged.

tubulin (ONCOGENE Research Products, San Diego, CA, USA) at 4℃. Solutions were washed three times with 50 mM sodium-phosphate buffer (pH 7.0), and treated with a 1:200 dilution of Alexa-488-conjugated goat anti-mouse IgG (Wako Pure Chemical Industries, Ltd, Osaka Japan) for 2 h. Cells were then washed 3× with 50 mM sodium-phosphate buffer (pH 7.0), and mounted in a Mowiol solution[14] with 0.1% *p*-phenylenediamine dihydrochloride for examination with a BX50 type (Olympus, Tokyo, Japan) epifluorescence microscope. Images were recorded by CCD camera (DP70, Olympus, Tokyo, Japan), stored as TIFF files and processed with Adobe Photoshop 5.0. Microtubules in a single cell having typical orientation patterns were analyzed on a photograph. The orientation of MTs in *Spirogyra* cells is normally perpendicular to the longitudinal cell axis. Following a degradation of MTs with an anti-MT reagent, different orientations may occur as MTs are reorganized in certain solutions. As the concentration of mannitol or alkali metal ion in these solutions increases, orientation changes

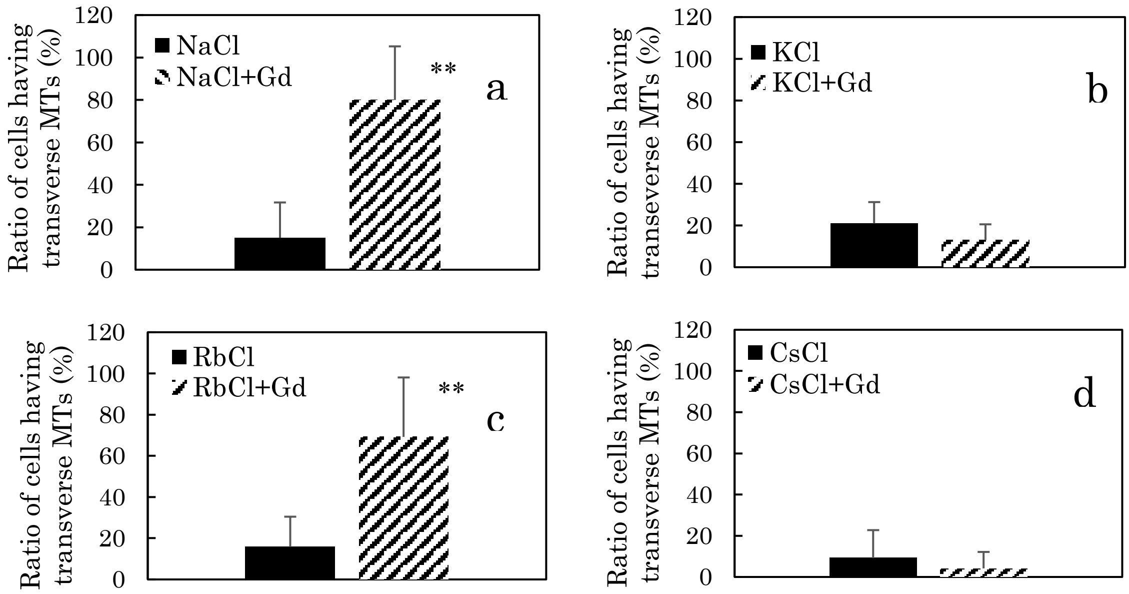

Fig. 4 Ratio of cells having transverse MTs reorganized in 50 mM NaCl(a), KCl(b), RbCl(c), CsCl(d), with or without 10μM $GdCl_3$. Experiments were repeated five times. 20 cells were counted in each experiment. **P<0.01 (t-test).

from transverse to oblique, then to longitudinal[7,8].

When the angle of MTs to longitudinal cell axis was 80~90°, 10~80°, or 0~10°, respectively, MT orientation were regarded as transverse, oblique, or longitudinal (Fig.1).

Chemicals

Sodium chloride (NaCl), potassium chloride (KCl), rubidium chloride (RbCl), cesium chloride (CsCl), $GdCl_3$, sodium orthovanadate, mannitol, sorbitol, glucose, arabinose, sucrose, maltose, lactose and trehalose were purchased from Wako Pure Chemical Co.; ethylene glycol tetraacetic acid (EGTA) was obtained from Sigma-Aldrich.

Results and Discussion

As already reported, different alkali metal ions (Na^+ or K^+) alone have a similar effect on MT orientation, specifically the appearance of oblique or longitudinal MTs[7,8]. On this basis, the effect of a combination of alkali metal ions (Na^+ and K^+), and a chemical affecting the plasma membrane on MT orientation in *Spirogyra* cells, was investigated. Specific chemicals used in the study included a mechanosensing receptor inhibitor ($GdCl_3$), inhibitor of ATPase (sodium orthovanadate) and a Ca^{2+} chelator (EGTA).

In the case of APM treatment for 1 h, effects of Na^+ and K^+ were as follows. Together with $GdCl_3$, Na^+ increased transverse MT ratio, while K^+ did not. When Na^+ and K^+ was used with sodium orthovanadate, transverse MT orientation ratio was not affected. A decrease of this ratio occurred for a combination of EGTA and Na^+. On the other hand, K^+ damaged cells thus MT orientation could not be assessed (Fig.2).

In the case of APM treatment for 24 h, transverse NTs were not observed. Na^+ and K^+ had the following effects. Longitudinal MT ratio increased when Na^+ was used with $GdCl_3$, while K^+ damaged cells making MT observation impossible. When used with sodium orthovanadate, longitudinal MT orientation ratio was not affected, while this ratio was elevated by a combination of Na^+ and K^+ and EGTA. (Fig.3). Results are summarized in Table 1. The combination of $GdCl_3$ with 1 h APM treatment was selected to test our hypothesis.

Transversely oriented MT ratio was increased by Na^+ and Rb^+ together with $GdCl_3$ compared to treatment without $GdCl_3$, while no changes in this ratio was caused by K^+ and Cs^+ compared to treatment without $GdCl_3$ (Fig.4). This phenomenon is considered the first report of Na^+ and Rb^+ having the same effect, while K^+ and Cs^+ a different effect on living things including MT

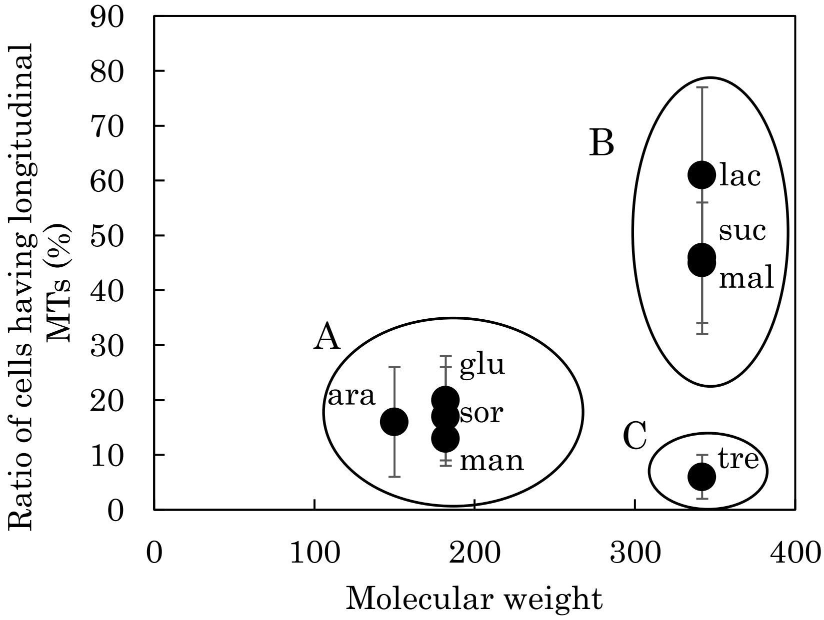

Fig. 5 Relationship between molecular weight of sugar and ratio of cells having longitudinal MTs reorganized in solution of each sugar. A, B and C indicate sugar alcohol and mono-saccharide, di-saccharide and trehalose, respectively. Man, sor, glu and ara in A indicate mannitol, sorbitol, glucose and arabinose, respectively. Suc, lac and mal in B indicate sucrose, lactose and maltose, respectively. Tre in C indicates trehalose. Experiments were repeated five times. 20 cells were counted in each experiment.

orientation in *Spirogyra* cells.

Stretch-activated channels open when the plasma membrane is subjected to mechanical stimuli[16]. Therefore, there is a possibility that Na^+ and Rb^+ could stimulate the plasma membrane mechanically, whereas K^+ and Cs^+ could not. Accordingly, there might be differences between Na^+/Rb^+ and K^+/Cs^+, respectively, with respect to energy exchange between ion and plasma membrane. If NMR data (from full width at half maximum of data of ^{17}O-NMR, water movement was estimated) are related to this phenomenon[1], an energy exchange between ion and ceramic-treated water might be reflected in such data. Water movement decreased with far-infrared radiating ceramics in NaCl/RbCl solutions, while it increased in KCl/CsCl solutions. These facts suggest that Na^+/Rb^+ tend to absorb some energy while K^+/Cs^+ radiate. It is necessary to do further research on the relationship between alkali metal ions, far-infrared radiating ceramics, and MT orientation in *Spirogyra* cells to investigate this further. It has been reported that far-infrared irradiating materials partially cancel the effect of NaCl on cortical microtubule orientation in *Spirogyra* cells[11]. Research, therefore, using far-infrared radiating ceramic seems promising.

Mainly mannitol has been used among sugars for studying cortical MTs so far[5,8]. Differences in effects of alkali metal ions on MT orientation in certain conditions were revealed in the current study. Thereby, variations in effects of different sugars were also investigated.

Sugar alcohols used were mannitol (182.17 g/mol) and sorbitol (182.17 g/mol); monosaccharides used were glucose (180.16 g/mol) and arabinose (150.16 g/mol); disaccharides used were sucrose (342.30 g/mol) lactose (342.30 g/mol) maltose (342.3 g/mol), trehalose (342.30 g/mol) in concentrations of 0.30 M. Cells treated with sugar alcohol and a monosaccharide had generally 13~20% of longitudinal MTs, whereas the number of cells with longitudinal MTs treated with a disaccharide having was much higher than for sugar alcohol and a monosaccharide (45~61%), except for trehalose (6%) (Fig.5). There was an apparent difference between result of monosaccharide and that of disaccharide. Therefore, relationship between ratio of cells having longitudinal MTs and molecular weight of each sugar was shown in Fig.5. The effect of trehalose is quite different from that of other sugars. It is known as a multifunctional molecule[17]. It might be of interest to study which known feature of the molecule plays a role in this anomaly.

It has already been reported that the ratio of cells with longitudinal MTs increases together with mannitol concentration. Therefore, the effect of molecular weight could be similar to that of concentration.

When MTs were reorganized in mannitol solution, longitudinal MT ratio decreased with $GdCl_3$, a mechanosensing receptor inhibitor (data not shown). This suggests that mannitol might cause mechanical stress to the plasma membrane. Therefore, it is likely that trehalose causes lower mechanical stress than mannitol or has the ability to ease mechanical stress.

In this paper, the first example of the phenomenon —as well as a verification of our hypothesis — that Na^+/ Rb^+ have the same effect while K^+/Cs^+ has a different effect on *Spirogyra* cells, was provided. In addition, newly described processes related to MT orientation in *Spirogyra* cells were also presented. Hopefully these findings will be useful for future research on effects of silicate, water, alkali metal ions and sugars on plant cells.

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