Axopodial Contraction in the Heliozoon Raphidiophrys contractilis Requires Extracellular Ca2

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Axopodial Contraction in the Heliozoon *Raphidiophrys contractilis* Requires Extracellular Ca^{2+}

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ABSTRACT—Axopodial contraction of the centrohelid heliozoon *Raphidiophrys contractilis* was induced by mechanical or electrical stimulation. For inducing contraction, extracellular Ca^{2+} was required. The threshold level of extracellular Ca^{2+} was between 10^{-6}–10^{-7} M. The speed of axopodial contraction was faster than 3.0 mm/sec. Re-elongation of axopodia started just after contraction, and its initial velocity was ~ 0.30 μm/sec. Electron microscopic observations were carried out using an improved fixative that contained 1 mg/ml ruthenium red and 15 μM Taxol. This fixative prevented artificial retraction of axopodia and resulted in better fixation. A bundle of hexagonally-arranged microtubules was observed in each axopodium, but no other filamentous structures were detected, suggesting that the contractile machinery of axopodia in *R. contractilis* may be different from that in actinophryid heliozoons in which Ca^{2+}-dependent contractile filaments are employed for contraction.

Key words: contraction, microtubules, contractile tubules, heliozoa, calcium

INTRODUCTION

Some protozoans are known to show characteristic cell movements that are controlled by intracellular calcium ions. They include coiling of the stalk in vorticellid ciliates (Katoh and Kikuyama, 1997; Moriyama *et al*., 1998), cell body contraction in heterotrichous ciliates (Huang and Pitelka, 1973; Yogosawa-Ohara *et al*., 1985; Ishida *et al*., 1996), contraction of the flagellar root fiber in algal cells (Salisbury *et al*., 1984; Salisbury, 1998), and contraction of the nucleus (Arikawa *et al*., 2003). Rapid axopodial contraction in actinophryid heliozoons also belongs to this class of motility (Arikawa and Suzaki, 2002; Arikawa *et al*., 2002). In actinophryid heliozoons such as *Actinophrys* and *Echinosphaerium*, rapid axopodial contraction occurs when a prey protozoan attaches to the tip of an axopodium, and the food organism is conveyed by the contraction toward the cell surface (Ockleford and Tucker, 1973; Suzaki *et al*., 1980, Kinoshita *et al*., 2001). Recently, we have shown that Ca^{2+}-dependent contraction of the "contractile tubules structure (CTS)" is responsible for the axopodial contraction in *Actinophrys sol* and *Echinosphaerium akamae* (Arikawa and Suzaki, 2002; Arikawa *et al*., 2002). The CTS has the appearance of a bundle of tubules that becomes conglobated to form a mass of agglomerated granules in a Ca^{2+}-dependent manner (Arikawa *et al*., 2002).

Centrohelid heliozoons are regarded as a distinct group of heliozoa that are possibly phylogenetically distant from actinophryids, as there are morphological differences in mitochondria and also in patterns of axonemal microtubules (Kinoshita *et al*., 1995, 2001). In spite of these differences, centrohelid heliozoons resembles actinophryids in many respects, including their overall spherical appearance with radiating axopodia and their ability to capture food organisms by axopodial contraction (Kinoshita *et al*., 1995; Sakaguchi *et al*., 2002). Moreover, the centrohelid *Raphidiophrys contractilis* also shows rapid axopodial contraction when a food particle is attached to the axopodial surface, followed by ingestion by food-cup-forming pseudopodia in the same manner as seen in actinophryid heliozoons (Kinoshita *et al*., 2001). In the present study, ultrastructure and axopodial contraction of *R. contractilis* were further examined and compared with those in actinophryid heliozoons.

MATERIALS AND METHODS

Organism and Culture

The heliozoon *Raphidiophrys contractilis* was originally collected from a brackish pond in Shukkei-en Garden, Hiroshima City, Japan. Organisms were cultured monoxenically at 20±1°C in a cul-

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ture medium based on 10% artificial sea water. The culture medium consisted of 47 mM NaCl, 1.1 mM KCl, 1.2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 2.5 mM $MgSO_4$, 1 mM Tris-HCl (pH 7.8), 0.74 mM sodium acetate, 0.01% polypeptone, 0.02% tryptone and 0.02% yeast extract plus the food flagellate *Chlorogonium elongatum* (Sakaguchi and Suzaki, 1999). Subculturing was carried out at intervals of about 3 weeks.

Axopodial contraction induced by external stimuli

Prior to experiments, the heliozoons were transferred to a Petri dish and left for 1 hr at room temperature until the cells were attached to the substratum. Then the heliozoons were washed with a large quantity of artificial brackish water (ABW; 47 mM NaCl, 1.1 mM KCl, 1.2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 2.5 mM $MgSO_4$ and 1 mM Tris-HCl at pH 7.8) and finally with calcium-depleted ABW containing 5 mM hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), 2 mM ethylene glycol bis (β-aminoethylether)-N ,N ,N' ,N' -tetraacetic acid (EGTA) and various concentrations of $CaCl_2$, adjusted to pH 7.0. The concentration of free calcium ions was calculated by an iterative procedure (Suzaki and Williamson, 1986). Mechanical shocks were applied by flicking a clip attached to the microscope stage, and electric shocks were applied by a home-made electrical stimulator. To determine the refractory period, two successive mechanical shocks were delivered at different time intervals. If a cell did not respond to the second shock, the cell was regarded to be still in the refractory period.

Light microscopy of contraction and re-elongation of axopodia

Heliozoons were placed on a glass slide and surrounded with rectangular mounting ridges of petroleum jelly. The cells were left still for 5–10 min to recover from axopodial disturbance caused by pipetting. Mechanical shocks were applied at different time intervals, and axopodial contraction and re-elongation were recorded on S-VHS video tape using a video recorder (BR-S822, Victor, Tokyo, Japan). Light microscope observation was carried out with Nomarski differential interference optics (BX50, Olympus, Tokyo, Japan), and photographs were taken with a digital camera (DP-11, Olympus, Tokyo, Japan).

Electron microscopy

To find better fixation conditions for electron microscopy, *R.contractilis* cells were fixed with an improved glutaraldehyde prefixative that has been modified from the conventional fixative for actinophryid heliozoons. With the conventional glutaraldehyde prefixative composed of 3% glutaraldehyde (TAAB Laboratories Equipment Ltd., Reading, UK) in 50 mM cacodylate buffer (pH 7.0) containing 0.02 mM $MgSO_4$ and 2 mM sucrose, almost all axopodia became retracted**.** Therefore, cells were fixed with an improved prefixative that contained 1 mg/ml ruthenium red (Katayama Chemical, Osaka, Japan) and 15 μM Taxol (Biomol Research Laboratories, Inc., Plymouth Meeting, USA) added to the conventional fixative. After fixation for 3 min at room temperature, the cells were rinsed with 50 mM cacodylate buffer (pH 7.0) containing 0.02 mM $MgSO_4$ and 2 mM sucrose, and post-fixed with 0.5% OsO_4 in the same buffer for 30 min. The fixed cells were dehydrated through a graded ethanol series (50%, 70%, 90%, 95%, 99% and 100%) and embed-

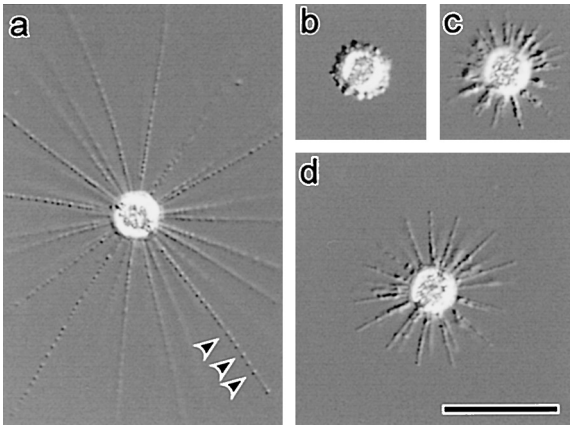

Fig. 1. Light micrographs of *Raphidiophrys contractilis* showing contraction and re-elongation of axopodia. (a) *R. contractilis* before mechanical stimulation, with fully-elongated axopodia. Arrowheads show kinetocysts. (b) *R. contractilis* just after stimulation. The axopodial contraction took place within one frame of the video recording, i.e., within 1/30 sec after application of the mechanical shock. (c) Re-elongation stage of axopodia after stimulation. (d) Ongoing re-elongation stage of axopodia. Bar=50 μm.

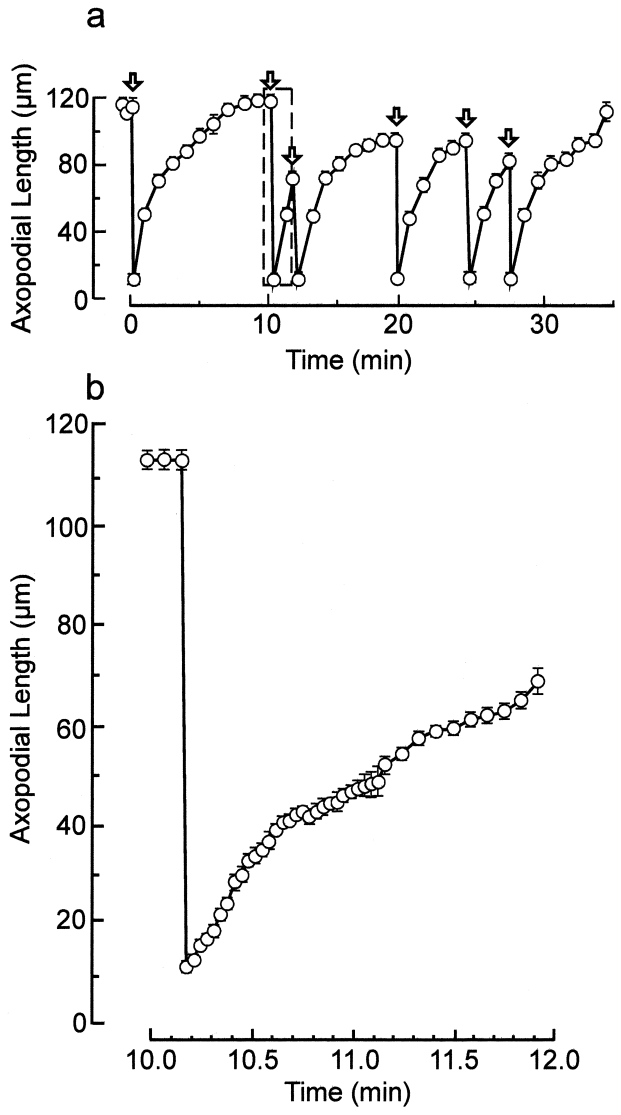

Fig. 2. Axopodial contraction induced by external stimulation in the heliozoon *R. contractilis*. Data represent mean±S.D. from 10 axopodia. (a) Axopodial contraction and re-elongation in response to mechanical shocks with different time intervals between successive stimulations. Arrows indicate application of mechanical shocks. (b) Detailed trace of the axopodial length showing a sequence of axopodial contraction and subsequent re-elongation. This graph corresponds to the rectangle in (a) indicated by a broken line.

ded in Spurr's low-viscosity embedding resin (Spurr, 1969). Ultrathin sections were stained with 3% aqueous uranyl acetate for 15 min and Reynolds' lead citrate stain (Reynolds, 1963) for 5 min at room temperature, and observed with a Hitachi S-7100 transmission electron microscope.

RESULTS

Raphidiophrys contractilis has a spherical cell body with many radiating axopodia (120 μm in length on average) around the cell (Fig. 1a). Each axopodium has 10–20 granular kinetocysts (Fig. 1a, arrowheads) which lie beneath the plasma membrane and have been implicated in the process of food uptake. In this study, we found that axopodial contraction of *R. contractilis* can be induced by mechanical or electrical stimulation in the presence of extra-cellular Ca^{2+}. After applying a mechanical shock, for example, all axopodia disappeared at once (Fig. 1b). Re-elongation of axopodia started immediately after the contraction, and continued at an initial speed of about 0.3 μm/sec. It took about 10 min

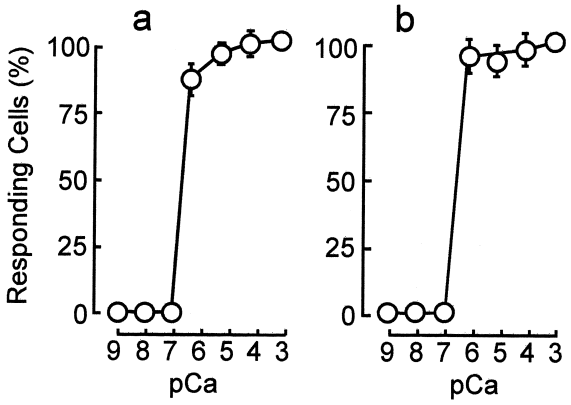

Fig. 3. Ca^{2+}-dependency of axopodial contraction in *R. contractilis*. Axopodial contraction was evoked by electrical (a) or mechanical (b) shock, and the ratios of the cells that showed axopodial contraction were determined. Data represents average±S.D. from 10-20 independent determinations with 5–10 cells in each experiment. Both graphs show a similar profile of dependency on extra-cellular Ca^{2+} concentration, with a threshold level between 10^{-6}–10^{-7} M. At free Ca^{2+} concentrations lower than 10^{-7} M, axopodial contraction was not induced at all.

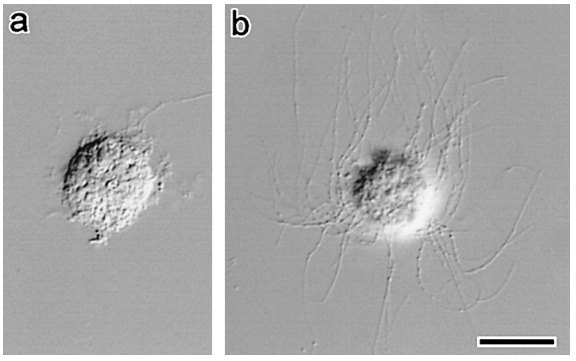

Fig. 4. Light micrographs of the heliozoon *Raphidiophrys contractilis* treated with a conventional glutaraldehyde fixative (a) or the improved glutaraldehyde fixative containing 1 mg/ml ruthenium red and 15 μM Taxol (b). Axopodia were disrupted by the conventional fixation, while they were well preserved with ruthenium red and Taxol. Bar=20 μm.

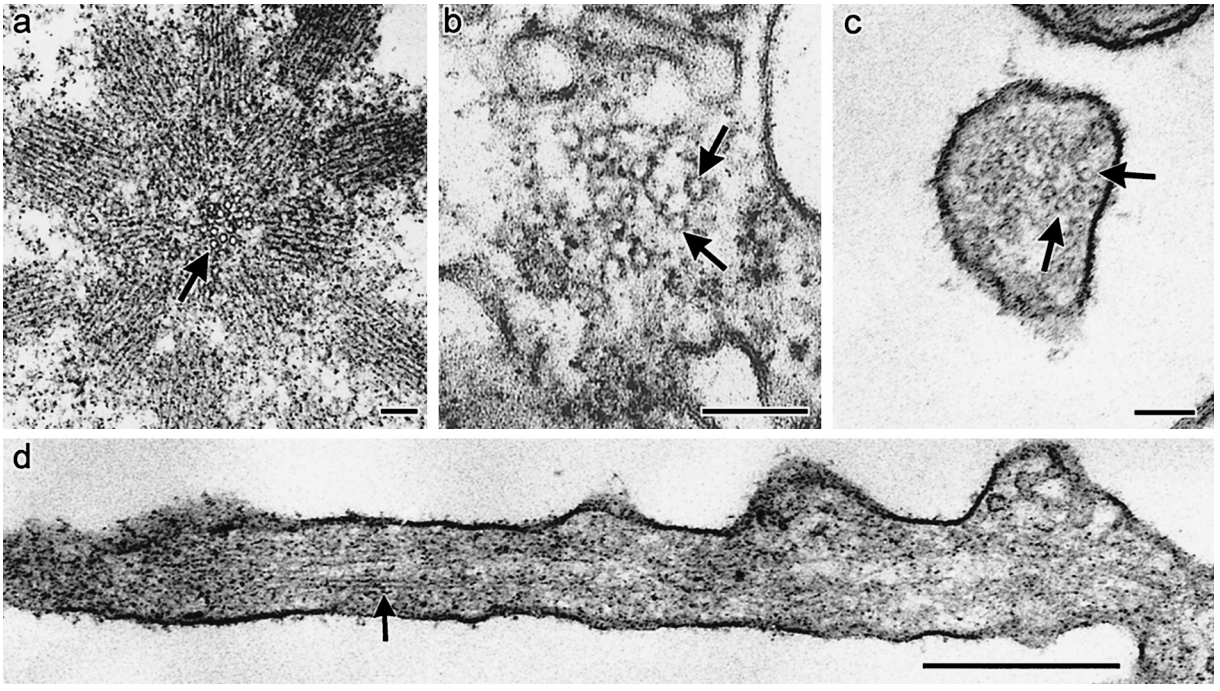

Fig. 5. Electron micrographs of *R. contractilis*. Bundles of microtubules are shown by arrows. (a) Cross section of bundles of microtubules near the central region of the cell body. (b) Cross section of microtubules located around the periphery of the cell body. (c) Cross section of an axopodium. (d) Longitudinal section of an axopodium. No filamentous structures other than microtubules were present in the cytoplasm near the microtubule bundles. Bars=100 nm (a–c), 500 nm (d).

before the axopodia reached their full length (Fig. 1b–d, and Fig. 2). Immediately after the contraction, a refractory period of about 5 sec was present. Within this period, no further contraction of the re-elongating axopodia could be induced at all. After 5 sec, a part of the growing axopodia started to show contraction in response to the second stimulus, and it took about 10 sec until all the axopodia regained the ability to respond to the stimulus. Mechanical shocks were applied at different time intervals (Fig. 2a, arrowheads) and the resulting axopodial contraction and re-elongation were monitored. The axopodial contraction took place within one frame (Fig. 1b) of the video recording, which indicates that the velocity of the contraction was greater than 3.0 mm/sec. Contraction was observed even when stimulation was applied during axopodial recovery. The cells responded to the electrical shock in the same way as to mechanical stimulation. In the absence of extra-cellular Ca^{2+}, axopodial contraction did not occur. The proportion of cells that exhibited axopodial contraction upon either mechanical or electrical stimulation was dependent on the extra-cellular Ca^{2+} concentration, with a threshold level between 10^{-6} to 10^{-7} M. In higher concentrations of Ca^{2+}, all cells showed axopodial contraction (Fig. 3a and Fig. 3b).

Electron microscopy was used to identify the cytoskeletal component that is responsible for axopodial contraction. Almost all axopodia were disrupted when a conventional glutaraldehyde pre-fixative was used (Fig. 4a), but addition of ruthenium red and Taxol was found to be effective in preserving axopodia (Fig. 4b). Bundles of microtubules radiate from the central region of the cell body (Fig. 5a). In cross sections of a bundle of microtubules located in the peripheral region of a cell body (Fig. 5b) and in an axopodium (Fig. 5c), a hexagonal pattern of microtubules was clearly observed, but no other structures like the CTS of actinophryid heliozoa were observed. Longitudinal sections of an axopodium also showed no indication of the presence of any other filamentous element in the axopodium (Fig. 5d).

DISCUSSION

In heliozoans, rapid contraction of axopodia is one of the most important cell functions, as they capture and ingest food organisms by using axopodia as tentacles for trapping passing food. In *R. contractilis* the axopodial contraction took place within one frame of the video recording and the velocity of the contraction was calculated to be more than 3.0 mm/sec, within which the microtubule-containing axopodia completely disappear. Similar phenomena have been described in other heliozoon species such as *Echinosphaerium nucleofilum*, *Actinophrys sol* and *Actinocoryne contractilis* (Febvre-Chevalier, 1980; Febvre-Chevalier and Febvre, 1992; Suzaki *et al.*, 1992; Kinoshita *et al.*, 2001). Also in these species, axopodia or the axopodium-like stalk show contraction at similarly rapid rates (1–10 mm/sec). In animal and plant cells, the velocity of microtubule disassembly shows a distribution ranging from 0.2 to 3.3 μm/sec (Moore *et al.*, 1997; Vorobjev *et al.*, 1999; Bray, 2001). When compared with these cases, contraction of the heliozoan axopodia and concomitant breakdown of the microtubules are regarded to occur in a very rapid way, at speed more than 1,000 times quicker than the normal microtubule disassembly. As the normal microtubule breakdown cannot explain such a rapid contraction in heliozoan axopodia, one may have to look for some additional mechanism for facilitating the rate of microtubule breakdown. Although further studies should be made to understand the mechanism of microtubule breakdown in *R. contractilis*, involvement of microtubule-severing proteins such as centrin (Salisbury *et al.*, 1986; Baron and Salisbury, 1988) or katanin (McNally *et al.*, 2002) is one of the possible explanations that may account for such a rapid disorganization of the microtubular system. In this research, we reported that axopodial contraction of the centrohelid heliozoon *R. contractilis* requires extracellular Ca^{2+}. To the best of our knowledge, it has not previously been reported. Although future research is also needed, involvement of intracellular Ca^{2+} in the microtubule disorganization is also probable.

In actinophryid heliozoons such as *Actinophrys sol* and *Echinosphaerium akamae*, axopodial contraction can be induced only when a prey cell attaches to the axopodial surface, and mechanical stimulation is not effective in inducing contraction (Kitching, 1960; Ockleford and Tucker, 1973; Suzaki *et al.*, 1980). In centrohelid heliozoons such as *Ciliophrys* and *Heterophrys*, on the contrary, axopodial contraction has also been reported to be induced by mechanical stimulation (Davidson, 1969, 1973). When *Ciliophrys* receives a mechanical shock, all of the axopodia contract simultaneously within a fraction of a second. Re-extension of axopodia takes about a minute and about the first 45 seconds of this is a refractory period during which further shocks will not cause another contraction. As shown in *R. contractilis* in this study, axopodial contraction did not occur in *Ciliophrys* in calcium-free artificial sea water (Davidson, 1969). In *R. contractilis*, axopodial extension required about 10 min, and a refractory period is also present but it is shorter (~ 5 sec) as compared with *Ciliophrys*.

The velocity of microtubules elongation in different animal and plant cells have been reported to be 0.017–0.5 μm/sec (Vorobjev *et al.*, 1999; Bray 2001; Wilde *et al.*, 2001; Stepanova *et al.*, 2003). In *R. contractilis*, re-elongation of axopodia took place with an initial speed of 0.30 μm/sec, which is among the range of reported values for microtubule assembly. Thus, it is suggests that the basis for the axopodial re-elongation may lie in the extension of the supporting microtubular bundles in axopodia.

In actinophryid heliozoa such as *Actinophrys sol* and *Echinosphaerium akamae*, the driving force for axopodial contraction is thought to be generated by transformation of the CTS in a Ca^{2+}-dependent manner (Suzaki *et al.*, 1994; Arikawa and Suzaki, 2002; Arikawa *et al.*, 2002). In centrohelid heliozoons, however, the existence of similar filamentous structures has not been reported so far. Since the

axopodia of centrohelids are susceptible to glutaraldehyde and easily disrupted, the contractile structure might have been destroyed during preparation. Thus, the fixation protocol has been improved, and better fixation was achieved by including ruthenium red and Taxol in the fixative. Ruthenium red is an effective reagent that has long been used for fixing actinophryid heliozoons, and possibly works by blocking presumptive calcium channels on the axopodial membrane and thereby preventing axopodial contraction and microtubule disassembly (Suzaki *et al*., 1980). Taxol is also known to prevent microtubule disassembly in heliozoa (Hausmann *et al*., 1983; Hauser, 1986). The improved fixation was successfully applied to *R. contractilis*; no artificial breakdown of axopodia was observed and microtubules were well preserved. Nevertheless, no filamentous elements other than microtubules were observed in any region of the cell. Although neither the force-producing structure nor the mechanism of axopodial contraction have been shown, these observations at least suggest that the mechanism of axopodial contraction in *R. contractilis* may be a Ca^{2+}-dependent phenomenon unique to this group of heliozoons which is different from the CTS-based machinery in the actinophryids.

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