Calmodulin and Ca^{2+} /Calmodulin-Binding Proteins are Involved in *Tetrahymena thermophila* Phagocytosis

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ABSTRACT. The ciliated protist, *Tetrahymena thermophila*, possesses one oral apparatus for phagocytosis, one of the most important cell functions, in the anterior cell cortex. The apparatus comprises four membrane structures which consist of ciliated and unciliated basal bodies, a cytostome where food is collected by oral ciliary motility, and a cytopharynx where food vacuoles are formed. The food vacuole is thought to be transported into the cytoplasm by a deep fiber which connects with the oral apparatus. Although a large number of studies have been done on the structure of the oral apparatus, the molecular mechanisms of phagocytosis in *Tetrahymena thermophila* are not well understood. In this study, using indirect immunofluorescence, we demonstrated that the deep fiber consisted of actin, CaM, and Ca^{2+} /CaM-binding proteins, p85 and EF-1 α , which are closely involved in cytokinesis. Moreover, we showed that CaM, p85, and EF-1 α are colocalized in the cytostome and the cytopharynx of the oral apparatus. Next, we examined whether Ca^{2+} /CaM signal regulates *Tetrahymena thermophila* phagocytosis, using Ca^{2+} /CaM inhibitors chlorpromazine, trifluoperazine, N-(6-aminohexyl)-1-naphthalenesulfonamide, and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl. In *Tetrahymena*, it is known that Ca^{2+} /CaM signal is closely involved in ciliary motility and cytokinesis. The results showed that one of the inhibitors, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl, inhibited the food vacuole formation rather than the ciliary motility, while the other three inhibitors effectively prevented the ciliary motility. Considering the colocalization of CaM, p85, and EF-1 α to the cytopharynx, these results suggest that the Ca^{2+} /CaM signal plays a pivotal role in *Tetrahymena thermophila* food vacuole formation.

Key words: actin/calmodulin/EF-1 α /phagocytosis/p85/*Tetrahymena*

The ciliated protist, *Tetrahymena*, has one oral apparatus for phagocytosis, in the anterior cell cortex. The oral apparatus is a funnel-like structure, and mainly comprises three membranelles, an undulating membrane, and a ribbed wall which exists in between the three membranelles and the undulating membrane (Fig. 1A, B) (Frankel, 1999). The membrane

structures of the oral apparatus consist of ciliated and unciliated basal bodies (Frankel, 1999). In addition, underlying the ribbed wall is a crescent-shaped structure (Fig. 1A, B) (Williams and Bakowska, 1982). In the innermost part of the funnel-like structure, a cytostome, a cytopharynx, and a cytostomal lip, which is a projection separating the cytostome from the cytopharynx, can be seen adjacent to the ribbed wall (Fig. 1B) (Elliott and Kennedy, 1973). It is thought that in *Tetrahymena*, food is gathered into the cytostome by ciliary motility of the oral membrane structures, and then a food vacuole is formed in the cytopharynx (Elliott and Kennedy, 1973; Nilsson, 1979). Although the system for transporting the food vacuole into the cytoplasm is not well understood, a deep fiber which courses inward from the cytostome may be involved.

The architecture of the oral apparatus is classified into a microtubular structure and nonmicrotubular components. The microtubular structure includes connectives among the three membranelles and the undulating membrane (Kiers-

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Abbreviations: CaM, calmodulin; CaR1, affinity-purified rabbit anti-*Tetrahymena* calmodulin antibody 1; CPZ, chlorpromazine hydrochloride; DMSO, dimethyl sulfoxide; EF-1 α , elongation factor 1 α ; EFR1, affinity-purified rabbit anti-*Tetrahymena* EF-1 α antibody 1; F-actin, filamentous actin; NP-40, Nonidet P-40; p85GP1, guinea pig anti-p85 antiserum 1; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TAGP5, guinea pig anti-*Tetrahymena thermophila* actin antiserum 5; TFP, trifluoperazine hydrochloride; W5, N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl.

Fig. 1. Schematic diagrams of the cortical organization of *T. thermophila*. (A) Cortical structure. (B) Three-dimensional structure of the oral apparatus. M1-3, three membranelles. UM, undulating membrane. These membrane structures are mainly composed of ciliated and unciliated basal bodies. CS, crescent-shaped structure. CyS and CyP show cytostome and cytopharynx, respectively, and indicate the region where these structures exist. DF, deep fiber. The deep fiber extends from the cell cortex into the cytoplasm. BB, basal body which possesses cilia. CVP, contractile vacuole pores. RW, ribbed wall. ORM, oral-rib microtubules. CyL, cytolipid.

nowska and Golinska, 1996; Williams and Luft, 1968). In addition, there are the oral-rib microtubules in the ribbed wall and the microtubules in the deep fiber (Kiersnowska and Golinska, 1996; Williams and Luft, 1968). Williams and Bakowska demonstrated that the deep fiber microtubules are inward continuations of the oral-rib microtubules (Williams and Bakowska, 1982). Previous studies showed that some proteins existed in the oral apparatus as nonmicrotubular components. In *Tetrahymena pyriformis* (*T. pyriformis*), actin was localized in the three membranelles and the undulating membrane (Hirono *et al.*, 1987) and coexisted with the actin-modulating protein, fimbrin, in the deep fiber (Watanabe *et al.*, 1998; Watanabe *et*

al., 2000). By immunofluorescence staining of *T. pyriformis*, CaM was observed in the three membranelles, the undulating membrane, and the crescent-shaped structure (Suzuki *et al.*, 1982). Honts and Williams isolated filament-forming proteins, tetrins, from *T. pyriformis* cortical cytoskeleton and showed that they were localized in the oral apparatus (Honts and Williams, 1990). In addition, Numata *et al.* found that *T. pyriformis* citrate synthase formed 14-nm filaments and that immunofluorescent staining for this protein was seen in the τ -shaped region which corresponds to the root part of the deep fiber (Numata *et al.*, 1983; Numata, 1996). In *Tetrahymena thermophila* (*T. thermophila*), actin was detected ultrastructurally in the basal body cages of isolated oral apparatus, by the immunogold technique (Hoey and Gavin, 1992). Fimbrin was also localized in *T. thermophila* deep fiber (Watanabe *et al.*, 1998). These findings provide useful information on the structure of the oral apparatus. However, the molecular mechanisms of *Tetrahymena* phagocytosis have not been well understood.

Phagocytosis also has been studied well in other eukaryotic cells. In *Dictyostelium discoideum* (*D. discoideum*), it was shown that profilin acted as a negative regulator of phagocytosis, since profilin-null cells were more efficient in phagocytosis than wild type cells (Temesvari *et al.*, 2000). In addition, a number of key phagosomal pathway regulatory proteins have been identified in *D. discoideum*, including actin (Jenne *et al.*, 1998), α -actinin (Furukawa and Fehcheimer, 1994), coronin (Maniak *et al.*, 1995), and low-molecular-weight GTPase Rab7 (Buczynski *et al.*, 1997), RacC (Seastone *et al.*, 1998), and Rap1 (Seastone *et al.*, 1999). In the mouse macrophage cell line J774.A1, the colocalization of Arp2/3 complex and actin was observed in the phagosome during Fc γ receptor and complement receptor-mediated phagocytosis (May *et al.*, 2000), and the recruitment of Arp2/3 and actin to phagosome was controlled by the small GTPase Rho, Cdc42, and Rac1 (May *et al.*, 2000). Moreover, phagocytosis of macrophage cell line J774.16 cells was inhibited by Ca^{2+} /CaM inhibitor TFP and W7 (Horwitz *et al.*, 1981). From these results, it is thought that the actin cytoskeleton, small GTPase, and Ca^{2+} /CaM signal play an important role in eukaryotic phagocytosis.

We have used *Tetrahymena* for studying cytokinesis. In the cytokinesis of animal cells, first, a division plane is determined at the cellular equator and then an actomyosin-based contractile ring appears at the division plane. The contractile ring constricts, generates a division furrow, and divides the cell (Satterwhite and Pollard, 1992; Schroder, 1968). We previously reported that actin (Gonda *et al.*, 1999a) and actin-modulated protein, EF-1 α (Numata *et al.*, 2000), were localized in a furrow in *T. thermophila*. EF-1 α binds to F-actin and induces F-actin bundles *in vitro* (Kurasawa *et al.*, 1996). Therefore, EF-1 α may bundle F-actin in the contractile ring. EF-1 α also binds to CaM in a Ca^{2+} -dependent manner, and Ca^{2+} /CaM inhibits the formation of F-actin bundles by EF-1 α (Kurasawa *et al.*, 1996). *T.*

thermophila p85 is localized to the presumptive division plane before the formation of the contractile ring (Gonda *et al.*, 1999a; Numata *et al.*, 1995; Ohba *et al.*, 1986), and the contractile ring is thought to be formed where p85 is localized in the division plane (Gonda *et al.*, 1999a). In addition, Ohba *et al.* reported immunofluorescent staining of p85 in the membrane structures and the crescent-shaped structure of the *T. thermophila* oral apparatus (Ohba *et al.*, 1986). Recently, we found that p85 interacted directly with CaM in a Ca^{2+} -dependent manner, and that both proteins colocalized in the division furrow during cytokinesis (Gonda *et al.*, 1999a; Gonda *et al.*, 1999b). Moreover, we showed that Ca^{2+} /CaM inhibitor W7 inhibited the direct interaction between p85 and Ca^{2+} /CaM, the localization of p85 and CaM to the division plane, and some events downstream of p85, such as the formation of a contractile ring and division furrow (Gonda *et al.*, 1999a). Therefore, the direct interaction of p85 and Ca^{2+} /CaM may determine the division plane and play a crucial role in the formation of the contractile ring.

In this study, to clarify the mechanisms of *T. thermophila* phagocytosis, we further examined the localization of actin, CaM, and Ca^{2+} /CaM-binding proteins, p85 and EF-1 α , to the oral apparatus in interphase cells, using various immunofluorescence procedures. The results showed that actin is localized in the deep fiber, and that CaM, p85, and EF-1 α are colocalized in the crescent-shaped structure, the cytostome, the cytopharynx, and the deep fiber in *T. thermophila*. Moreover, we report that Ca^{2+} /CaM inhibitor W7 inhibits the food vacuole formation in *T. thermophila*. We also discuss the mechanisms of phagocytosis regulated by CaM and Ca^{2+} /CaM-binding proteins.

Materials and Methods

Cell culture

Cultivation of *Tetrahymena thermophila* (B24964WT) was performed as described previously (Watanabe *et al.*, 1994).

Antibodies

A guinea pig anti-p85 antiserum p85GP1 (Ohba *et al.*, 1986), a guinea pig anti-*T. thermophila* actin antiserum TAGP5 (Gonda *et al.*, 1999a), an affinity-purified rabbit anti-*Tetrahymena* CaM antibody CaR1 (Gonda *et al.*, 1999a), and an affinity-purified rabbit anti-*Tetrahymena* EF-1 α antibody EFR1 (Numata *et al.*, 2000) were prepared as described previously.

Immunofluorescence

Immunofluorescence microscopy was performed using two different techniques. First, to immunostain actin, the cells were washed with S buffer (7.5 mM Na_2HPO_4 , 2.5 mM NaH_2PO_4 , and 100 mM NaCl, pH 7.2), incubated in S buffer for 5 min at room temperature, air-dried on slides, and fixed with methanol for 30 min at -80°C . After a wash with S buffer, the cells were incubated

at room temperature with 5% nonfat-dried milk in S buffer for 30 min, and then with TAGP5 (diluted 1:100 in S buffer) for 2 hours. After a wash with S buffer, they were incubated with rhodamine-conjugated anti-guinea pig IgG antibodies (KPL, Gaithersburg, MD, USA; diluted 1:100 in S buffer) for 2 hours at room temperature. After a final wash with S buffer, the cells were observed with a Karl Zeiss Axio fluorescence microscope and photographed with Tri-X Pan 400 film (Kodak, Rochester, NY, USA). Second, to double-immunostain p85 and CaM or p85 and EF-1 α , an immunofluorescent technique using a cell model was applied (Goode-nough, 1983). The cells were treated with 0.25% NP-40, air-dried on slides, and then fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at 4°C . After a wash with PBS, the cells were incubated sequentially with 0.1 M glycine in PBS for 30 min at 4°C , with 5% nonfat-dried milk in PBS for 30 min at room temperature, with p85GP1 (diluted 1:50 in PBS) and CaR1 (diluted 1:4 in PBS) or p85GP1 (diluted 1:50 in PBS) and EFR1 (diluted 1:4 in PBS) for 2 hours at room temperature, and with rhodamine-conjugated anti-guinea pig IgG antibodies (KPL; diluted 1:100 in PBS) for p85 and FITC-conjugated anti-rabbit IgG antibodies (KPL; diluted 1:500 in PBS) for CaM and EF-1 α , for 2 hours at room temperature. The cells were washed with PBS after each incubation.

Inhibitory experiments on Ca^{2+} /CaM inhibitors CPZ, TFP, W5, and W7

To observe the effect of CPZ (Wako, Osaka, Osaka, Japan), TFP (SIGMA, St. Louis, MO, USA), W5 (SIGMA), and W7 (ICN, Aurora, OH, USA) on the phagocytosis, these inhibitors dissolved in 0.2% DMSO were added to log-phase cultures, and the cultures incubated at 26°C . After incubation for 15 min, 2% red India ink (Kaimei, Urawa, Saitama, Japan) was added to the culture and the ratio of the cells forming carbon-containing food vacuoles and the number of food vacuoles in a cell were then determined.

Results

Localization of Actin, CaM, p85, and EF-1 α in *T. thermophila* Oral Apparatus

In *T. pyriformis*, previous studies revealed that actin and CaM were localized to the membranelles and the undulating membrane of the oral apparatus and the deep fiber (Fig. 1A, B) (Hirono *et al.*, 1987; Suzuki *et al.*, 1982). CaM was also localized in the crescent-shaped structure of the oral apparatus (Fig. 1A) (Suzuki *et al.*, 1982). In *T. thermophila*, we showed that the Ca^{2+} /CaM-binding and actin-crosslinking protein EF-1 α was localized to the crescent-shaped structure of the apparatus (Numata *et al.*, 2000), and that the localization of p85 was observed in the membranelles, the undulating membrane, and the crescent-shaped structure (Ohba *et al.*, 1986). In addition, Hoey and Gavin reported that actin was localized in the basal body cages of the isolated oral apparatus (Hoey and Gavin, 1992). However, the

functions of actin, CaM, p85, and EF-1 α in phagocytosis still remain unknown. To understand the molecular mechanisms of phagocytosis, we reinvestigated the localization of these proteins in the *T. thermophila* oral apparatus, using various immunofluorescence procedures.

In *T. thermophila*, it was not known whether actin exists in the deep fiber and the division furrow. We recently prepared a guinea pig anti-*T. thermophila* actin antiserum TAGP5 and succeeded in immunostaining actin filaments of the contractile ring in *T. thermophila* with TAGP5, using cells permeated and fixed with methanol (Fig. 2A) (Gonda *et al.*, 1999a). We next examined the localization of actin in *T. thermophila* interphase cells, utilizing TAGP5. *T. thermophila* actin was detected in the deep fiber in interphase (Figs. 1A, B, 2B) and, as in yeast, actin patches formed during the cell cycle (Fig. 2A, B). Since the deep fiber degenerates during cytokinesis, the localization of actin in the deep fiber was not observed during cytokinesis (Fig. 2A). These observations for actin could not be made by indirect immunofluorescence microscopy using a guinea pig preimmune serum (data not shown).

Next, to investigate the localization of CaM and Ca²⁺/CaM-binding proteins, p85 and EF-1 α in *T. thermophila* oral apparatus, double immunostaining for p85 and either CaM or EF-1 α was carried out, using cells fixed with 3% paraformaldehyde after permeation with 0.25% NP-40. In interphase cells, p85 and CaM (Fig. 3A, B) or p85 and EF-1 α (Fig. 3C, D) colocalized in the deep fiber. These results show that actin, CaM, p85, and EF-1 α colocalize in the

Fig. 2. Immunofluorescent localization of actin during the cell cycle. Immunofluorescent images for actin with TAGP5 in the middle phase of cytokinesis (A) and interphase (B) are represented. CR and DF show the contractile ring and the deep fiber, respectively. In all photographs, the anterior regions of the cells are aligned on the upper side.

Fig. 3. Immunofluorescent localization of p85, CaM, and EF-1 α in interphase cells. Double-immunofluorescent images for p85 (A) and CaM (B), and those for p85 (C) and EF-1 α (D) are shown. DF, deep fiber. The anterior regions of the cells are aligned on the upper side.

deep fiber of *T. thermophila*. In addition, immunofluorescence for p85, CaM, and EF-1 α was seen in the membranelles, the undulating membrane, and the crescent-shaped structure of the oral apparatus (Figs. 1A, B, 3A–D). The localization of CaM was also observed in cilia (Fig. 3B) (this data will be reported in another paper), as in *T. pyriformis* (Suzuki *et al.*, 1982). Immunofluorescence of EF-1 α was also seen in the macronucleus (Fig. 3D). More noteworthy is that CaM and Ca²⁺/CaM-binding proteins, p85 and EF-1 α , colocalized in the cytostome and the cytopharynx of the oral apparatus where the deep fiber connects (Figs. 1A, B, 3A–D). In *T. thermophila* phagocytosis, it is thought that food is collected into the cytostome by the oral ciliary motility and then a food vacuole is formed in the cytopharynx. Therefore, CaM, p85, and EF-1 α may cooperate in regulating the formation of the food vacuole. These

localizations were not detected by indirect immunofluorescence using a rabbit and a guinea pig control sera (data not shown).

T. thermophila phagocytosis is inhibited by Ca^{2+} /CaM inhibitor W7

Since CaM and two Ca^{2+} /CaM-binding proteins, p85 and EF-1 α , colocalized in the cytostome and the cytopharynx of the oral apparatus which is closely involved in *T. thermophila* phagocytosis, we next investigated whether Ca^{2+} /CaM signaling actually regulates *T. thermophila* phagocytosis, using CPZ, TFP, W5, and W7. CPZ and TFP, phenothiazine-derivatives, and W5 and W7, naphthalenesulfonamide-derivatives, are known well as Ca^{2+} /CaM inhibitors. These drugs bind to CaM in a Ca^{2+} -dependent manner and inhibit the binding of Ca^{2+} /CaM to its binding protein (Craven *et al.*, 1996; Hidaka *et al.*, 1981; Levin and Weiss, 1976; Osawa *et al.*, 1998; Palacios *et al.*, 1993). To observe the effect of these inhibitors on the phagocytosis, the inhibitors dissolved in DMSO were added to log-phase cell cultures and the cells were incubated at 26°C. After incubation for 15 min, India ink was added to the cell culture and then the effect of inhibitors on phagocytosis was investigated. The ratio of cells forming carbon-containing food vacuoles decreased depending on the dosage of inhibitor (Fig. 4). Although the treatment with 50 μM CPZ, 30 μM TFP, 800 μM W5, and 150 μM W7 did inhibit the ciliary motility, some activity remained (data not shown). Moreover, while, in the cells treated with CPZ, TFP, and W5, the ratio of cells forming carbon-containing vacuoles increased gradually, in the cells treated with 150 μM W7, it did not increase and was steady at approximately 66% (125 μM) and 44% (150 μM) (Fig. 4). From these results, we conclude that W7 is more effective for observing Ca^{2+} /CaM signaling in *T. thermophila* phagocytosis, a mat-

ter to be considered further in the Discussion.

Next, in the cells treated with 50 μM CPZ, 30 μM TFP, 800 μM W5, 150 μM W7, and DMSO alone, we examined the number of food vacuoles in each cell. The results showed that the number of food vacuoles was smaller in the cells treated with inhibitors than in the cells treated with DMSO alone (Fig. 5, 6).

In the cells treated with 50 μM CPZ, 30 μM TFP, 800 μM W5, and 150 μM W7, carbon-containing food vacuoles were not accumulated in the region connecting the deep fiber with the cytopharynx (Fig. 6). Thus, it is thought that these inhibitors do not prevent the transport of the food vacuole from the cytopharynx to cytoplasm. Considering the colocalization of CaM, p85, and EF-1 α in the cytopharynx of the oral apparatus, these results suggest that the Ca^{2+} /CaM signal is involved in the food vacuole formation. In addition, when the cells treated with 150 μM W7 were transferred to fresh medium at 40 min after the treatment with W7, they performed phagocytosis normally. Thus, the effect of W7 on phagocytosis is reversible.

Finally, to examine the effect of 150 μM W7 on the localization of CaM and Ca^{2+} /CaM-binding proteins, p85 and EF-1 α , to the oral apparatus, we observed the localization of CaM, p85, and EF-1 α in the cells in which W7 completely inhibited the formation of carbon-containing food vacuoles. The results showed that W7 did not disturb the localization of CaM, p85, and EF-1 α in the oral apparatus and the deep fiber (Fig. 7). In addition, we previously showed that 100 μM W7 inhibited the binding of Ca^{2+} /CaM and p85 and thereby prevented initiation of cytokinesis. Therefore, these results suggest that the treatment with W7 does not affect the structure of the oral apparatus, though it prevents the binding of Ca^{2+} /CaM and its target protein in the structure. In these inhibitory experiments on Ca^{2+} /CaM inhibitors, the treatment with DMSO alone had no effect on phagocytosis.

Fig. 4. Effects of Ca^{2+} /CaM inhibitors on the ratio of the cells forming carbon-containing food vacuoles. Log-phase cell cultures were incubated with various concentrations of Ca^{2+} /CaM inhibitor at 26°C. After incubation for 15 min, India ink was added to the culture and the cells were then fixed with 1.9% formalin at the indicated time. CPZ: ▲, 30 μM ; ■, 50 μM . TFP: ▲, 20 μM ; ■, 30 μM . W5: ▲, 600 μM ; ■, 800 μM . W7: ▲, 100 μM ; ●, 125 μM ; ■, 150 μM . In all line graphs, open circles (○) with dashed lines show the cells treated with DMSO alone. The abscissa represents time (min) after the addition of India ink and the ordinate shows the population of cells ingesting carbon. At the indicated time at various inhibitor concentrations, 200–300 cells were counted. The results are representative of three independent experiments.

Fig. 5. Effects of Ca^{2+} /CaM inhibitors on the number of carbon-containing food vacuoles in each cell. Cell cultures were incubated with 50 μM CPZ, 30 μM TFP, 800 μM W5, 150 μM W7, or DMSO alone at 26°C. After incubation for 15 min, India ink was added to the culture, as in Fig. 4. In each histogram, at 20 min after the addition of India ink, the number of carbon-containing food vacuoles in the cells was counted. In CPZ: av.=2.8, n=124. In TFP: av.=2.5, n=93. In W5: av.=2.8, n=77. In W7: av.=3.3, n=94. In DMSO: av.=6.8, n=106. The abscissa shows the number of carbon-containing food vacuoles in the cells and the ordinate the population of cells. The results are representative of three independent experiments.

Discussion

In *T. thermophila* phagocytosis, first, food is collected into the cytostome of the oral apparatus by ciliary motility (Fig. 1A, B). Next, to ingest the food, vacuoles are formed in the cytopharynx of the oral apparatus (Fig. 1A, B), and then transported into the cytoplasm by an unknown mechanism. The deep fiber which connects with the oral apparatus may be involved in the transport (Fig. 1A, B).

In this study, to clarify the molecular mechanisms of phagocytosis, we investigated the localization of CaM and Ca^{2+} /CaM-binding proteins, p85 and EF-1 α , in the *T. thermophila* oral apparatus. The results showed that CaM, p85, and EF-1 α colocalized in the cytostome and the cytopharynx of the oral apparatus (Fig. 3A–D). Therefore, it is thought that the Ca^{2+} /CaM signal is closely involved in the formation of food vacuoles in *T. thermophila*. To investigate whether the Ca^{2+} /CaM signal regulates the food vacuole formation, we examined the effect of Ca^{2+} /CaM inhibitors CPZ, TFP, W5, and W7 on *T. thermophila* phagocytosis using India ink. The results showed that the ratio of the cells forming carbon-containing food vacuoles and the number of food vacuoles in each cell decreased de-

pending on the dosage of inhibitors (Figs. 4–6). It is known that the Ca^{2+} /CaM signal is involved in the ciliary motility (Kink *et al.*, 1990; Suzuki *et al.*, 1982), and that the motility is important for collecting the food into the oral apparatus. Therefore, although it is possible that inhibition of the ciliary motility by inhibitors prevents the food vacuole formation, we suppose that W7 affects the formation rather than the motility, while CPZ, TFP, and W5 affect the motility rather than the formation, for the following reason. Although the treatments with 50 μM CPZ, 30 μM TFP, 800 μM W5, and 150 μM W7 partially inhibited the activity of ciliary motility, some activity remained. In the cells treated with CPZ, TFP, and W5, the ratio of cells forming carbon-containing food vacuoles increased gradually (Fig. 4). These results suggest that, since the formation of food vacuoles is little affected by CPZ, TFP, and W5, the cells treated with these inhibitors can gradually form food vacuoles using the remaining activity of ciliary motility. In other words, it is thought that, in the cells treated with CPZ, TFP, and W5, the inhibition of food vacuole formation is caused only by the inhibition of the ciliary motility. Likewise, Suzuki *et al.* previously showed that in *T. pyriformis* cells treated with TFP, the ratio of cells forming carbon-containing vacuoles

increased gradually (Suzuki *et al.*, 1982), as in this study. In contrast, in the cells treated with 150 μM W7, the ratio did not increase and was steady at approximately 44% (Fig. 4). This result suggests that, since the food vacuole formation is inhibited by W7, the ratio in the cells treated with W7 did not increase and was steady in spite of the activity of ciliary motility. In summary, it is supposed that W7 prevents the formation of food vacuoles more effectively than ciliary motility in phagocytosis. Therefore, we proposed that W7 is more suitable for observation of the $\text{Ca}^{2+}/\text{CaM}$ signal in *Tetrahymena* phagocytosis than other $\text{Ca}^{2+}/\text{CaM}$ inhibitors. The effectiveness of a $\text{Ca}^{2+}/\text{CaM}$ inhibitor is thought to be related to its ability to permeate the cell membrane and oral apparatus. Moreover, we think that W7 does not affect the transport of the food vacuoles. If W7 does inhibit the transport, the untransported vacuoles ought to be accumulated in the connecting region between the deep fiber and the cytopharynx. However, no such accumulation was observed in the inhibitory experiments with W7 (Fig. 6), indicating that W7 inhibits the formation but does not affect the transport of the food vacuoles. These results suggest that the $\text{Ca}^{2+}/\text{CaM}$ signal regulates the formation of food vacuoles, and that p85 and EF-1 α are candidates for the target protein of the $\text{Ca}^{2+}/\text{CaM}$ signal in *T. thermophila* phagocytosis.

Actin, CaM and two $\text{Ca}^{2+}/\text{CaM}$ -binding proteins, p85 and EF-1 α , colocalized in the deep fiber (Figs. 2B, 3A–D). In *T. thermophila* cytokinesis, it is thought that p85 and $\text{Ca}^{2+}/\text{CaM}$ cooperatively regulate the assembly of actin filaments in the division plane (Gonda *et al.*, 1999a), and that EF-1 α may bundle the actin filaments in the contractile ring, the bundling activity being regulated by $\text{Ca}^{2+}/\text{CaM}$ (Kurasawa *et al.*, 1996; Numata *et al.*, 2000). Thus, p85 and EF-1 α may be involved in the formation of the deep fiber under

regulation by $\text{Ca}^{2+}/\text{CaM}$. Since $\text{Ca}^{2+}/\text{CaM}$ inhibits the F-actin bundling activity of EF-1 α , actin filaments in the deep fiber may not be bundled. In addition, since the $\text{Ca}^{2+}/\text{CaM}$ inhibitor W7 did not affect the transport of the food vacuole (Fig. 6), it is thought that the $\text{Ca}^{2+}/\text{CaM}$ in the deep fiber is not involved in the transport.

In our previous study, since 100 μM W7 prevented the binding of p85 and $\text{Ca}^{2+}/\text{CaM}$, we treated the cells with 100 μM W7 before the formation of the contractile ring. The result showed that W7 prevented the appearance of p85 in the presumptive division plane (Gonda *et al.*, 1999a). However, in this study, we treated the cells with 150 μM W7 after the formation of the oral apparatus and the deep fiber. At this concentration, W7 did not affect the localization of CaM, p85, and EF-1 α in the oral apparatus and the deep fiber (Fig. 7). Therefore, it is supposed that, while W7 affects the interaction among CaM, p85, and EF-1 α in the oral apparatus and the deep fiber, treatment with W7 does not disturb the localization of these proteins in either structure. CaM, p85, and EF-1 α are probably being incorporated into a stable structure of the oral apparatus after the formation of the apparatus. In this inhibitory experiment, we treated *T. thermophila* cells with 150 μM W7. In CHO-K₁ cells, 100 μM W7 completely inhibited the cell proliferation (Hidaka *et al.*, 1981). Since *Tetrahymena* possesses three membrane structures, a cell membrane, an outer alveolar membrane, and an inner alveolar membrane (Frankel, 1999), it is thought that the sensitivity to W7 in *Tetrahymena* is lower than that in culture cells. Therefore, we believe that the W7 concentration used in this experiment is not abnormal. In addition, the applied concentration of naphthalenesulfonamid-derivatives W5 and W7 was higher than that of phenothiazine-derivatives CPZ and TFP. Thus, it is thought that cell permeation by naphthalenesulfonamid-

Fig. 6. Nomarski differential interference-contrast images of the cells treated with 50 μM CPZ, 30 μM TFP, 800 μM W5, 150 μM W7, and DMSO alone. These images show the cells at 20 min after the addition of India ink.

Fig. 7. Immunofluorescent localization of p85, CaM, and EF-1 α in the cells treated with 150 μ M W7. A log-phase cell culture was incubated with 150 μ M W7, and, after incubation for 15 min, India ink was then added to the culture, as in Figs. 4–6. Immunofluorescence was carried out using cells in which W7 completely inhibited the formation of carbon-containing food vacuoles at 20 min after the addition of India ink. Double-immunofluorescent images for p85 (A) and CaM (B), and those for p85 (C) and EF-1 α (D) are shown. DF, deep fiber. The anterior regions of the cells are aligned on the upper side.

derivatives is weaker than that by phenothiazine-derivatives in *T. thermophila*.

The Ca^{2+} /CaM signal is also involved in the phagocytosis of other eukaryotic cells. The phagocytosis of Kupffer cells (Watanabe *et al.*, 1988) and macrophage line J774.16 (Horwitz *et al.*, 1981) cells was prevented by Ca^{2+} /CaM inhibitors TFP and W7, suggesting that Ca^{2+} /CaM signal regulates the phagocytosis of these cells. In *Dictyostelium*, the Ca^{2+} -binding and actin-crosslinking protein, α -actinin, is localized in the phagocytic cup and is thought to be involved in the phagocytosis (Furukawa and Fechheimer, 1994). These results indicate that Ca^{2+} /CaM and Ca^{2+} -binding protein are generally crucial to phagocytosis in eukaryotic cells.

Recently, Lee *et al.* demonstrated that the *T. thermophila* cytoplasmic dynein heavy chain (Dyh1) regulates phagocytic activity, using gene knockout cells of Dyh1 (Lee *et al.*, 1999). Dynein is a molecular motor that transduces the chemical energy of ATP hydrolysis into mechanical motion along microtubules. In the knockout cells, although the formation of food vacuoles was inhibited, no accumulation of untransported food vacuoles was observed in the connective region between the deep fiber and the cytopharynx. Therefore, Dyh1 may function in the formation of food vacuoles in *T. thermophila* cells, as well as in Ca^{2+} /CaM signaling. Lee *et al.* did not discuss the molecular mechanisms of phagocytosis in detail (Lee *et al.*, 1999), and the relation between Dyh1 and CaM in the vacuole formation is unknown. It is important to elucidate the relation among Dyh1, CaM, and Ca^{2+} /CaM-binding proteins in the formation of food vacuoles in *T. thermophila*.

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