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The role of the cytoskeleton in the ecdysis of the dinoflagellate *Prorocentrum minimum*

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Summary

Actin filaments (actomyosin system) and microtubules form highly organized cytoskeleton structures in unicellular organisms, particularly in dinoflagellates. The cytoskeleton organization of *Prorocentrum minimum* (Pavillard) Schiller, a bloom forming species, differs from that of most other studied dinoflagellates. While the cortical microtubules are absent, a dense layer of actin filaments is located beneath the cell covering. A previous study has shown that cytoskeleton likely plays an essential role in the ecdysis, the process of cell covering reorganization including shedding of the plasma membrane, outer amphiesmal vesicle membranes and thecal plates, of *P. minimum*. In this study, the involvement of cytoskeleton in the ecdysis process was investigated using specific drugs influencing cytoskeleton arrangement and functioning. *P. minimum* cells were treated with cytochalasin D, 2,3-butanedione monoxime (BDM) or nocodazole, and ecdysis was induced by centrifugation, a potent ecdysis trigger. The application of drugs significantly affected the rate of ecdysis induced by centrifugation. Cytochalasin D, an inhibitor of actin polymerization, impaired the last step of ecdysis, i.e. leaving the old thecal plates. BDM, a myosin function inhibitor, and nocodazole, a microtubule-disrupting agent, delayed this process, but did not inhibit it. Taken together, our data suggest that in *P. minimum* the cortical actin is a crucial cytoskeleton element, which is required for the final step of the ecdysis process. Apparently, it could enable opening of the old thecal plates, and squeezing of the ecdysing cell between them.

Key words: 2,3-butanedione monoxime, cytochalasin D, cytoskeleton, ecdysis, nocodazole, *Prorocentrum minimum*

Introduction

Dinoflagellates possess a complex cell covering called amphiesma. It consists of a continuous plasma membrane and flattened membrane vesicles (amphiesmal vesicles) laying beneath it. Amphiesmal vesicles of armored dinoflagellates contain cellulosic thecal plates, whereas amphiesmal vesicles of naked dinoflagellates lack them. In addition to the structures mentioned above, dinoflagellates have a complex cortical cytoskeleton. It includes microtubules and actin filaments which arrangement varies in different species of dinoflagellates (Roberts et al., 1992). Many dinoflagellates possess well-organized

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cortical microtubule rows spanning a cell from the apical and antapical poles to the cingulum (Brown et al., 1988; Roberts et al., 1988; Perret et al., 1993; Sekida et al., 2012).

Actin cytoskeleton can form a continuous cortical layer or, in some species, its organization pattern is more diffused and confined in flagellar or cingular areas (Roberts and Roberts, 1991). Actin network is important for cell functioning since it serves as a track system for various membrane organelles that move along actin microfibers via myosin. It is required for the transport of endocytic vesicles and interaction of cellular organelles. In dinoflagellates, the actomyosin complex is involved in the translocation of chloroplasts and scintillons and participates in mechanically induced bioluminescence (Heimann et al., 2009; Villanueva et al., 2015; Stires and Latz, 2018). Both cortical microtubules and actin network are essential for the cell shape maintenance and correct development of amphiesma during cell division and ecdysis (Schnepf, 1988; Schnepf et al., 1990; Sekida et al., 2012).

Ecdysis is a process of active cell covering rearrangement in response to various stressors, such as mechanical perturbation or the application of certain chemical agents, which is unique to dinoflagellates. During this process, amphiesmal vesicles fuse, a cell loses motility and sheds its plasma membrane and outer amphiesmal vesicle membrane, while the inner amphiesmal vesicle membrane develops into a new plasma membrane (Sekida et al., 2001; Pozdnyakov and Skarlato, 2012; Pozdnyakov et al., 2014). An immotile cell stays covered with thecal plates and/or pellicle layer. After a resting period a cell builds new flagella and leaves its old covering, and the new full amphiesma matures (Sekida et al., 2001; Chan et al., 2019).

Recently it was shown that the actin cytoskeleton is involved in ecdysis of *Prorocentrum minimum* (Pavillard) Schiller, a harmful bloom-forming species. Specifically, treatment with the actin depolymerizing agent latrunculin B leaded to a sharp decrease in the thecae shedding rate (Berdieva et al., 2018). In this study, we investigated the effect of other cytoskeleton-specific drugs, i.e. actin depolymerizing agent cytochalasin D, uncompetitive inhibitor of myosin function 2,3-butanedione monoxime (BDM), and microtubule stabilizing compound nocodazole, on the stressor-induced ecdysis process.

Table 1. List of the cytoskeletal drugs used in the study.

Drug	Stock solution	Final concentration
Cytochalasin D (Sigma-Aldrich, USA)	2 mg/ml in DMSO	5 µg/ml
2,3-butanedione monoxime (Sigma-Aldrich, USA)	50,5 mg/ml in 25 f/2 medium	1 µg/ml
Nocodazole (Sigma-Aldrich, USA)	5 mg/ml in DMSO	10 µg/ml

Material and methods

Culture maintenance and growth conditions

A clonal culture of *Prorocentrum minimum*, strain CCAP1136/16, was maintained in artificial seawater-based f/2 medium (Guillard and Ryther, 1962) without silicate at salinity adjusted to 25. Cells were grown at 25 ˚C under a 12 h/12 h light/dark illumination cycle and 100 µmol photons m^{-2} s^{-1}.

For experiments, cells were inoculated into 100 ml glass flasks at a cell concentration of 3×10^4 cells ml^{-1} and then allowed to grow for 6-7 days to reach an exponential growth phase (Berdieva et al., 2020).

Experimental procedures

Prior the induction of ecdysis, *P. minimum* cells were incubated with drugs for 20 min at room temperature. The list of drugs used in this study is shown in the Table 1. Cells incubated with the same volume of drug solvent (DMSO or f/2 medium depending on the chemical agent) were used as control. Following the incubation, ecdysis was induced by centrifugation at 10000 g for 5 min. After that, cells were resuspended in the same medium by short vortexing and kept at usual culturing conditions. The fraction of cells that successfully discarded their old thecal plates – ecdysis level – was estimated in 2, 4, and 6 h after centrifugation. Two additional cell samples were taken and kept at the same conditions to serve as an untreated (no chemical treatment, no centrifugation) control reflecting the natural level of ecdysis in the cultures. This natural level of ecdysis in untreated cells was estimated at the time points 0 and 6 h. All experiments were performed in triplicates.

Analytical procedures

To determine the fraction of cells that entered ecdysis (shedding of plasma membrane and outer

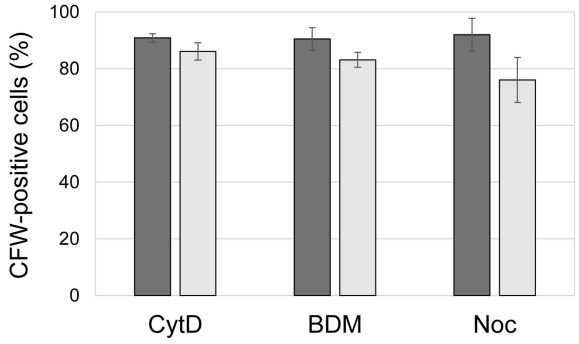

Fig. 1. Effect of cytochalasin D, BDM, and nocodazole on the number of cells that entered ecdysis immediately after centrifugation. Shown is the percentage of Calcofluor White M2R (CFW)–positive cells. Dark gray bars correspond to the mock-treated cells, light gray bars – to the cells treated with the indicated drugs. Hereinafter, error bars represent standard error of the mean values, CytD – cytochalasin D, BDM - 2,3-butanedione monoxime, Noc – nocodazole.

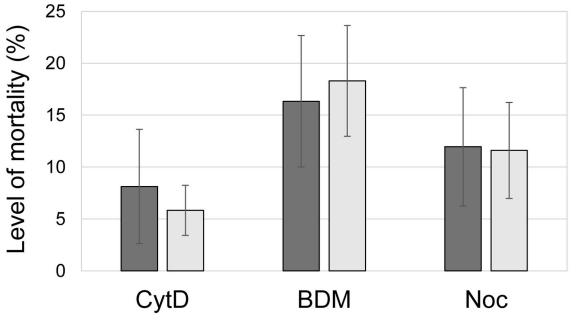

Fig. 2. The level of cell mortality 6 h after the induction of ecdysis. Shown is the percentage of propidium iodide-positive cells. Dark gray bars correspond to the mock-treated cells, light gray bars – to the cells treated with the indicated drugs.

amphiesmal vesicle membrane), the cell samples were taken immediately after centrifugation and stained by the cellulose-specific fluorescent dye Calcofluor White M2R (Sigma-Aldrich, St. Louis, MO, USA). The final concentration of dye was 5×10^{-5} %. Calcofluor White M2R is membrane-impermeable. Therefore, cellulosic thecal plates of the cells with intact plasma membrane are not stained, while the cells that entered ecdysis by discarding their plasma and outer amphiesmal vesicle membranes demonstrate bright fluorescence. Cell viability was evaluated in 6 h after the induction of ecdysis by the propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) staining. The fraction of Calcofluor White M2R- and propidium iodide-positive cells was estimated using the Countess II FL Automated Cell Counter (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with appropriate light cubes: 1) Ex (nm) 357/44, Em (nm) 447/60 for Calcofluor White M2R, and 2) Ex (nm) 531/40, Em (nm) 593/40 for propidium iodide assay.

The proportion of cells that successfully discarded their old thecal plates was analyzed in 2, 4 and 6 h after centrifugation. The number of cells and empty thecal plates were counted using a Fuchs-Rosenthal counting chamber. At least 200 cells per sample were counted. Since *P. minimum* cells have two big thecal plates, the ecdysis level was evaluated as a number of empty thecal plates divided by a duplicated total number of cells.

Results

Centrifugation at 10000 g for 5 min induced shedding of the plasma membrane and outer amphiesmal vesicle membrane in more than 90% of cells. Treating with cytochalasin D, BDM, and nocodazole insignificantly decreased the number of cells entering ecdysis relative to a control (Fig. 1). Moreover, incubation with drugs had no influence on the cell viability as compared to control experiments (Fig. 2), whereas high-speed centrifugation caused 10–15% cell mortality.

The fraction of cells subjected to ecdysis in the absence of drugs was about 10–15% in 2 h after centrifugation and reached 30–40% at the time points 4 and 6 h (Fig. 3A–C). The treatment with cytochalasin D sharply decreased the ecdysis level. The shedding of thecal plates was strongly inhibited and the ecdysis rate did not exceed the background level (Fig. 3, A). Interestingly, BDM delayed the shedding of old thecal plates. In 2 and 4 h after the centrifugation, the ecdysis level in the BDM-treated samples was two times lower than in the untreated ones, but almost reached the control level at 6 h (Fig. 3, B). A similar, but less pronounced tendency was observed for the treatment with nocodazole (Fig. 3, C). The background level of ecdysis in untreated (no chemical treatment, no centrifugation) cells was low: ca. 1% in the culture before the experiment and ca. 5% in 6 h after transferring into an Eppendorf tube (Fig. 3, A–C).

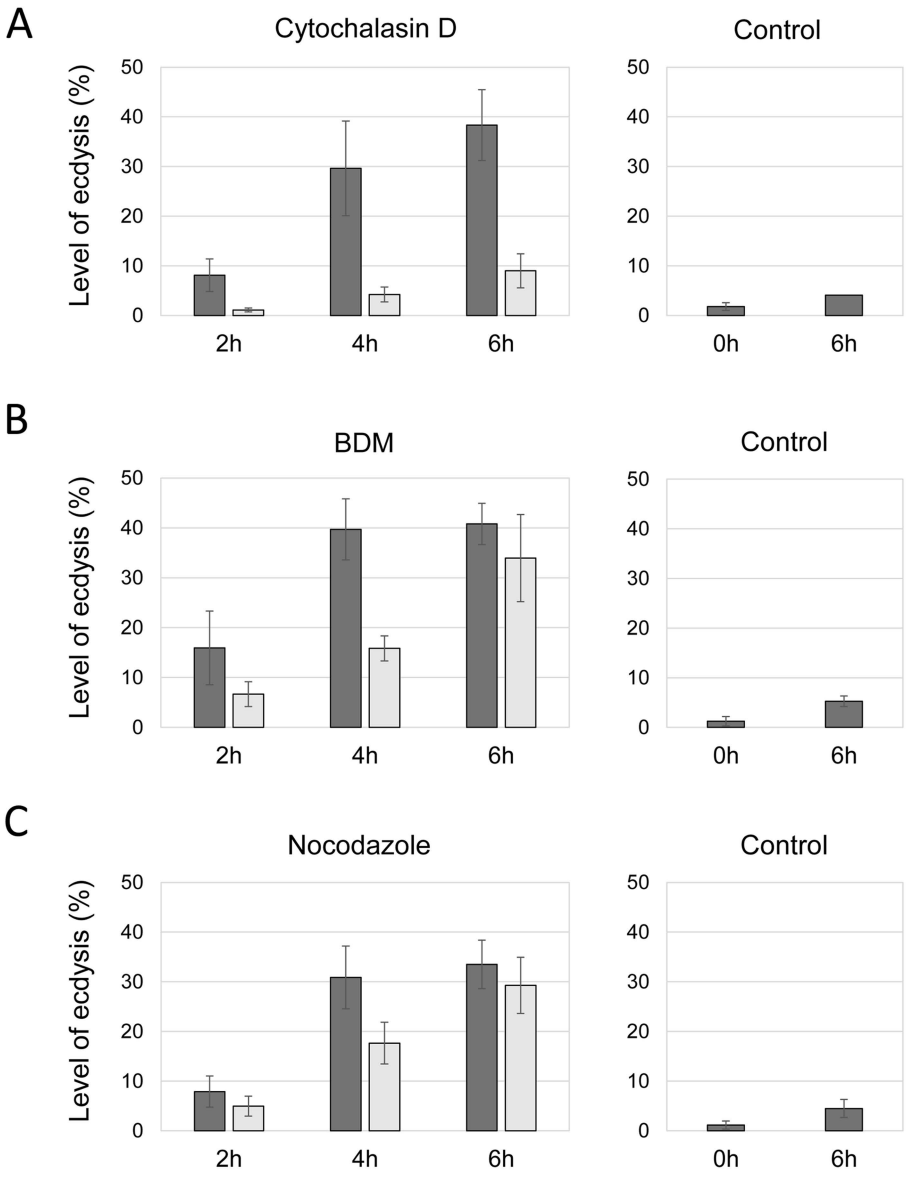

Fig. 3. The effect of cytochalasin D (A), BDM (B), and nocodazole (C) on the stressor-induced ecdysis level. Shown is the percentage of releases from the old thecal plates. Dark gray bars correspond to the mock-treated cells, light gray bars – to the cells treated with the indicated drugs. Results for the untreated cultures (no centrifugation, no drugs) that served as a reference (control) are shown in the right-side panels.

Discussion

The cell covering organization in Prorocentrales differs from that in other dinoflagellate groups. Amphiesma contains two big thecal plates (valves) and several small platelets surrounding the flagellar pore (Hoppenrath et al., 2013). Cortical microtubules are absent in *Prorocentrum minimum, P. micans*, and, presumably, in the other members of this genera. Instead, a dense cortical F-actin layer is present beneath the amphiesma (Schnepf et al., 1990; Soyer-Gobillard et al., 1996; Berdieva et al., 2018). Apparently, it undertakes a primary role in keeping membrane stiffness during cell division and ecdysis.

Treatment with the actin depolymerizing agent cytochalasin D results in immediate disruption of the ordered microfilament network (Schliwa, 1982). In both armored and naked dinoflagellates,

it inhibits the final step of cytokinesis leading to the formation of "Siamese twins" during mitosis. Moreover, disruption of the actin filaments results in malformation of new thecal plates in *P. micans* and *Scrippsiella acuminata* (Schnepf, 1988; Schnepf et al., 1990). In this study, cytochalasin D had no effect on discarding of the plasma membrane and outer amphiesmal vesicle membrane, but, similarly to another actin depolymerizing drug latrunculin B (Berdieva et al., 2018), strongly inhibited shedding of the old thecal plates during ecdysis. It is probable that the cortical actin network provides contractile activity ensuring the opening of cellulosic valves, changing of a cell shape, squeezing through an opening and leaving the old theca. We suggest that myosin is likely involved in this process.

BDM has been mainly considered as a non-competitive inhibitor of the skeletal muscle myosin-II functioning which blocks its ATPase activity (Higuchi and Takemori, 1989; Ostap, 2002; Bond et al., 2013). Its effect on the non-muscle myosins was demonstrated in various cellular processes and in different organisms (reviewed in Forer and Fabian, 2005). BDM was shown to inhibit the contractile activity, cell motility, and cell division processes, as well as organelle translocation and vesicular transport (Forer and Fabian, 2005). This drug was used in experiments with vertebrates, plant cells and unicellular eukaryotes, including microalgae. In particular, it disrupted cytomorphogenesis, nuclear migration and chloroplast distribution in the desmidian *Micrasterias denticulata* (Oertel et al., 2003). BDM treatment led to disorganization of the actin network in the dinoflagellate *Symbiodinium kawagutii* (Villanueva et al., 2015). Furthermore, it inhibited mechanically inducible bioluminescence in *Pyrocystis lunula* in a dose-dependent manner (Heimann et al., 2009). Myosin is supposed to be involved in the ecdysis process. However, surprisingly, only a delay but not inhibition of thecal plates shedding was observed in the BDM-treated cultures. A dose-dependent manner of the BDM action is one of the possible explanations of this phenomenon, which can be tested, albeit increasing the concentration of BDM should be applied with caution due to its probable toxic effect, which can influence the outcome of experiments.

Another plausible scenario of role of the actomyosin complex in the cell covering rearrangement is that it participates in the transport of the material for the newly forming plasma membrane and juvenile amphiesmal vesicles. According to this hypothesis, complete disruption of the actin filaments by cytochalasin D leads to the arrest of the plasma membrane maturation. Therefore, a cell undergoing ecdysis cannot leave the valves protecting it. Apparently, in our experiments, BDM did not inhibit, but delayed this process.

Besides, the reports about the effect of BDM on calcium currents and oscillations in a cell, in particular, its ability to induce calcium release from intracellular depos (Phillips and Altschuld, 1996), should also be taken in consideration. The ecdysis process appeared to be dependent on the elevation of intracellular calcium ion concentration, as it was shown in the dinoflagellate *Alexandrium catenella* (Tsim et al., 1997).

Nocodazole affects the dynamics of microtubule assembly/disassembly by binding to free tubulin dimers and preventing them from incorporation into newly forming microtubules. It is widely used as an antiproliferative agent. Nocodazole reversi-bly prolongs the cell cycle progression in the dinoflagellate *Crypthecodinium cohnii* affecting the mitotic spindle (Yeung et al., 2000). In spite of the fact that cortical microtubules are absent in *P. minimum*, we suggested that nocodazole could inhibit or prolong the formation of new flagella during ecdysis and leaving the old valves. In the present study, treatment with nocodazole delayed but not inhibited this process. We observed significant amount of motile cells in 6 h after the induction of ecdysis. The explanation could be that the action of this drug is reversible and a cell can readjust to a new equilibrium of tubulin (Yeung et al., 2000). Moreover, there are microtubule-stabilizing proteins such as MAP6 and MAP6d1 that make microtubules resistant to nocodazole. MAP6-related proteins are well studied in vertebrates, but not in protists (Dacheux et al., 2012).

Based on the obtained results, we conclude that cytoskeleton plays an important role in releasing from the old thecal plates during ecdysis in *P. minimum*. The involvement of the actomyosin complex in maturation of the new amphiesma should be confirmed in future studies

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