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Ca^{2+}- and glycoconjugates-dependent prey capture in the heliozoon *Actinophrys sol*

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Summary

Exocytosis of extrusomes, secretory granules found in protozoa, is involved in prey capture by the heliozoon *Actinophrys sol*. Here we show that extracellular Ca^{2+} is necessary for exocytosis and prey capture in *A. sol*. We found that *A. sol* could not capture prey cells in a Ca^{2+}-free solution. L-type Ca^{2+} channel blockers and a calmodulin antagonist also inhibited the capture of prey. These results suggest that Ca^{2+} influx via L-type Ca^{2+} channels plays a crucial role in exocytosis in *A. sol*. Concanavalin A (Con A) also inhibited prey capture, and the inhibition was relieved by the addition of its hapten sugar, α-mannoside, suggesting that Con A-binding glycoconjugates are implicated in exocytosis of extrusomes and the adhesion of prey cells.

Keywords: calcium ion influx, Con A, exocytosis, extrusome, glycoprotein, secretory granule

Introduction

The actinophryid heliozoon *Actinophrys sol* is a predatory protozoan that has a spherical cell body and radiating stiff pseudopodia called axopodia. *A. sol* captures prey organisms with its axopodia. When a prey organism makes contact with the axopodia, the prey becomes trapped by adhesion and is conveyed closer to the cell body by contraction of the axopodia. Beneath the plasma membrane, there are many electron-dense granules, called extrusomes. It was observed that the contents of the extrusomes were discharged when *A. sol* captures prey organisms (Patterson and

Hausmann 1981). It was also reported that the contents of extrusomes were labeled with Con A and that extrusomes contained a 40-kDa glycoprotein (gp40) that specifically binds to Con A (Sakaguchi et al. 2001). These findings suggest that extrusomes and gp40 play a crucial role in prey capture.

Secretory granules, or dense-core granules, have been studied extensively in metazoan cells, and the role of Ca^{2+} in secretion of granules is well documented (Burgoyne and Morgan 2003). Appropriate stimuli cause Ca^{2+} influx into cells, where it interacts with Ca^{2+} sensor proteins and induces exocytosis of the granules. Among unicellular organisms, exocytosis of trichocysts, the extrusomes of *Paramecium*, has been shown to be dependent on Ca^{2+} (Plattner and Kissmehl 2003). However, with respect to the function of Ca^{2+} in heliozoa, only Ca^{2+}-dependent contraction of axopodia has so far been reported (Matsuoka and Shigenaka 1984).

In this study, we examined the role of extracellular Ca^{2+} in exocytosis and prey capture in *A. sol*. In addition, we investigated the effect of Con A on this process. We discuss the possible role of extrusomes and gp40 in prey capture.

Materials and methods

Organism and culture

A. sol was cultured monoxenically as described by Sakaguchi and Suzaki (1999). The prey flagellate *Chlorogonium* sp. was cultured axenically as described by Sakaguchi et al. (1998). Both cell types were collected by centrifugation and washed

with 10% artificial seawater (10% ASW; 47 mM NaCl, 1.1 mM KCl, 1.1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 2.5 mM $MgSO_4$ and 1 mM Tris-HCl at pH 7.8) or with Ca^{2+}-free 10% ASW before they were used for experiments.

Prey capture assays

All experiments were carried out under a stereoscopic microscope (Nikon SMZ1500), and photographs were taken with a DP11 digital camera on a microscope (Olympus BX50). Prior to the experiments on Ca^{2+} dependency, *A. sol* cells were washed twice with Ca^{2+}-free 10% ASW. $CaCl_2$ was added to Ca^{2+}-free 10% ASW to final concentrations of 0, 0.25, 0.50, 0.75, or 1.00 mM. *A. sol* cells that had been washed with Ca^{2+}-free 10% ASW were then mixed with these solutions on depression slides and incubated for 5 min. *Chlorogonium* cells were then added to the solutions which were incubated for another 5 min. Finally, *A. sol* cells were transferred to new depression slides, and the number of cells per 20 cells that had captured prey was counted.

Other experiments were conducted by the same method, with reagents added to 10% ASW containing 1 mM Ca^{2+}. EGTA was added at 1.1 mM in order to remove Ca^{2+}, and the final concentration of free Ca^{2+} was calculated by an iterative procedure (Suzaki and Williamson 1986). Verapamil (50 µM; Nacalai Tesque, Japan), nifedipine (100 µM; Nacalai Tesque, Japan), ω-conotoxin GVIA (200 nM; Alomone Labs, Israel), $NiCl_2$ (100 µM), $LaCl_3$ (40 µM) and ruthenium red (2 µM) were used as Ca^{2+} channel blockers. The calmodulin antagonists W-7 and W-5 (BIOMOL International, LP, USA) were used at 20 µM. Calcium ionophore A-23187 (Nacalai Tesque, Japan) was used at

2 μM. Nifedipine, W-7, W-5 and A-23187 were dissolved in dimethylsulfoxide (DMSO), the final concentrations of which was less than 0.1%. Chloral hydrate was used at 10 mM to disturb the extrusomes.

The lectin Con A was used at 20 μg/ml, with or without its hapten sugar α-methyl-D-mannoside, at 20 mM. The effects of a pretreatment with Con A were also examined. *A. sol* cells or *Chlorogonium* cells were incubated for 5 min in solution containing 20 μg/ml Con A. The cells were then washed with 10% ASW, and new untreated prey or predator cells, respectively, were added. After 5 min, the number of *A. sol* cells that had captured prey was counted.

The cells did not appear to be damaged by any of these treatments, except chloral hydrate (discussed below). For each experiment, data were taken from at least five experiments and results were expressed as mean ± standard error of the mean (S.E.M.).

Results and Discussion

Extrusomes are secretory granules that are present under the cell membrane of many protozoa. Extrusomes have various roles, such as offensive or defensive functions; those in ciliates have been the most studied. For example, trichocysts of *Paramecium* and cortical granules of *Blepharisma* were reported to have defensive functions (Miyake 2002), while toxicysts of *Didinium* and *Homolozoon* are known to have offensive functions (Hausmann 2002). In the actinophryid heliozoa, the extrusomes have been implicated in prey capture (Patterson and Hausmann 1981; Sakaguchi et al. 2001). Unlike those in ciliates, the contents of the extrusomes in

actinophryid heliozoa are not ultrastructurally organized; they have an amorphous appearance, with electron-dense material, and resemble secretory granules of metazoa.

To determine the effect of extracellular Ca^{2+} concentration on prey capture by *A. sol*, we supplied prey to cells at various concentrations of Ca^{2+}, and counted cells that captured prey. In the Ca^{2+}-free solution, *A. sol* did not capture any prey (Figs. 1 and 2), and very few prey were captured at 0.25 mM Ca^{2+} (Fig. 2). We observed similar Ca^{2+}-dependency for prey capture in a related heliozoon species *Echinosphaerium akamae* (data not shown). The concentration of Ca^{2+} required for prey capture by *A. sol* is relatively high compared to that required for exocytosis of trichocysts of *Paramecium* (Kerboeuf and Cohen 1990). Perhaps this is because the *A. sol* used here was originally collected from brackish water.

Chelation of Ca^{2+} by EGTA also inhibited prey capture (Fig. 2), although not completely as in Ca^{2+}-free solution. It is possible that EGTA chelated other cations or intracellular Ca^{2+}, and consequently some extracellular Ca^{2+} would remain even when it was theoretically absent. Capturing ability was incompletely restored by the addition of Ca^{2+} (Fig. 2). Because of these effects, we could not use Ca^{2+}/EGTA buffer to examine dose dependency or the threshold value. Nevertheless, these results indicate that extracellular Ca^{2+} is necessary for prey capture in *A. sol*. In the slime mold *Dictyostelium discoideum*, it was reported that Ca^{2+} was needed for phagocytosis, although whether influx of Ca^{2+} was also required was not determined (Yuan et al. 2001).

To rule out the possibility that Ca^{2+}-dependent adhesion molecules are responsible for prey capture, we used Ca^{2+} channel blockers. Verapamil, nifedipine and $LaCl_3$ inhibited prey capture, although conotoxin GVIA, $NiCl_2$ and ruthenium red did

not affect it (Fig. 3). From these results, it is unlikely that prey adhesion is merely due to Ca^{2+}-dependent adhesive substances on the cell surface. The Ca^{2+} channel blockers verapamil and nifedipine that inhibited prey capture in *A. sol* are selective inhibitors for L-type Ca^{2+} channels. Therefore, the results suggest that an L-type Ca^{2+} channel is involved in prey capture. Influx of Ca^{2+} causes release of secretory granules in both metazoa and other protozoa, and earlier studies showed that granule secretion occurred during prey capture by *A. sol* (Patterson and Hausmann 1981). In *Paramecium*, it was observed that L-type Ca^{2+} channel blockers inhibited secretion of trichocysts (Maleki et al. 1987; Satir et al. 1988). In mammalian cells, L-type channels are also known to play crucial roles in granule secretion in some cell types, including dendritic cells, cytotoxic T lymphocytes and pancreatic B cells (Gardella et al. 2000; Lyubchenko et al. 2001; Mears 2004). Ca^{2+} influx is also necessary for granule secretion in invertebrates (e.g. Johansson and Söderhäll 1985). Several proteins that are components of metazoan exocytosis were also found in *Paramecium* (Plattner and Kissmehl 2003). It seems likely that similar mechanisms are employed in exocytosis in both multicellular- and unicellular organisms, including *A. sol*.

The calcium ionophore A-23187 inhibited prey capture (Fig. 4). This could possibly be due to depletion of extrusomes. A-23187 might cause Ca^{2+} influx, elevating intracellular Ca^{2+} concentration, and finally resulting in unregulated secretion of extrusomes and inhibition of prey capture, although we have not obtained evidence for this possibility, so far.

Extracellular Ca^{2+} and its influx seem to be necessary for prey capture by *A. sol*, and the secretion of extrusomes is probably involved. However, the detailed mechanism of prey capture remains unknown. It is possible that intracellular Ca^{2+} stores may also

be involved in the secretion of extrusomes, as putative Ca^{2+} stores were observed in *E. akamae* (Matsuoka and Shigenaka 1984).

In the experiments using calmodulin antagonists, capture was inhibited by W-7, but not by W-5 at the same concentration (Fig. 4). These drugs bind to calmodulin and inhibit its activity. W-7 is, however, more specific and effective on calmodulin than W-5 (Hidaka et al. 1981). A similar inhibition effect was reported in *Paramecium* (Garofalo et al. 1983). Calmodulin is not considered to be essential for exocytosis in metazoan secretory cells; nevertheless it may play a regulatory role in exocytosis (Burgyone and Morgan 1998; Burgoyne and Morgan 2003; Easom 1999). Chloral hydrate also inhibited prey capture. In the heliozoon *Echinosphaerium akamae*, it was previously observed that extrusomes, described as dense granules, were detached from the plasma membrane by chloral hydrate (Suzaki and Shigenaka 1982). The inhibition by chloral hydrate was therefore possibly due to effects on granule secretion. However, as it was observed that longer incubation in chloral hydrate disturbed the cell surface of *A. sol* (data not shown), we cannot exclude the possibility that the inhibition effect was a non-specific effect of chloral hydrate.

In Con A-containing solution, prey capture was almost completely inhibited, but prey capture was not affected when its specific hapten sugar, α-methyl-D-mannoside, was also present (Fig. 5). Furthermore, the addition of mannoside after incubation in Con A allowed *A. sol* to again capture prey (data not shown). In the pretreatment experiments, inhibition was achieved when *A. sol* cells were pretreated with Con A solution (Fig. 5). In contrast, pretreatment of the prey did not strongly affect prey capture. The Con A pretreatment results indicate that the glycoconjugates that bind to Con A are localized on the cell surface of *A. sol*. Considering that gp40 is a dominant

protein that binds to Con A (Sakaguchi et al. 2001), it would be reasonable to surmise that gp40 is the targeted protein in these experiments. Gp40 is, however, considered to reside in the extrusomes, and it seems unlikely that Con A would penetrate into the extrusomes. There may therefore be other Con A-binding glycoconjugates on the cell surface that participate in prey capture. Alternatively, parts of extrusomes could be secreted constitutively and adhesive substances might be conveyed to the cell surface of *A. sol*. However, the results from the Ca^{2+} experiments show that regulated exocytosis is probably necessary for prey capture. Therefore, it is possible that cell surface gp40, or some other Con A-binding glycoconjugate, act as receptors and is used for prey recognition, and that a receptor-operated ion channel is involved in induction of exocytosis of extrusomes as in the case of *Paramecium* (Hennessey et al. 1995). In *Euplotes mutabilis*, it has been implied that lectin-binding regions were involved in food selection (Wilks and Sleigh 2004).

To elucidate the secretion and prey capture mechanism in *A. sol*, we are trying to prepare an antibody against gp40 and to clone the gp40 gene. Such an antibody would also provide an excellent molecular marker for secretion of extrusomes and prey capture in *A. sol*.

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Figure legends

Fig. 1. Prey capture by *Actinophrys sol*. (A) In solution containing 1 mM Ca^{2+}, *A. sol* (a) captured prey organisms *Chlorogonium* (c) with its axopodia (arrowheads). (B) In a Ca^{2+}-free solution, *A. sol* did not capture prey. Scale bars = 50 µm.

Fig. 2. Prey capture was dependent on extracellular Ca^{2+} concentration. (A) Prey cells were supplied to *A. sol* in solutions containing Ca^{2+} at concentrations from 0 to 1 mM. The percentages of cells that captured prey were determined. Prey capture decreased at lower Ca^{2+} concentrations. When the concentration was lower than 0.25 mM, capture was strongly inhibited (*n*=5). (B) In the presence of EGTA, prey capture was inhibited, although not completely. The addition of Ca^{2+} partially removed the inhibition (*n*=5). Theoretical Ca^{2+} concentrations were 0 and 1 mM, respectively. Error bars are S.E.M. *n* indicates number of experiments.

Fig. 3. Effect of Ca^{2+} channel blockers on prey capture. Heliozoons were incubated with Ca^{2+} channel blockers and the percentages of cells that captured prey were determined. (A) Control, *n*=10. (B) 50 µM verapamil, *n*=5. (C) 100 µM nifedipine, *n*=5. (D) 40 µM $LaCl_3$, *n*=5. (E) 100 µM $NiCl_2$, *n*=5. (F) 2 µM ruthenium red, *n*=5. (G) 200 nM ω-conotoxin GVIA, *n*=6. $LaCl_3$ and the L-type Ca^{2+} channel blockers, verapamil and nifedipine (D, B, C), inhibited capture. Other drugs did not cause significant inhibition. Error bars are S.E.M. *n* indicates number of experiments.

Fig. 4. Effect of various drugs on prey capture. Heliozoons were incubated with drugs

and the percentages of cells that captured prey were determined. (A) Control, *n*=10. (B) 20 µM W-5, *n*=5. (C) 20 µM W-7, *n*=7. (D) 2 µM A-23187, *n*=7. (E) 10 mM chloral hydrate, *n*=11. The calmodulin antagonist W-7 inhibited prey capture, while its less efficient analog W-5 did not. The calcium ionophore A-23187 and chloral hydrate also inhibited prey capture. Error bars are S.E.M. *n* indicates number of experiments.

Fig. 5. Effect of Concanavalin A on prey capture. Control (A, *n*=7). In the presence of 20 µg/ml Con A, prey capture was inhibited (B, *n*=7). Addition of 20 mM mannoside relieved the inhibition completely (C, *n*=7). When *Actinophrys sol* cells were pretreated with 20 µg/ml Con A, prey capture was inhibited (D, *n*=8), whereas the pretreatment of prey cells did not cause strong inhibition (E, *n*=8). Error bars are S.E.M. *n* indicates number of experiments.

Figure 1

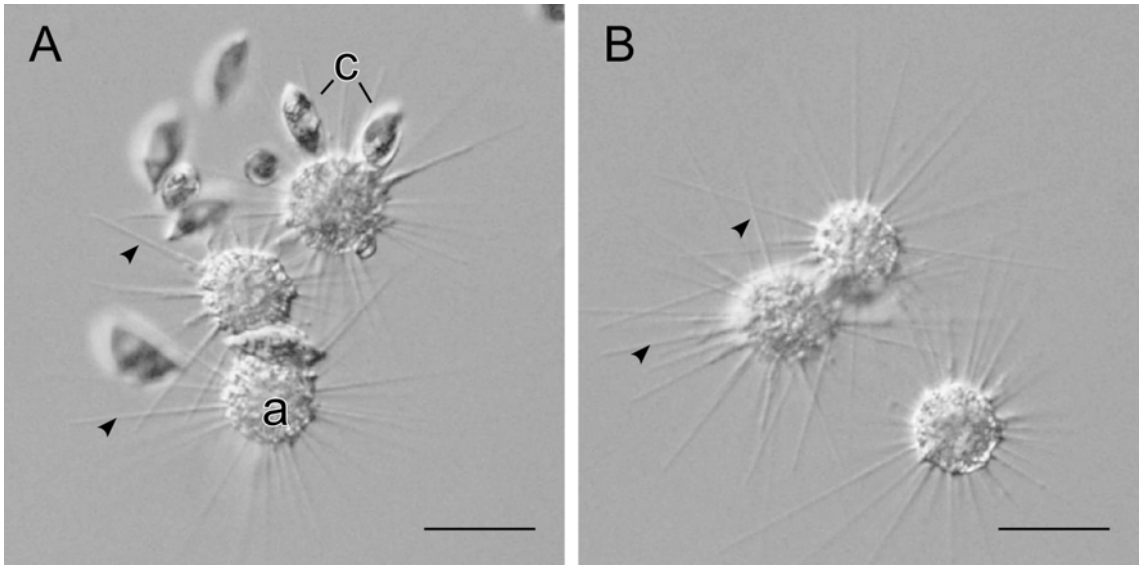

Figure 2

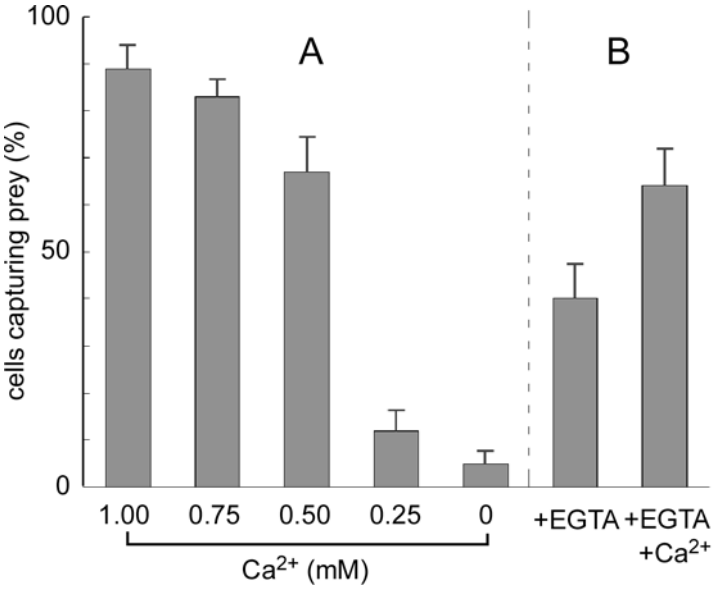

Figure 3

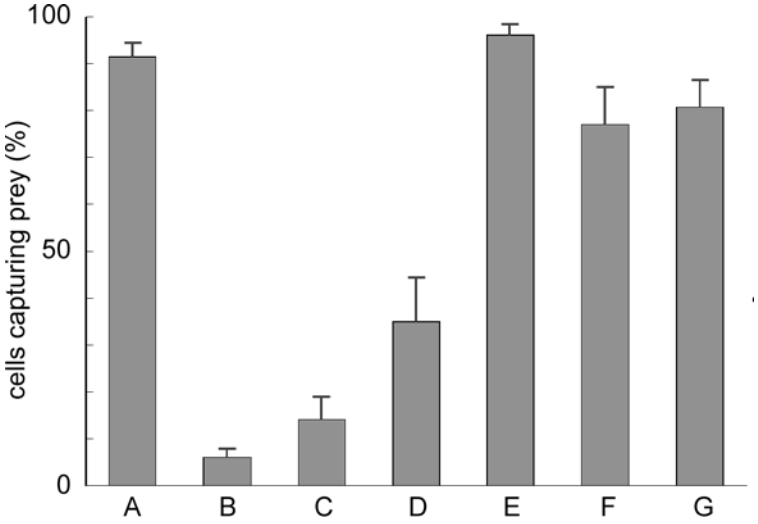

Figure 4

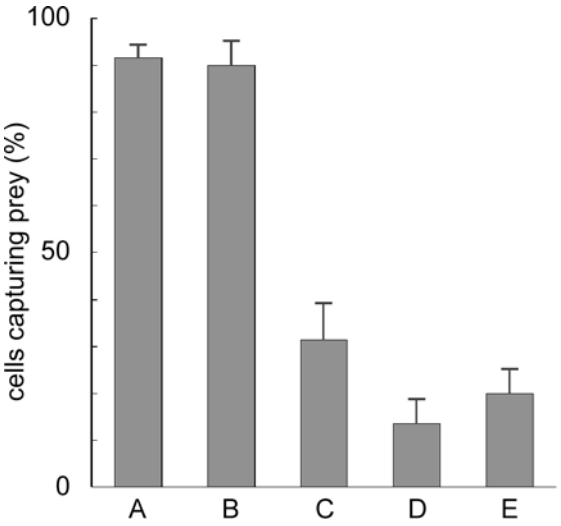

Figure 5