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Rapid downregulation of the Ca^{2+}-signal after exocytosis stimulation in *Paramecium* cells: Essential role of a centrin-rich filamentous cortical network, the infraciliary lattice

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Summary We analysed in *Paramecium tetraurelia* cells the role of the infraciliary lattice, a cytoskeletal network containing numerous centrin isoforms tightly bound to large binding proteins, in the re-establishment of Ca^{2+} homeostasis following exocytosis stimulation. The wild type strain d4-2 has been compared with the mutant cell line Δ-PtCenBP1 which is devoid of the infraciliary lattice (''Δ-PtCenBP1'' cells). Exocytosis is known to involve the mobilization of cortical Ca^{2+}-stores and a superimposed Ca^{2+}-influx and was analysed using Fura Red ratio imaging. No difference in the initial signal generation was found between wild type and Δ-PtCenBP1 cells. In contrast, decay time was greatly increased in Δ-PtCenBP1 cells particularly when stimulated, e.g., in presence of 1 mM extracellular Ca^{2+}, $[Ca^{2+}]_o$. Apparent halftimes of f/f_0 decrease were 8.5 s in wild type and ~125 s in Δ-PtCenBP1 cells, requiring ~30 s and ~180 s, respectively, to re-establish intracellular $[Ca^{2+}]$ homeostasis. Lowering $[Ca^{2+}]_o$ to 0.1 and 0.01 mM caused an acceleration of intracellular $[Ca^{2+}]$ decay to $t_{1/2}$ = 33 s and 28 s, respectively, in Δ-PtCenBP1 cells as compared to 8.1 and 5.6, respectively, for wild type cells. We conclude that, in *Paramecium* cells, the infraciliary lattice is the most efficient endogenous Ca^{2+} buffering system allowing the rapid downregulation of Ca^{2+} signals after exocytosis stimulation.

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Introduction

In eukaryotic cells, the universal second messenger, Ca^{2+}, can be activated from different sources and then activates a variety of processes including secretion [1—4]. Thereby cells operate with a very large excess of Ca^{2+} to achieve sufficient local concentrations although few molecules suffice for local activation [5—7]. Spatio-temporal precision is a prerequisite of specific functional activation, as is the subsequent downregulation of the Ca^{2+} signal. Depending on the system, during stimulated exocytosis, rapidly increasing values of intracellular Ca^{2+} concentrations, $[Ca^{2+}]_i$, can be counteracted by widely different mechanisms, such as Ca^{2+}-pumps (Ca^{2+}-ATPases in the cell membrane and in the membranes of Ca^{2+}-stores), cation exchangers (notably Na^+/Ca^{2+} exchangers) and/or binding to buffer proteins such as calsequestrin and calreticulin in the Sarcoplasmatic and Endoplasmic Reticulum, respectively, as well as calbindin or parvalbumin and some others in the cytosolic compartment [1,8—13]. Within the cytosol, due to Ca^{2+}-binding proteins, only <0.1 to <1% of the Ca^{2+} remains in free form under steady state conditions [14,15].

We here propose a special mechanism for *Paramecium* cells where a large subcortical network, the infraciliary lattice (ICL), displays a Ca^{2+}-dependent contractility [16—18]. The ICL is formed mainly of numerous centrin isoforms bound to specific binding proteins and this network provides a potential system with high capacity of Ca^{2+}-binding [19—22]. Stimulation by the secretagogue, aminoethyldextran (AED) which triggers massive exocytosis of dense core-vesicles (''trichocysts'') [23,24] also triggers ICL contraction—a Ca^{2+}-dependent process inducible also by Ca^{2+} injection [16]. Furthermore, cells devoid of ICL, although performing exocytosis upon stimulation by AED, are not able to contract [25].

In a primary step, during AED stimulated exocytosis, Ca^{2+} is mobilized from ''alveolar sacs'' (established subplasmalemmal Ca^{2+}-stores [26—28]), and this process is superimposed by a Ca^{2+}-influx from the medium [29—33]. Exocytosis as well as exocytosis-coupled endocytosis are accelerated with increasing extracellular Ca^{2+} concentrations, $[Ca^{2+}]_o$ [34].

The motivation for the present work was as follows. First, we observed that wildtype (wt.) *Paramecium* cells contract upon $[Ca^{2+}]_i$ increase upon exocytosis stimulation. Second, this is caused by the ICL. Third, the observation that the ICL was a dispensable organelle, at least under laboratory conditions [25], offered the possibility to assess its function in vivo. Investigating the role of the ICL in buffering Ca^{2+} influx was all the more interesting as pilot calculations have shown that none of the established Ca^{2+}-pumps in *Paramecium* can account for the rapid downregulation of the Ca^{2+} signal after exocytosis stimulation [33]. With the technique formerly established for fluorochrome analyses with *Paramecium* cells [32,35], we here compare $[Ca^{2+}]_i$ dynamics during AED stimulation in wt. cells and in Δ-PtCenBP1 cells stably devoid of ICL following deletion of the gene for the centrin-binding protein, *PtCenBP1* [21,22]. In addition, the two cell types, both carrying the *nd7-1* mutation which prevents exocytosis performance upon AED stimulation [29], were also studied. We find that Δ-PtCenBP1 cells, whether of the *ND7-1+* or *ND7-1* genotype, show the same greatly retarded downregulation of $[Ca^{2+}]_i$ after AED stimulation as compared to wt. cells. These observations demonstrate that the infraciliary lattice is a most important component for re-setting $[Ca^{2+}]_i$ after exocytosis stimulation. This is in contrast to some recent reviews that discuss merely Ca^{2+} transport mechanisms [36] along these lines.

Materials and methods

Strains and culture conditions

Stock d4-2 of *Paramecium tetraurelia*, the wt. reference strain, was used in all experiments. The following mutant strains, all derived from the wt. stock, were also used. The mutant nd7-1 carries a recessive mutation preventing trichocyst discharge [37]. The strain Δ-PtCenBP1 carries a macronuclear deletion of the gene *PtCenBP1* encoding a large centrin-binding protein shown to form the backbone of the ICL [21,22]. The deleted strain was obtained after autogamy under conditions of RNAi for the *PtCenBP1* gene, according to the protocol of Garnier et al. [38]. The strain Δ-PtCenBP1/nd7-1 was obtained in the progeny of a cross between the strains Δ-PtCenBp1 and nd7-1 (J. Beisson, unpublished). Cells were cultivated monoxenically, at 25 °C, in a decoction of dry lettuce inoculated with *Enterobacter aerogenes* as a food organism, as previously described [35].

Immuno-fluorescence microscopy

Immuno-staining of cells was carried out as previously described [39]. The monoclonal antibody 1A9 raised against *Paramecium* ICL [25] was used at a dilution 1:200, and the secondary anti-mouse antibody from Jackson ImmunoResearch Labs (West Grove, PA) at a dilution of 1:500. Cells were observed under a Zeiss Axioskop 2-plus fluorescence microscope equipped with a Roper Coolsnap-CF intensifying camera (Zeiss, Oberkochen, Germany). Images were processed using the Metamorph software (Universal Imaging, Downington, Pennsylvania).

Cell fractionation, electrophoresis and immuno-blotting

Wildtype and mutant cells at the end of logarithmic growth phase were harvested and washed twice in 10 mM phosphate buffer pH 7.0. The cell pellet was mixed with 1 volume of buffer A (0.25 M sucrose, 50 mM potassium phosphate, pH 7.0). The cells were homogenized in a Dounce homogenizer at 4 °C, and first centrifuged at 30,000 × *g* for 20 min. The supernatant was recentrifuged at 100,000 × *g* for 1 h, yielding a postmicrosomal supernatant. This fraction was stored at −80 °C until used. SDS-PAGE was performed as previously described [19] on 12% or on 6—15% gradient acrylamide gels (37.5:1, acrylamide:bis-acrylamide). Upon completion of migration, proteins were electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) in 0.05 M Tris, 0.05 M boric acid for 2 h at 35 V. The transferred proteins were stained with 1% Ponceau S Red in 7% acetic acid. The filters were saturated with 5% non-fat dry milk in 0.01 M Tris—HCl pH 7.4,