Possible involvement of a calmodulin regulated Ca^{2+}-ATPase in exocytosis performance in *Paramecium tetraurelia* cells

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Surface membrane fractions from *Paramecium tetraurelia* cells contain a calmodulin-stimulated Ca^{2+}-ATPase responding to low levels of free Ca^{2+} and with features characteristic of a membrane-bound ATPase. Among the different strains analyzed this enzyme was practically absent selectively from the 'non-discharge' mutant *nd9–28°C* (from J. Beisson); if cultured at a permissive temperature (18°C), this strain showed identical values of calmodulin-stimulated Ca^{2+}-ATPase activity as wild-type cells (*7S*) or strains with mutations which do not affect exocytosis performance. We conclude that this calmodulin-stimulated Ca^{2+}-activated ATPase might be a prerequisite for membrane fusion in the course of exocytosis performance.

Adenosinetriphosphatase *Calcium* *Calmodulin* *Exocytosis* *Membrane fusion* Paramecium

1. INTRODUCTION

The mechanism by which Ca^{2+} acts during 'stimulus–secretion coupling' is largely unknown. Only a short time ago the first evidence was found for a calcium- and calmodulin-regulated protein phosphorylation step during exocytosis [1–3]. Working with *Paramecium tetraurelia* cells we could localize cytochemically a Ca^{2+}-dependent ATPase on the pre-formed 'trichocyst' exocytosis sites selectively in those strains which are capable of exocytosis performance, whereas 'non-discharge' mutations (in spite of the occurrence of docked 'trichocysts'; cf. [4]) were negative [5]. A previous study on the Ca^{2+}-ATPase activity of isolated surface membranes had already revealed a consistent, though small, difference between 'normal' and 'non-discharge' strains [6]. Here, we analyze the possible involvement of a calmodulin-stimulated Ca^{2+}-ATPase in exocytosis regulation in *Paramecium* cells.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

Paramecium tetraurelia, strains *K401, 7S* (wild type) and *d4–500r* [7] were cultured monoxenically with *Enterobacter agglomerans* added (25°C; cf. [5]). Strain *nd9* [4] was cultured at 18°C (permissive) or 28°C (non-permissive for exocytosis performance), either monoxenically or as sterile cultures in a synthetic medium [8]. Cells were always harvested at early stationary phase and washed twice with 20 mM Tris–maleate (pH 7.8)–3 mM EGTA.

2.2. *Cell fractionation*

We followed essentially our original method [6], but with the following modifications: For homogenization and fractionation the same buffer was used as for washing. A cocktail of freshly dissolved proteinase inhibitors (50 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml *p*-tosyl-L-arginine methylester, 2.5 μl/ml Trasilol (Aprotinin), all from Sigma (St Louis MO) was added at each preparation step (cf. [7]). Centrifuge tubes (20 ml each) were filled with layers of 9.5 ml 1.9 M sucrose, 8.5 ml 1.4 M sucrose and 1–1.5 ml homogenate

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containing 8–12 mg protein. Centrifugation at 650×g for 2 h (Minifuge II, Heraeus-Christ) resulted in pure surface membrane fractions at the 1.4/1.9 M sucrose interface.

2.3. *Ca^{2+}-ATPase assays*

Measurements of Ca^{2+}-stimulated adenosine triphosphate (ATP) phosphohydrolase (EC 3.6.1.3) were made at 22°C and at the pH optimum of 7.8. To 0.6 ml of 50 mM Tris–maleate buffer (with 3.5 mM KCl, 3 mM EGTA, 1 mM Tris–ATP from Sigma, $CaCl_2$) we added 0.1 ml sample containing 70–80 μg protein. EGTA (ethyleneglycol–bis-(β-aminoethyl ether)-*N,N'*-tetraacetate) and CDTA (cyclohexane-1,2-diamine-*N,N,N',N'*-tetraacetate) were from Sigma. The free Ca^{2+}-concentration was measured under assay conditions with the use of a Ca^{2+}-selective electrode (Radiometer F 2112 Ca). The calmodulin used was from bovine (Fluka, Buchs) or porcine brain (Boehringer, Mannheim) or from *Paramecium tetraurelia* (Dr J. Schultz, Tübingen).

Sodium dodecyl sulphate–polyacrylamide gel (10%) electrophoresis, protein and inorganic phosphate (P_i) determination was done as in [6].

3. RESULTS

Fig.1 documents the absence of any significant amounts of secretory proteins (M_r ~17 000 [9]) and of ciliary tubulin (M_r 55 000) from the surface membrane fractions. Equally pure fractions were obtained from all *P. tetraurelia* strains analyzed (unpublished), so that enzyme activities are directly comparable. Table 1 shows the inhibitory effect of a non-hydrolyzable ATP analog (cf. [10]), of vanadate (cf. [11]) and of ruthenium red (cf. [12]) and also documents the stimulating effect of calmodulin as well as its abolition by calmodulin-directed drugs (trifluoperazine or compound R 24571; see section 4). Although the data presented come from our most widely used strain *K401*, they are representative also of any other strain with the capability of normal exocytosis performance. Almost maximal Ca^{2+}-ATPase activity is achieved with 10^{-7} M free Ca^{2+} (table 2) and with 10 μM calmodulin/mg protein (fig.2). The presence of some endogenous calmodulin can be inferred from the basal Ca^{2+}-ATPase activity without calmodulin added (table 2) and from the inhibitory effect

Fig.1. SDS–PAGE of subcellular fractions isolated from strain *K401*; 2% mercaptoethanol; 10% gel; Coomassie blue staining. (A) Surface membranes; (B) uppermost gradient layer enriched in secretory contents ('trichocysts'); (C) cilia; (D) markers (M_r), (a) cytochrome *c*, 12 300; (b) myoglobin, 17 800; (c) chymotrypsinogen, 25 000; (d) ovalbumin, 45 000; (e) bovine serum albumin, 67 000; (f) aldolase, 140 000).

of R 24571 in the absence of exogenous calmodulin (table 1A). Calmodulins of different origin resulted in an identical stimulation rate (unpublished).

Cells of strains *K401, 7S* and *nd9* (if cultured at a permissive temperature of 18°C; [4]), which are all capable of normal exocytosis performance, show an identical stimulatory effect of calmodulin on the Ca^{2+}-ATPase activity of their surface membranes (fig.2, table 2). After culturing at a non-permissive temperature, strain *'nd9–28°C'* [4]

Table 1

Inhibition of Ca^{2+}-ATPase activity of surface membranes from strain *K401*[a]

Experimental conditions	Ca^{2+}-ATPase (μmol P_i . mg protein^{-1} . min^{-1})
(A) No calmodulin added	
without inhibitor	1.19
0.1 mM ATP analog[b]	0.78
50 μM ruthenium red	0.12
40 μM R 24571	0.48
100 μM vanadate	0.89
3 mM CDTA	0.67
1 mM Mg^{2+}	1.13
(B) With calmodulin added (10 μmol/mg protein)	
without inhibitor	4.84
0.1 mM ATP analog [b]	1.40
50 μM ruthenium red	0.18
40 μM R 24571	0.89
100 μM vanadate	1.12
1 μM trifluoperazine	4.20
10 μM trifluoperazine	1.30
100 μM trifluoperazine	1.05
3 mM CDTA	0.86
1 mM Mg^{2+}	4.13

[a] Monoxenic cultures. Each value is on the average from 3 duplicate determinations. Each assay contained 1 mM ATP + 1 mM free Ca^{2+}

[b] *S*-Dinitrophenyl-6-mercaptopurine riboside 5′-triphosphate [10]

shows a much lower 'basal' and calmodulin-stimulated Ca^{2+}-ATPase activity. The same difference between *nd9–18°C* and *nd9–28°C* was found when surface membranes were analyzed from sterile cultures (table 2), so that any possible contamination of surface membrane fractions by digesting vacuole membranes can be excluded as a source of these differences. Strain *d4–500r* was analyzed because its Ca^{2+}-regulating system in its ciliary membranes is defective (cf. [7]), whereas its exocytosis performance is normal. Its Ca^{2+}-ATPase activities are much closer to those of the other strains with normal exocytosis performance than to those of the 'non-discharge' strain *nd9–28°C*. This makes it unlikely that the ciliary system would be a source of the differences found between 'discharge' and 'non-discharge' strains.

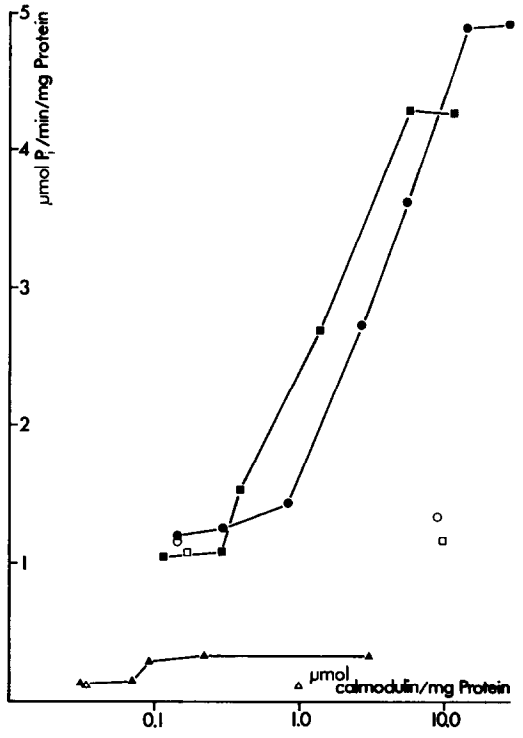

Fig.2. Calmodulin-dependent stimulation of Ca^{2+}-ATPase activity of surface membranes from strain *K401* (•), *nd9–18°C* (▪) and *nd9–28°C* (▲). Open symbols are with 10^{-4} M trifluoperazine added. Each value is from duplicate measurements.

4. DISCUSSION

In various exocytotic systems, including endo- and exocrine pancreas [13–15], adrenals [16] and mast cells [17], various authors found:

(i) Proteins undergoing phosphorylation in a Ca^{2+}-, calmodulin- and ATP-dependent way [14,18];

(ii) The suppression of phosphorylation by phenothiazines [14];

(iii) The simultaneous inhibition of exocytosis performance by these drugs [15–18].

Phenothiazines are known to bind selectively to calmodulin [19]. In mast cells the degree of exocytosis inhibition by phenothiazines was strictly parallel to the potency of reducing calmodulin-mediated functions [17]. In another approach, calmodulin-directed antibodies abolished the exocy-

Table 2

Ca^{2+}-ATPase activity of surface membranes from different strains

Culture conditions and strains used	Ca^{2+}-ATPase (μmol P_i . mg protein^{-1} . min^{-1})			
	10^{-7} M Ca^{2+} −calmodulin	10^{-3} M Ca^{2+} −calmodulin	10^{-7} M Ca^{2+} + calmodulin[a]	10^{-3} M Ca^{2+} + calmodulin[a]
(A) Monoxenic cultures				
K401	0.86	1.19	3.66	4.84
7S	0.71	1.11	2.94	4.04
nd9–18° C	0.54	0.95	2.15	3.88
nd9–28° C	0.08	0.15	0.13	0.32
d4–500r	nt[b]	0.71	nt[b]	2.00
(B) Sterile cultures				
nd9–18° C	nt[b]	0.95	nt[b]	4.15
nd9–28° C	nt[b]	0.30	nt[b]	0.60

[a] 10 μmol calmodulin/mg protein
[b] Not tested
[c] Each data point represents at least 3 duplicate measurements

tosis capacity in sea urchin oocytes [20]. All these results can be seen in line with our present findings with exocytosis-competent or -incompetent *P. tetraurelia* strains.

We used inhibitors of calmodulin-regulated ATPase activity: trifluoperazine [12] and compound R 24571 for its similar, but considerably more specific effect [21]. Thus we found some evidence for endogenous calmodulin (see section 3). Mg^{2+} is required for this Ca^{2+}-ATPase activity, since the use of CDTA (a Mg^{2+}- and Ca^{2+}-chelator; cf. [13]) instead of EGTA (a specific Ca^{2+}-chelator) in the assay (while keeping the free Ca^{2+}-concentration at identical levels) entailed a considerable reduction of the Ca^{2+}-ATPase activity. Similar observations were reported for other systems [13] and may be explained by the inhibitory effect of high Mg^{2+}-levels, i.e., in the usual mM level, on Ca^{2+}–calmodulin interaction [22].

Our own data obtained with the different *P. tetraurelia* strains (including discharge mutations) would be compatible with a concept of exocytotic membrane fusion which assigns an active role to a membrane-bound calmodulin-stimulated Ca^{2+}-ATPase system at a physiologically low intracellular Ca^{2+}-concentration.

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