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Phosphorylation-Dephosphorylation is Involved in Ca^{2+}-controlled Cytoplasmic Streaming of Characean Cells

Y. TOMINAGA[1], R. WAYNE[2], H. Y. L. TUNG[3], and M. TAZAWA[4,*]

[1] St. Agnes' Jr. College for Women, Kamikyo-ku, Kyoto
[2] The Department of Botany, The University of Texas, Austin. The Department of Botany, The University of Massachusetts, Amherst
[3] Clayton Foundation Biochemical Institute, The University of Texas, Austin
[4] Department of Botany, Faculty of Science, University of Tokyo, Hongo, Tokyo

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Summary

The mechanism of Ca^{2+} regulation of the cytoplasmic streaming in characean cells was studied in relation to protein phosphorylation and dephosphorylation. A tonoplast-free cell model was developed which was sensitive to Ca^{2+}. Protein phosphatase-1 and its inhibitor-1 were applied into the tonoplast-free cells. A synthetic inhibitor of protein phosphatase, α-naphthylphosphate, was applied either to tonoplast-free cells from inside or to the outside of plasmalemma-permeabilized cells which are known to be very sensitive to Ca^{2+}. ATP-γ-S applied to permeabilized cells strongly inhibited the recovery of the streaming which had been stopped by 10 μM Ca^{2+}. Both inhibitor-1 and α-naphthylphosphate inhibited the streaming even in the absence of Ca^{2+}. On the other hand, protein phosphatase-1 recovered the streaming even in the presence of Ca^{2+}.

The results indicate that characean streaming is regulated by the phosphorylation state of a regulatory and/or motile protein component. Streaming is activated when the component is dephosphorylated and inactivated when the component is phosphorylated. Ca^{2+} is assumed to stimulate both phosphorylation and dephosphorylation of the component. Involvement of Ca^{2+}-calmodulin in the streaming recovery was discussed in terms of the stimulation of dephosphorylation.

Keywords: Characeae; Ca^{2+}; Cytoplasmic streaming; Protein phosphatase; Protein phosphatase inhibitor.

Abbreviations: ATP-γ-S, Adenosine-5'-O-(3-thiotriphosphate); α-NP, α-naphthylphosphate; EGTA, ethylenglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid).

1. Introduction

It has been established that the motile system of cytoplasmic streaming in characean cells is an actomyosin-ATP system (KAMIYA 1981). Bundles of actin filaments are attached to the immobile cortical gel, while myosin is assumed to be present in the streaming endoplasm (PALEVITZ *et al.* 1974, CHEN and KAMIYA 1975, KATO and TONOMURA 1977, SHEETZ and SPUDICH 1983). The cytoplasmic streaming of characean cells is inhibited by an increase in the Ca^{2+} concentration of the cytoplasm (HAYAMA *et al.* 1979, WILLIAMSON and ASHLEY 1982, KIKUYAMA and TAZAWA 1982, 1983). The plasmalemma of characean cells can be permeabilized by plasmolysis in media containing EGTA at low temperature (SHIMMEN and TAZAWA 1983). The streaming of permeabilized cells is as sensitive to Ca^{2+} as that of intact cells. The streaming almost stops at 1 μM Ca^{2+} (TOMINAGA *et al.* 1983). The tonoplast of characean cells can be easily removed by internal perfusion of the vacuole with media containing the Ca^{2+} chelator EGTA (WILLIAMSON 1975, TAZAWA *et al.* 1976). Cytoplasmic streaming of tonoplast-free cells is far less sensitive to Ca^{2+} than that of plasmalemma-permeabilized cells (TOMINAGA and TAZAWA 1981). In tonoplast-free cells the endoplasm which is assumed to contain some Ca^{2+}-sensitizing component is detached from the cortical gel with time, while in plasmalemma-permeabilized cells the endoplasm remains intact (TOMINAGA *et al.* 1983, TOMINAGA *et al.* 1985).

The possibility that calmodulin is the Ca^{2+}-sensitizing component was tested in our previous paper (TOMINAGA *et al.* 1985). Calmodulin inhibitors applied to permeabilized cells did not affect the Ca^{2+}-sensitive

* Correspondence and Reprints: Department of Biology, Faculty of Science, The University of Tokyo, Hongo, Tokyo 113, Japan.

streaming cessation but completely inhibited the streaming recovery which otherwise occurred by removing Ca^{2+} (TOMINAGA *et al.* 1985). Probably, calmodulin with Ca^{2+} plays an important role for releasing the streaming from Ca^{2+} inhibition. Therefore, another yet unidentified Ca^{2+}-sensitizing component, which is involved in streaming cessation, must exist also in the endoplasm.

It is well-known that Ca^{2+}/calmodulin-dependent phosphorylation of myosin light chain of smooth muscle cells occurs via activation of light chain kinase (HARTSHORNE 1982). The movement of latex beads coated with *Physarum* myosin is inhibited by micromolar concentrations of Ca^{2+} (KOHAMA and SHIMMEN 1985). The Ca^{2+}-sensitivity of the ATPase was observed only when the myosin was phosphorylated (KOHAMA and KENDRICK-JONES 1986). The assembly of myosin into thick filament is possible only with the phosphorylated heavy chain of myosin in *Physarum* (OGIHARA *et al.* 1983) and with the dephosphorylated one in *Dictyostelium* (KUCZMARSKI and SPUDICH 1980). Myosin appears to be regulated through phosphorylation in a species-specific manner. Thus involvement of phosphorylation or dephosphorylation of some motile component in the control of cytoplasmic streaming should be examined also in *Characeae* cells. In 1984 we (TOMINAGA *et al.* 1984) speculated the possible involvement of phosphorylation and/or dephosphorylation in the Ca^{2+}-controlled streaming from the observation that ATP-γ-S inhibited the recovery of the streaming once inhibited by Ca^{2+}. To confirm this hypothesis, we applied protein phosphatase and its inhibitor which had been isolated from rabbit skeletal muscle directly into tonoplast-free cells. An artificial phosphatase inhibitor was also tested for its effect on the cytoplasmic streaming.

2. Materials and Methods

2.1. Materials

Internodal cells of *Chara australis* (= *C. corallina*), *Nitella axilliformis*, and *Nitella expansa* were used. Culture conditions of algae were described before (TOMINAGA and TAZAWA 1981, TOMINAGA *et al.* 1983). *Chara* cells were used for tonoplast-free (TAZAWA *et al.* 1976) and plasmalemma-permeabilized (SHIMMEN and TAZAWA 1983) cell models, and cells from both species of *Nitella* were used for the permeabilized cell model. We refer to the plasmalemma-permeabilized cell simply as permeabilized cell.

2.2. Preparation of Tonoplast-free Cells by Intracellular Perfusion

The vessel shown in Fig. 1 was designed for intracellular perfusion under continuous observation with a microscope (TAZAWA 1968, TOMINAGA and TAZAWA 1981). An internode was placed on a glass slide that contained spacers made from two pieces of another glass slide which prevented the internode from being pressed by the cover glass. Two plexiglass blocks (B) were placed on the cell in such a way that the cell was fitted into the groove (g) of the block. The cell was exposed to air until its turgor was lost due to evaporation of water that adhered to the cell surface. Then liquid paraffin was poured to the central part of the cell positioning between the two spacers, and a cover glass was put on the spacers. The blocks insulated the central part of the cell from the perfusion medium present outside the blocks. A slightly hypertonic solution (300 mOsm) was irrigated on one cell end which then was amputated. The solution was removed and the hypotonic perfusion medium (220 or 250 mOsm) was irrigated in both cell ends. The other closed cell end was amputated and the cell was perfused several times with an interval of several tens of seconds. The amount of perfusion medium used for one perfusion was only 5–10 µl. After several minutes the tonoplast was broken (details in 3.2.1.).

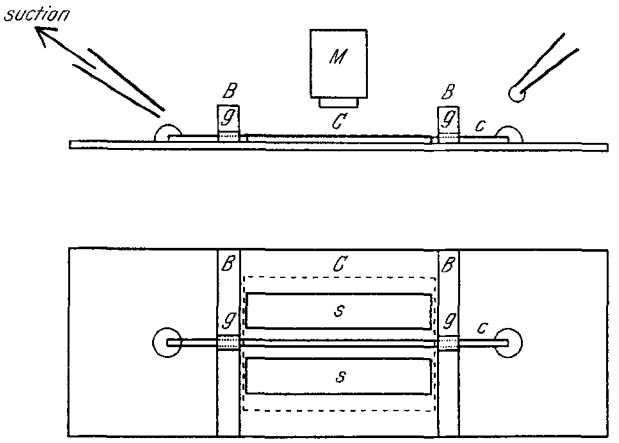

Fig. 1. Vessel for intracellular perfusion. Internodal cell (*c*) is placed between two spacers (*s*) on a glass slide. *B* plexiglass block with groove (*g*), *M* microscope, *C* cover glass. For further explanation, see the text

2.3. Perfusion Media

The perfusion medium Mg·ATP contained 5 mM EGTA, 30 mM PIPES, 6 mM $MgCl_2$, 1 mM ATP, 67–71 mM K^+, and variable concentrations of sorbitol (pH 7.1) (TOMINAGA *et al.* 1983). Mg-ATP-γ-S medium contained 1 mM ATP-γ-S instead of 1 mM ATP. The perfusion medium lacking ATP was named Mg medium. Two $[Ca^{2+}]$, 10 µM and 1 mM, were used for controlling cytoplasmic streaming. The former was adjusted by adding 4.94 mM $CaCl_2$ to Mg or Mg·ATP medium, and the latter by adding 1 mM $CaCl_2$ to EGTA-free Mg or Mg·ATP medium. The Ca^{2+}-EGTA buffered medium was made using the association constant of $10^{6.9}$ at pH 7.1 (PORTZEHL *et al.* 1964). The concentration of free Ca^{2+} in the 10 µM Ca^{2+} media was checked with a calcium-sensitive electrode (F2112Ca, Radiometer, Copenhagen).

2.4. Preparation of Plasmalemma-permeabilized Cells

We slightly modified the method of SHIMMEN and TAZAWA (1983). A cell was placed on a glass slide on ice and was superfused with cold, slightly hypertonic Mg medium. In the original method (SHIMMEN and TAZAWA 1983) the cell was dipped into a beaker containing the cold hypertonic medium. Transfer of the permeabilized turgor-less

cell from the beaker to a glass slide often caused bending of the cell due to the surface tension of the medium. Such a bending could be avoided in our modified method.

2.5. Preparation of Phosphatase-1 and Inhibitor-1

Protein phosphatase-1 and active phosphorylated form of inhibitor-1 were isolated and purified to homogeneity from rabbit skeletal muscle as described before (TUNG *et al.* 1984, NIMMO and COHEN 1978). Activities of the enzymes were assayed after HEMMINGS *et al.* (1982) and FOULKES and COHEN (1980). One unit (U) of phosphatase-1 activity was that amount of enzyme which catalysed the release of 1.0 nmol phosphate·minute from phosphorylase *a*. Stock solutions of phosphatase-1 at 260 U/ml and inhibitor-1 at 56 nmol/ml were prepared. Inhibitor-1 at 10–25 nM inhibited phosphatase-1 at 0.015 U by 100% in a 60 µl standard assay (TUNG *et al.* 1984). The stock solutions were diluted with perfusion media.

The effect of phosphatase on [Ca^{2+}] was checked. Phosphatase at 1 U/ml (final concentration) was added to solutions containing various concentrations of free Ca^{2+}. [Ca^{2+}] was measured with a Ca^{2+}-sensitive electrode (F2112Ca, Radiometer, Copenhagen). Phosphatase did not affect the free Ca^{2+} concentrations of any of these solutions (data not shown), which indicated that phosphatase did not chelate Ca^{2+}.

2.6. Others

ATP-γ-S was purchased from Boehringer and Fluka, and α-NP from Sigma.

Low molecular mass substances (ATP-γ-S and α-NP) were applied to either inside of the tonoplast-free cell by perfusion or the outside of permeabilized cells. High molecular mass substances (protein phosphatase and its inhibitor) which can not penetrate the cell wall were applied only to the inside of tonoplast-free cells.

All the perfusion experiments were done at room temperature (20–25 °C).

3. Results

3.1. Effect of ATP-γ-S on Streaming Cessation Induced by Ca^{2+} and on Recovery

In Fig. 2 a permeabilized cell of *Nitella axilliformis* was first bathed in Mg medium. The streaming was completely inhibited due to lack of ATP (0 time). Preincubation of 1 mM ATP-γ-S together with 0.5 mM ATP before application of Ca^{2+} caused complete activation of streaming. Subsequent removal of the ATP-γ-S and application of 10 µM Ca^{2+} stopped the streaming in 6.6 minutes (average of 3 cells) even in the presence of enought ATP (0.5 mM and more, 1 mM in this case), indicating that the Ca^{2+}-sensitive mechanism was intact. No spontaneous recovery of streaming was observed, unless Ca^{2+} was removed by superfusing permeabilized cells with Mg·ATP medium (TOMINAGA *et al.* 1983). But in Fig. 2 where the cell was pretreated with ATP-γ-S, removal of the Ca^{2+} did not recover the streaming. When in Fig. 2 cells were incubated in 1 mM

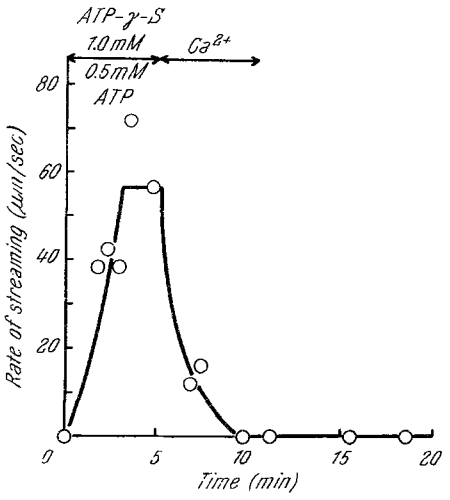

Fig. 2. Effect of ATP-γ-S given before Ca^{2+} application on recovery of streaming in *N. axilliformis*. At time zero a permeabilized cell bathed in Mg medium was superfused with Mg·ATP medium containing 0.5 mM ATP and 1 mM ATP-γ-S. After reactivation of streaming the cell was superfused with Mg·ATP medium containing 1 mM ATP and 10 µM Ca^{2+}. The streaming was completely inhibited. Removal of the Ca^{2+} caused no recovery of the inhibited streaming

ATP-γ-S together with 1 mM ATP, the inhibition of recovery was incomplete (data not shown, Table 1 *C*). When 1 mM ATP-γ-S was applied simultaneously with 10 µM Ca^{2+} in the presence of 1 mM ATP to cells whose streaming had been reactivated with Mg·ATP medium, the streaming stopped in 7.2 minutes (average of 3 cells) (Fig. 3, Table 1 *D*). The time for cessation of streaming seems to be unaffected by ATP-γ-S, since this stopping time was similar to that in Fig. 2 where Ca^{2+} was applied without ATP-γ-S. Removing both ATP-γ-S and Ca^{2+} did not recover the streaming, similarly as in Fig. 2. When the concentration of ATP-γ-S was reduced from 1 mM to 0.1 mM, partial recovery of streaming was observed (data not shown, Table 1 *E*). A complete and irreversible inhibition of streaming was also observed when 1 mM ATP-γ-S was applied at the time of Ca^{2+} removal (Fig. 4, Table 1 *F*). When the concentration of ATP-γ-S was reduced to 0.1 mM, partial recovery of the streaming was also observed (Table 1 *G*). In Fig. 5 replacement of Mg medium with Mg·ATP-γ-S medium could not reactivate the streaming. Exchange of the Mg·ATP-γ-S medium with Mg·ATP medium reactivated the streaming. The streaming stopped upon addition of Ca^{2+}. After removal of the Ca^{2+}, the streaming started but only for a few minutes. It is likely that in the absence of ATP and Ca^{2+} thiophosphorylation occurs less than in the presence of the two.

Table 1. *Recovery of streaming which was stopped by Ca^{2+} under various combinations of ATP and ATP-γ-S*

Expt.	Conditions			recovery of streaming	Fig.
	before Ca^{2+}-treatment	during Ca^{2+}-treatment	after Ca^{2+}-removal		
A	1 ATP	1 ATP	1 ATP	+ + + (complete)	
B	0.5 ATP 1 ATP-γ-S	1 ATP	1 ATP	—(no recovery)	2
C	1 ATP 1 ATP-γ-S	1 ATP	1 ATP	+ (partial)	
D	1 ATP	1 ATP 1 ATP-γ-S	1 ATP	—	3
E	1 ATP	1 ATP 0.1 ATP-γ-S	1 ATP	+	
F	1 ATP	1 ATP	1 ATP 1 ATP-γ-S	—	4
G	1 ATP	1 ATP	1 ATP 0.1 ATP-γ-S	+	
H	1 ATP-γ-S → 1 ATP	1 ATP	1 ATP	+	5

Numerals before ATP or ATP or ATP-γ-S mean concentrations in mM.

3.2. Cytoplasmic Streaming in Relation to Protein Phosphorylation and Dephosphorylation

3.2.1. Ca^{2+}-Sensitive Tonoplast-free Cell Model

The assumption that the Ca^{2+}-induced streaming inhibition and its recovery are caused by phosphorylation and dephosphorylation of some component, respectively, was tested by regulating the phosphorylation state of the component with protein phosphatase and its inhibitor. We used the tonoplast-free cell model. The cytoplasmic streaming of tonoplast-free cells prepared with the usual method is less sensitive to Ca^{2+}, since a Ca^{2+}-sensitizing component in the streaming endoplasm is dispersed after disintegration of the tonoplast (TOMINAGA and TAZAWA 1981, TOMINAGA *et al.* 1983); therefore, we developed a method to prepare tonoplast-free cells which were more sensitive to Ca^{2+}. Two important improvements were done for the perfusion: (1) use of slightly hypotonic media containing EGTA and (2) a slow perfusion of about 5 μl/a drop at intervals of several tens of seconds.

Usually in the open-vacuole condition (Fig. 1) the cell ends remained open and the tonoplast disintegrated only with the rapid perfusion which exerted a strong shear force on the tonoplast. On the other hand, perfusion of a hypotonic solution containing EGTA caused swelling of the cytoplasm due to the osmotic uptake of water and facilitated disintegration of the tonoplast even under low shear. The tonoplast-free cells thus prepared did not lose the endoplasm in which the Ca^{2+}-sensitizing component was assumed to be present (TOMINAGA and TAZAWA 1981, TOMINAGA *et al.* 1983, TOMINAGA *et al.* 1984, 1985). When the total amount of perfused medium attained the cell volume (about 20 μl) or exceeded it slightly, the tonoplast was broken. This was demonstrated by the fact that perfusion of Mg·ATP medium containing Ca^{2+} at higher than 1 μM stopped the streaming.

The spontaneous cessation of streaming was often observed before continuous smooth recovery occurred (Figs. 6, 7, 9, and 10). This may be caused by release of Ca^{2+} from internal stores in response to hypotonic

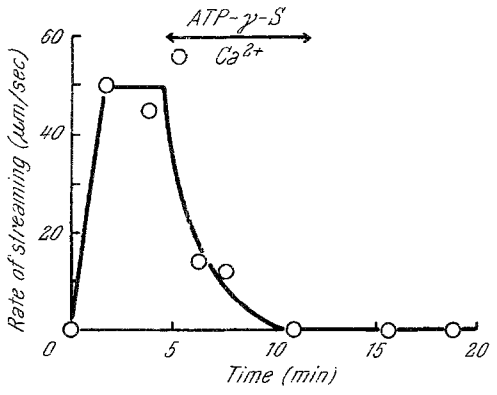

Fig. 3. Effect of ATP-γ-S applied with Ca^{2+} on recovery of streaming in *N. axilliformis*. A permeabilized cell bathed in Mg medium was superfused with Mg·ATP medium at time zero. The recovered streaming was inhibited by 1 mM ATP-γ-S plus 10 μM Ca^{2+}. Removal of both Ca^{2+} and ATP-γ-S did not recover the streaming

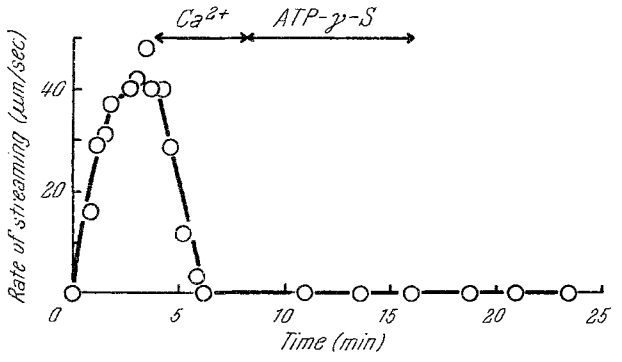

Fig. 4. Effect of ATP-γ-S given after application of Ca^{2+} on recovery of streaming in *N. axilliformis*. Streaming of a permeabilized cell bathed in Mg medium was reactivated by superfusing with Mg·ATP medium at time zero and then inhibited by 10 μM Ca^{2+}. When the Ca^{2+} was washed away with Mg·ATP medium containing 1 mM ATP-γ-S, the streaming did not recover

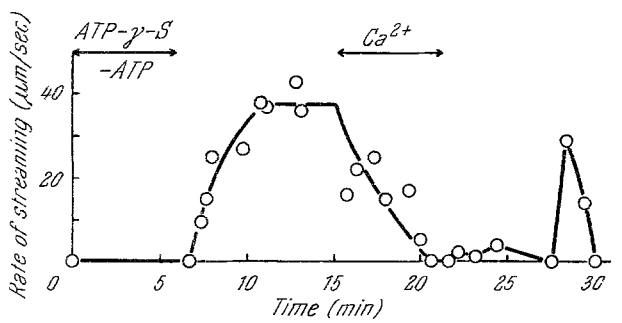

Fig. 5. Effect of pretreatment of ATP-γ-S in the absence of ATP before Ca^{2+} treatment on streaming recovery in *C. australis*. A permeabilized cell bathed in Mg medium was superfused with Mg medium containing 1 mM ATP-γ-S at time zero. The inhibited streaming was reactivated by superfusing Mg·ATP medium. The streaming was again inhibited by 1 mM $CaCl_2$. After removal of the Ca^{2+}, the streaming partially recovered only for several minutes

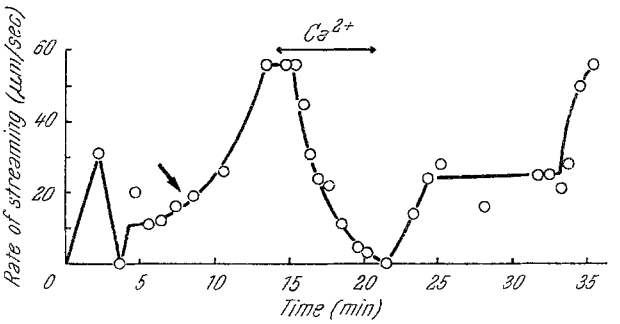

Fig. 6. Reversible inhibition of cytoplasmic streaming with 10 μM Ca^{2+} in a tonoplast-free cell prepared under slow perfusion of *C. australis*. The vacuole of an internode was perfused with a hypotonic (220 mOsm) Mg·ATP medium from time zero. The recovering streaming spontaneously stopped possibly due to an increase of cytoplasmic Ca^{2+} at 3.6 minutes. After recovery of the streaming 10 μM Ca^{2+} was added to Mg·ATP medium. The streaming stopped completely. Removal of the Ca^{2+} restored the original streaming rate. The arrow denotes continuous smooth recovery of streaming resulting from disintegration of the tonoplast

treatment. These Ca^{2+} may be chelated by EGTA after disintegration of the tonoplast, as was shown by the continuous recovery of the streaming. Fig. 6 shows such a model in which the streaming was reversibly inhibited by 10 μM Ca^{2+}. At time zero the perfusion of 220 mOsm Mg·ATP medium which is hypotonic to the intact cell sap was started. The spontaneous stop at 3.6 minutes is interpreted to be due to an increase in free Ca^{2+} resulting from the release of Ca^{2+} from Ca^{2+}-stores by hypotonic treatment. This interpretation is based on the phenomenon that in the transcellular osmosis (KAMIYA and KURODA 1956) spontaneous cessation of streaming often occurred only at the cell part where the cell sap is diluted by water entry (HAYAMA and TAZAWA 1978).

It is supposed that during the subsequent recovery of the streaming the tonoplast was disintegrated. This is supported by the fact that subsequent application of 10 μM Ca^{2+} stopped streaming. However, the immediate recovery of streaming due to the removal of Ca^{2+} was often partial, which is similar to the result reported previously (TOMINAGA and TAZAWA 1981).

3.2.2. Protein Phosphatase-1 and its Inhibitor

In Fig. 7 1 mM ATP was always included in the internal perfusion medium. At time zero cell ends were amputated and a slow perfusion with Ca^{2+} plus protein phosphatase-1 at 5 U/ml was started. Addition of protein phosphatase-1 to the internal perfusion medium containing 10 μM Ca^{2+} gradually reversed the

streaming inhibition which was caused either by spontaneous increase of Ca^{2+} or by the Ca^{2+} applied. Removal of the phosphatase-1 could not still induce inhibition of streaming by Ca^{2+}, indicating that this protein remained in the cell. However, the subsequent addition of 10 nmol/ml inhibitor-1, the natural protein inhibitor of phosphatase-1 slowed down the streaming. The inhibitor-1 alone inhibited not only the recovery of streaming induced by removing the Ca^{2+} (Fig. 7) similarly as ATP-γ-S did (Figs. 2–4), but also the streaming in the absence of Ca^{2+} (data not shown). In contrast to the inhibition with ATP-γ-S, this inhibition could be reversed as shown in Fig. 7, suggesting that the removal of the inhibitor might activate an endogenous phosphatase to overcome the activity of an endogenous

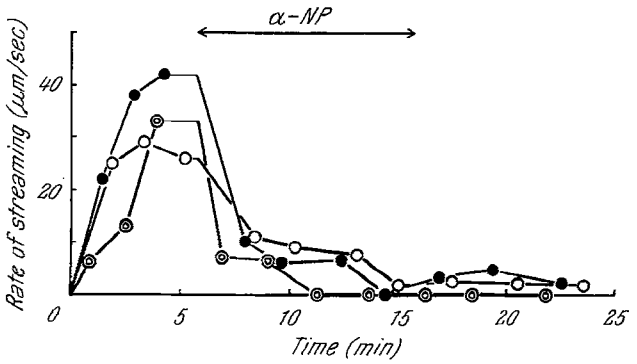

Fig. 8. Effect of α-naphthylphosphate (α-NP) on streaming in permeabilized cells of *N. expansa*. Three permeabilized cells bathed in Mg medium was superfused with Mg·ATP medium at time zero. The reactivated streaming was irreversibly inhibited by superfusing with Mg·ATP medium containing 10 mM α-NP

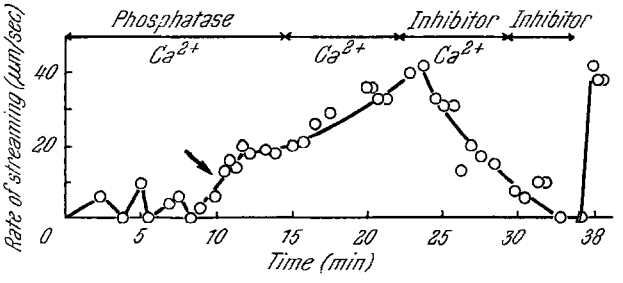

Fig. 7. Effect of protein phosphatase-1 and its inhibitor-1 on streaming in a tonoplast-free cell of *C. australis*. An internode was perfused with a hypotonic (220 mOsm) Mg·ATP medium containing 10 μM Ca^{2+} plus 5 U/ml phosphatase-1. The streaming recovered even in the presence of 10 μM Ca^{2+}. Removal of the phosphatase did not affect the streaming. The activated streaming was inhibited by perfusing intracellularly with Mg·ATP medium containing Ca^{2+} plus inhibitor-1 (10 nmol/ml) of protein phosphatase or inhibitor-1 alone. The inhibited streaming was reactivated by removing the inhibitor-1. The arrow denotes continuous smooth recovery of streaming resulting from disintegration of the tonoplast

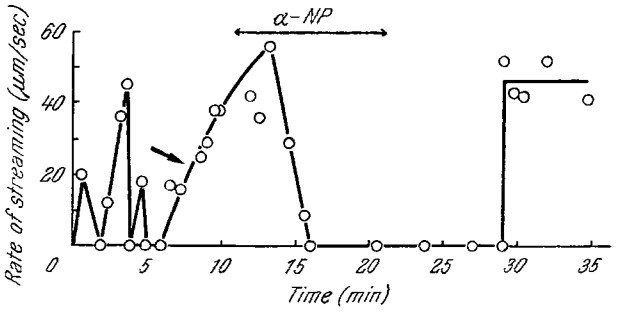

Fig. 9. Effect of α-NP on streaming in a Ca^{2+}-sensitive tonoplast-free cell of *C. australis*. An internode was perfused with a slightly hypotonic (250 mOsm) Mg·ATP medium from time zero. In about 6 minutes the streaming began to recover suggesting that the tonoplast was removed at that time. Then the cell was intracellularly perfused with 250 mOsm Mg·ATP medium containing 1 mM α-NP. The streaming was completely inhibited. Washing away the α-NP reactivated the streaming. The arrow denotes continuous smooth recovery of streaming resulting from disintegration of the tonoplast

kinase. The reversible inhibition of streaming with the protein phosphatase inhibitor is similar to that with Ca^{2+} (Fig. 6). The inhibition of streaming due to inhibitor-1 again supports the idea that the phosphorylation/dephosphorylation of some component may be involved in control of the streaming.

3.2.3. Synthetic Protein Phosphatase Inhibitor

A synthetic protein phosphatase inhibitor, α-NP at 1–10 mM, which is known to be a substrate of phosphatase, inhibited the streaming of both tonoplast-free and permeabilized cells. Removal of the drug did not allow the recovery of streaming in permeabilized cells (Fig. 8), but occasionally did in tonoplast-free cells (Fig. 9). Interestingly rapid perfusion of Mg·ATP medium made the streaming of tonoplast-free cells insensitive to the drug (Fig. 10), which will be discussed below.

4. Discussion

The plasmalemma-permeabilized cell is an excellent model for elucidating the mechanism of Ca^{2+} regulation of cytoplasmic streaming, since the streaming of this model is extremely sensitive to Ca^{2+} (TOMINAGA *et al.* 1983). A demerit of this model is that high molecular mass substances which can not permeate the cell wall can not be used. Present success of making tonoplast-free cells which are sensitive to Ca^{2+} opened

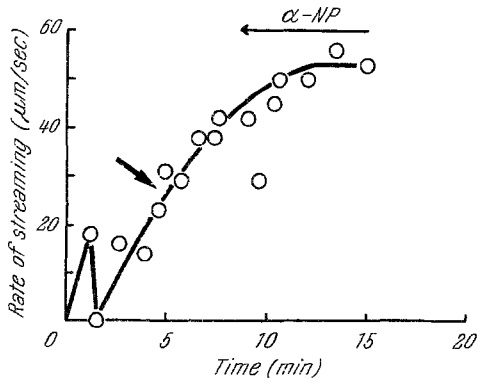

Fig. 10. Loss of sensitivity of cytoplasmic streaming to α-NP during rapid intracellular perfusion in *C. australis*. The vacuole of an internode was perfused with 100 μl of hypotonic (250 mOsm) Mg·ATP medium at time zero. The streaming rate which was zero was gradually increased by perfusing more 100 μl (total 200 μl). Subsequent intracellular perfusion of Mg·ATP medium containing 10 mM α-NP did not affect the streaming. The arrow denotes continuous smooth recovery of streaming resulting from disintegration of the tonoplast

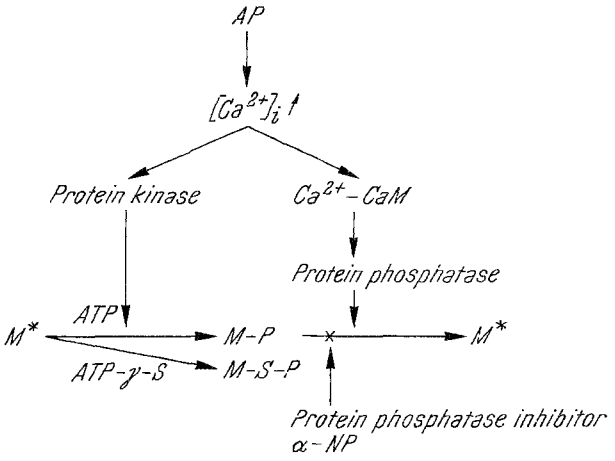

Fig. 11. A scheme for explanation of the Ca^{2+}-controlled cytoplasmic streaming in characean cells. *AP* action potential of membrane; $[Ca^{2+}]_i$: concentration of free cytoplasmic Ca^{2+}; *CaM* calmodulin; *M** active myosin; *M-P* phosphorylated inactive myosin; *M-P-S* thiophosphorylated inactive myosin. For further explanation, see the text

the way to study effects of high molecular mass chemicals on the Ca^{2+}-sensitive cytoplasmic streaming. Protein phosphatase-1 introduced into tonoplast-free cells inhibited the Ca^{2+} action and allowed cytoplasm to stream even in the presence of inhibitory concentration of Ca^{2+} (Fig. 7). On the other hand, the inhibitors of protein phosphatase-1 (inhibitor-1 and α-NP) inhibited streaming even in the absence of Ca^{2+} (Figs. 7, 8, and 9). Moreover, ATP-γ-S inhibited the recovery of streaming which had been stopped with Ca^{2+}. Proteins that are thiophosphorylated with ATP-γ-S are not dephosphorylated by phosphatases (GRATECOS and FISCHER 1974, MORGAN *et al.* 1976). Thus the present results indicate that the characean streaming is active when some component is in the dephosphorylated form and inactive when it is in the phosphorylated form, and also that endogenous phosphatase and kinase are preserved in the tonoplast-free cells prepared with slow perfusion. On the other hand, tonoplast-free cells prepared with rapid perfusion are less sensitive to Ca^{2+} (TOMINAGA and TAZAWA 1981). The endogenous kinase which is sensitive to Ca^{2+} might be washed away by the rapid perfusion. This is consistent with the result that in such cells phosphatase inhibitor, α-NP did not affect streaming (Fig. 10). If the kinase activity is extremely low, phosphorylation of the component leading to streaming cessation can not occur even if the phosphatase activity is suppressed by the inhibitor. Thus the low sensitivity of streaming to Ca^{2+} can be explained by the lowered kinase activity. ATP-γ-S did not inhibit streaming when given in the absence of Ca^{2+} (Fig. 2). By contrast, ATP-γ-S completely inhibited the streaming recovery no matter whether it was applied before, during or after Ca^{2+}-treatment (Table 1 *B*, *D*, and *F*; Figs. 2–4). Involvement of Ca^{2+} in phosphorylation, presumably via activation of a protein kinase, was supported by the fact that ATP-γ-S was very effective in inhibiting the recovery of streaming only when it coexisted with Ca^{2+} in the cytoplasm. When the external perfusion medium containing ATP-γ-S was replaced with the perfusion medium containing Ca^{2+}, ATP-γ-S was not immediately washed away (Fig. 2). Also in Fig. 4, ATP-γ-S coexisted with Ca^{2+} in the cytoplasm during the initial phase of perfusion with the medium containing ATP-γ-S. In a human lung fibroblast cell model ATP-γ-S thiophosphorylated the myosin light chain only in the presence of Ca^{2+} (MASUDA *et al.* 1984).

Based on the above discussions and the facts that in many motile systems the motility is regulated by myosin phosphorylation as mentioned before (KUCZMARSKI and SPUDICH 1980, HARTSHORNE 1982, OGIHARA *et al.* 1983, KOHAMA and KENDRICK-JONES 1986), we propose myosin as the most probable candidate for the substrate of phosphorylation-dephosphorylation, although there is also a possibility that the cytoplasmic streaming is controlled indirectly by phosphorylation of a regulatory protein. In Fig. 11 we depict schematically phosphorylation-dephosphorylation cycle of myosin for regulation of cytoplasmic streaming in

intact characean cells. Putative protein kinase and protein phosphatase are both activated with Ca^{2+} coming probably from the external medium upon membrane excitation (HAYAMA *et al.* 1979). Both protein kinase and protein phosphatase are assumed to be working even in the resting state where free Ca^{2+} concentration in the cytoplasm is very low, but their activities are considered to be weak under low Ca^{2+} concentration. To keep a high rate of streaming in the resting state, the phosphatase activity should be stronger than the kinase activity.

A sudden increase in Ca^{2+} concentration caused by membrane excitation activates the kinase stronger than does the phosphatase. Then myosin is phosphorylated and the streaming is stopped. An almost instantaneous cessation of streaming which occurs within 1 second after the onset of an action potential (TAZAWA and KISHIMOTO 1968) may be indicative of rapid phosphoralation of myosin. In our scheme shown in Fig. 11 the phosphatase is also activated by Ca^{2+} but its activity is considered to be too low to dephosphorylate myosin instantaneously, as envisaged by a very slow recovery of streaming which takes about 5 minutes (WILLIAMSON and ASHLEY 1982). On the other hand, the once elevated Ca^{2+} concentration quickly decreases and recovers its low resting level with 30 to 40 seconds (WILLIAMSON and ASHLEY 1982). This quick recovery will bring forth lowering of the kinase activity.

How can the Ca^{2+}-dependent protein phosphatase still retain a high activity under the low Ca^{2+} condition? This paradox may be solved, if the phosphatase activation is regulated by Ca^{2+} not directly but indirectly, namely via calmodulin which was demonstrated to exist in characean cells (TOMINAGA *et al.* 1985). In the previous paper we reported that calmodulin-binding drugs did not inhibit streaming in the absence of Ca^{2+} or Ca^{2+}-induced cessation of streaming but recovery of streaming in permeabilized cells. If the recovery is induced by Ca^{2+}/calmodulin, Ca^{2+}/calmodulin has to cause dephosphorylation of myosin via activating the phosphatase. Calmodulin, due to its cooperative binding of Ca^{2+} binds Ca^{2+} instantaneously and this Ca^{2+} remains bound even after the free Ca^{2+} concentration falls. This hysteresis would allow control of the phosphatase even after the free Ca^{2+} concentration has fallen.

SHIMMEN *et al.* (1984) permeabilized the plasmalemma of *Nitella* with electrical pulses which were given immediately after cessation of streaming. Presence of EGTA in the external medium accelerated the initial recovery of the streaming in the permeabilized cell presumably via depressing the kinase activity caused by rapid chelation of Ca^{2+}. But the time for complete recovery was equal to that found in intact cells. Thus the rate of Ca^{2+} removal is not critical for streaming recovery.

Protein phosphatase (2 B) (= calcineurin) is Ca^{2+}/calmodulin-dependent phosphatase. It dephosphorylates inhibitor-1 which in its phosphorylated form inhibits protein phosphatase-1 (PALLEN and WANG 1985, YANG *et al.* 1982, STEWART *et al.* 1983). If calcineurin-like phosphatase is present also in characean cytoplasm, Ca^{2+}/calmodulin may stimulate phosphatase activity by dephosphorylating its inhibitor.

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References

CHEN JWC, KAMIYA N (1975) Localization of myosin in the internodal cell of *Nitella* as suggested by differential treatment with N-ethylmaleimide. Cell Struct Function 1: 1–9

FOULKES JG, COHEN P (1980) The regulation of glycogen metabolism. Purification and properties of protein phosphatase inhibitor-2 from rabbit skeletal muscle. Eur J Biochem 105: 195–203

GRATECOS D, FISCHER EH (1974) Adenosine 5'-O-(3-thiotriphosphate) in the control of phosphorylase activity. Biochem Biophys Res Commun 58: 960–967

HARTSHORNE DJ (1982) Phosphorylation of myosin and the regulation of smooth-muscle actomyosin. In: DOWBEN RM, SHAY JW (eds) Cell and muscle motility II. Plenum, New York, pp 188–220

HAYAMA T, TAZAWA M (1978) Cessation of cytoplasmic streaming accompanying osmosis-induced membrane depolarization in *Nitella flexilis*. Cell Struct Function 3: 47–60

— SHIMMEN T, TAZAWA M (1979) Participation of Ca^{2+} in cessation of cytoplasmic streaming induced by membrane exitation in *Characeae* internodal cells. Protoplasma 99: 305–321

HEMMINGS BA, RESINK TJ, COHEN P (1982) Reconstitution of a Mg·ATP-dependent protein phosphatase and its activation through a phosphorylation mechanism. FEBS Lett 150: 319–324

KAMIYA N (1981) Physical and chemical basis of cytoplasmic streaming. Ann Rev Plant Physiol 32: 205–236

— KURODA K (1956) Artificial modification of the osmotic pressure of the plant cell. Protoplasma 46: 423–436

KATO T, TONOMURA Y (1977) Identification of myosin in *Nitella flexilis*. J Biochem 82: 777–782

KIKUYAMA M, TAZAWA M (1982) Ca^{2+} ion reversibly inhibits the cytoplasmic streaming of *Nitella*. Protoplasma 113: 241–243

— — (1983) Transient increase of intracellular Ca^{2+} during excitation of tonoplast-free *Chara* cells. Protoplasma 117: 62–67

KOHAMA K, KENDRICK-JONES J (1986) The inhibitory Ca^{2+}-regulation of the actin-activated Mg-ATPase activity of myosin from *Physarum polycephalum* plasmodia. J Biochem 99: 1433–1446

— SHIMMEN T (1985) Inhibitory Ca^{2+}-control of movement of beads coated with *Physarum* myosin along actin-cables in *Chara* internodal cells. Protoplasma 129: 88–91

KUCZMARSKI ER, SPUDICH JA (1980) Regulation of myosin self-assembly: phosphorylation of *Dictyostelium* heavy chain inhibits formation of thick filaments. Proc Natl Acad Sci USA 77(12): 7292–7296

MASUDA H, OWARIBE K, HAYASHI H, HATANO S (1984) Ca^{2+}-dependent contraction of human lung fibroblasts treated with Triton X-100: A role of Ca^{2+}-calmodulin-dependent phosphorylation of myosin 20.000-dalton light chain. Cell Motility 4: 315–331

MORGAN M, PERRY SV, OTTAWAY J (1976) Myosin light chain phosphatase. Biochem J 157: 687–697

NIMMO GA, COHEN P (1978) The regulation of glycogen metabolism. Purification and characterization of protein phosphatase inhibitor-1 from rabbit skeletal muscle. Eur J Biochem 87: 341–351

OGIHARA S, IKEBE M, TAKAHASHI K, TONOMURA Y (1983) Requirement of phosphorylation of *Physarum* myosin heavy chain for thick filament formation, actin activation of Mg^{2+}-ATPase activity, and Ca^{2+}-inhibitory superprecipitation. J Biochem 93: 205–223

PALEVITZ BA, ASH JF, HEPLER PK (1974) Actin in the green alga, *Nitella*. Proc Natl Acad Sci USA 71 (2): 363–366

PALLEN CJ, WANG JH (1985) A multifunctional calmodulin-stimulated phosphatase. Arch Biochem Biophys 237: 281–291

PORTZEHL H, CALDWELL PC, RÜEGG JC (1964) The dependence of contraction and relaxation of muscle fibres from the crab *Maia squinado* on the internal concentration of free calcium ions. Biochim Biophys Acta 79: 581–591

SHEETZ MP, SPUDICH JA (1983) Movement of myosin-coated structures on actin cables. Cell Motility 3: 485–489

SHIMMEN T, TAZAWA M (1983) Control of cytoplasmic streaming by ATP, Mg^{2+} and cytochalasin B in permeabilized *Characeae* cell. Protoplasma 115: 18–24

— TOMINAGA Y, TAZAWA M (1984) Involvement of Ca^{2+} and flowing endoplasm in recovery of cytoplasmic streaming after K^+-induced cessation. Protoplasma 121: 178–185

STEWART AA, INGEBRITSEN TS, COHEN P (1983) The protein phosphatases involved in cellular regulation. 5. Purification and properties of a Ca^{2+}-calmodulin-dependent protein phosphatase (2 B) from rabbit skeletal muscle. Eur J Biochem 132: 289–295

TAZAWA M (1968) Motive force of the cytoplasmic streaming in *Nitella*. Protoplasma 65: 207–222

— KIKUYAMA M, SHIMMEN T (1976) Electric characteristics and cytoplasmic streaming of *Characeae* cells lacking tonoplast. Cell Struct Funct 1: 165–176

— KISHIMOTO U (1968) Cessation of cytoplasmic streaming of *Chara* internodes during action potential. Plant Cell Physiol 9: 361–368

TOMINAGA Y, TAZAWA M (1981) Reversible inhibition of cytoplasmic streaming by intracellular Ca^{2+} in tonoplast-free cells of *Chara australis*. Protoplasma 109: 103–111

— SHIMMEN T, TAZAWA M (1983) Control of cytoplasmic streaming by extracellular Ca^{2+} in permeabilized *Nitella* cells. Protoplasma 116: 75–77

— MUTO S, SHIMMEN T, TAZAWA M (1984) Calcium control of cytoplasmic streaming and localization of Ca^{2+}-sensitizing component in endoplasm of characean cells. In: SENO S, OKADA Y (eds) International cell biology 1984. The Japan Society for Cell Biology, Kyoto, p 505

— — — — 1985: Calmodulin and Ca^{2+}-controlled cytoplasmic streaming in characean cells. Cell Struct Funct 10: 315–325

TUNG HYL, RESINK TJ, HEMMINGS BA, SHENOLIKAR S, COHEN P (1984) The catalytic subunits of protein phosphatase-1 and protein phosphatase 2 A are distinct gene products. Eur J Biochem 138: 635–641

WILLIAMSON RE (1975) Cytoplasmic streaming in *Chara*: a cell model activated by ATP and inhibited by cytochalasin B. J Cell Sci 17: 655–668

— ASHLEY CC (1982) Free Ca^{2+} and cytoplasmic streaming in the alga *Chara*. Nature 296: 647–651

YANG S-D, TALLANT EA, CHEUNG WY (1982) Calcineurin is a calmodulin-dependent protein phosphatase. Biochem Biophys Res Commun 106 (4): 1419–1425