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Ca^{2+} transport and chemoreception in *Paramecium*

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Abstract. Intracellular Ca^{2+} levels in *Paramecium* must be tightly controlled, yet little is understood about the mechanisms of control. We describe here indirect evidence that a phosphoenzyme intermediate is the calmodulin-regulated plasma membrane Ca^{2+} pump and that a Ca^{2+}-ATPase activity in pellicles (the complex of cell body surface membranes) is the enzyme correlate of the plasma membrane pump protein. A change in Ca^{2+} pump activity has been implicated in the chemoresponse of paramecia to some attractant stimuli. Indirect support for this is demonstrated using mutants with different modifications of calmodulin to correlate defects in chemoresponse with altered Ca^{2+} homeostasis and pump activity.

Key words: Ca^{2+}-ATPase – Ca^{2+} pump – Chemoreception – Calcium, *Paramecium*

Introduction

Paramecium is known for its graded Ca^{2+} action potentials that originate with the voltage-gated Ca^{2+} channels of the cilia (Eckert 1972). Among several other channel activities, Ca^{2+}-dependent K^+ and Na^+ conductances have been described (Saimi and Kung 1987; Preston et al. 1991). However, little is known about the pumps and transporters that tightly control internal Ca^{2+} levels. Recently, we described a Ca^{2+}-ATPase activity of the pellicles (a complex of cell body surface membranes) of *Paramecium* (Wright and Van Houten 1990). This Ca^{2+}-ATPase activity has all the hallmarks of a plasma membrane Ca^{2+} pump, and therefore, it may be one of the mechanisms responsible for the maintenance of low, Ca_i in *Paramecium*. Here we present correlative evidence that the Ca^{2+}-ATPase activity is, indeed, the Ca^{2+} pump of the plasma membrane and that this pump may be part of the chemosensory transduction pathway in *Paramecium*. A Ca^{2+} pump current could account for the hyperpolarizing conductance observed when cells are in attractant stimuli (Van Houten 1979; Preston and Van Houten 1987; Van Houten 1990, 1992).

The novel aspects of these findings are that the Ca^{2+}-ATPase and putative pump protein are not prevalent in the cilia, but rather are in the plasma membrane. Additionally, the Ca^{2+}-ATPase activity and pump are not identical to the Ca^{2+}-sequestering activity of the endoplasmic reticulum-like alveolar sacs. Thapsigargin can be used to inhibit alveolar uptake and study the Ca^{2+}-ATPase activity in the absence of a contribution from the alveoli transport. The Ca^{2+}-ATPase and pumping activities are implicated in chemoreception through the use of mutants with defects in calmodulin and concomitant defects in efflux and chemoresponse.

Abbreviations: EGTA, ethyleneglycol tetra-acetate; ER, endoplasmic reticulum; IBMX, isobutyl methylxanthine; I_{che}, index of chemokinesis; Mops, 3-[N-morpholino] propanesulfonic acid; PEI, phosphoenzyme intermediate; STEN, sucrose, TRIS, EDTA, sodium chloride; TCA, trichloroacetic acid; TRIS, tris[hydroxymethyl] aminomethane

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Materials and methods

Isolation of pellicle membranes. Pellicles, cell body surface membranes, are complex structures with associated cytoskeleton. The membranes can be isolated as large sheets with alveolar sacs attached, but in the relative absence of cilia, mitochondria, and other intracellular organelles. We use a modification of the method of Bilinski et al. (1981) and Wright and Van Houten (1990). This is a somewhat different procedure than in the preparation used for receptor purification (Van Houten et al. 1991).

Cilia preparation. Cells were harvested by centrifugation (350 *g*, IEC HNS–II) for 2 min, and resuspended in Dryl's solution (1 mmol · l^{-1} NaH_2PO_4, 1 mmol · l^{-1} Na_2HPO_4, 2 mmol · l^{-1} sodium citrate, 1.5 mmol · l^{-1} $CaCl_2$; pH 6.8) at room temperature. The cells were washed by three centrifugations in Dryl's solution, and the trichocysts (the fluffy layers on top of the cell pellets) were removed after each spin. The washed cells were resuspended in 50 ml ice-cold STEN (0.150 mmol · l^{-1} sucrose, 20 mmol · l^{-1} Trizma-base, 2 mmol · l^{-1} EDTA, and 6 mmol · l^{-1} NaCl; pH 7.5) and 50 ml room-temperature Dryl's solution. The cells were swirled on ice and observed for 10 min. 20 ml of fresh 180 mmol · l^{-1} KCl,

60 mmol · l^{-1} $CaCl_2$ solution was added to deciliate the cells. The cells were swirled on ice and observed using phase contrast microscopy for 5 min. The deciliated cells were centrifuged (1350 *g*, IEC HNS–II) twice for 2 min to remove the cell bodies. The second supernatant was centrifuged (29000 *g*, Beckman J2–21, JA–17 rotor) for 20 min at 4 °C. That supernatant was discarded and the pellet of cilia was resuspended in 1 ml buffer. This is a modified method of Aboutte et al. (1980).

A modified method for smaller volumes was used at times. This method substituted 1 mmol · l^{-1} KCl and 0.5 mmol · l^{-1} $CaCl_2$ solution for the 180 mmol · l^{-1} KCl and 60 mmol · l^{-1} $CaCl_2$, and the total volume of cells and deciliating solution was 30 ml.

Ca-ATPase assay. The (Ca^{2+} + Mg^{2+})-dependent ATPase activity was determined by measuring released inorganic phosphate by the method of Lanzetta et al. (1979). The medium contained, in a total volume of 250 µl, 20 mmol · l^{-1} Mops-TRIS (pH 7.0), 25 mmol · l^{-1} KCl, 3 mmol · l^{-1} $MgCl_2$, 1 mmol · l^{-1} TRIS-EGTA, 15–30 µg membrane protein, 2 mmol · l^{-1} Na-ATP, 2 mmol · l^{-1} NaN_3, and $CaCl_2$ to achieve the desired free Ca^{2+} concentration. Generally, 0.5 µmol · l^{-1} Ca^{2+} was used in the standard assay. The free Ca^{2+} was calculated by the method of Pershadsingh and McDonald (1980) with the aid of a computer program (Perrin and Sayce 1967). The reaction mixture was preincubated for 5 min at 30 °C before ATP was added to initiate the assay. After a 30-min incubation at 30 °C, the reaction was stopped by the addition of 2.5 ml color reagent (3 parts 0.045% malachite green hydrochloride to 1 part 4.2% ammonium molybdate in 4 mol · l^{-1} HCl with the addition of 0.4 ml 1% Sterox per 10 ml color reagent). This was followed by the addition of 325 µl 34% Na-citrate. Absorbance was measured spectrophotometrically at 660 nm according to the methods of Sanui (1974). The basal rate of ATP hydrolysis in the presence of Mg^{2+} was subtracted from the total rate in the presence of Mg^{2+} plus Ca^{2+} to determine the net Ca^{2+}-stimulated ATPase activity. Protein was assayed using the Pierce kit and gamma globulin as standard.

Ca^{2+}-efflux assay. 1.5 l of stationary phase cultures of paramecia were harvested by centrifugation at 350 *g* (IEC HNS–II centrifuge oil testing 100 ml tubes) and the final pellet was divided into two equal volumes. One was washed by centrifugation in 150 ml 10 mmol · l^{-1} NaCl buffer (1 mmol · l^{-1} citric acid monohydrate, 0.25 mmol · l^{-1} $Ca(OH)_2$, 5 mmol · l^{-1} NaCl; pH 7.05) and resuspended in 10 ml 10 mmol · l^{-1} NaCl solution. The second pellet was treated in the same manner, but in KCl buffers. $^{45}CaCl_2$, was added to both aliquots of cells to a final concentration of 2 µCi · ml^{-1} and the cells were placed in a circulating water bath (16 °C) overnight to come to equilibrium. Samples of 200 µl were placed in 10 ml of their respective NaCl or KCl buffers as described above and at appropriate times (0–15 min) were rapidly filtered on 5.0-µm Millipore nitrocellulose filters. The filters were then placed in 3.5 ml Ready Protein scintillation fluid (Beckman) and analyzed in a Beckman LS 7000 scintillation counter. The Medas curve-fitting program was used to analyze rate of efflux.

Ca^{2+}-uptake assay. Ca^{2+} uptake into isolated pellicles was performed as described by Wright and Van Houten (1990). Briefly, ^{45}Ca [2 µCi · ml^{-1} final concentration in each incubation] is taken up into pellicle membrane preparations and the amount taken up is quantified by filtering the pellicles onto Millipore filters (HAWP 0.45 µm) and counting the filters in a Beckman LS 7000 scintillation counter using Ready Protein scintillation fluid (Beckman). Nonspecific Ca^{2+} uptake in samples without ATP was subtracted from the experimental values.

Phosphoenzyme intermediate. The labeling of the phosphoenzyme intermediate and acid gel electrophoresis for autoradiography are described in detail in Wright and Van Houten (1990). Briefly, the pellicles or other membranes were incubated with [γ-^{32}P]-ATP (15 µCi · $nmol^{-1}$ per 0.2 ml reaction mixture) under conditions favorable for the formation of a stable phosphorylated enzyme intermediate, as modified from Wuytack et al. (1982). The incubation was performed in microcentrifuge tubes at 0 °C for 30 s after which the reaction was terminated with 10% cold trichloroacetic acid (TCA) containing 1 mmol · l^{-1} ATP and 50 mmol · l^{-1} H_3PO_4. After 10 min on ice, the TCA-precipitated proteins were pelleted by centrifugation for 4 min in a microfuge. The pellets were washed three times by resuspension in 0.5 ml TCA solution and recentrifugation. The final pellets were rinsed with 1 ml ice-cold water before dissolving in 25 µl of sample buffer which contained 50 mmol · l^{-1} NaH_2PO_4 (pH 6.3), 10% glycerol, 2% SDS, 5% β-mercaptoethanol. The electrode buffer also contained 100 mmol · l^{-1} $NaHPO_4$ and 0.1% SDS (Kosk-Kosika et al. 1986). The gels were run and prepared for autoradiography as described in Wright and Van Houten (1990). The dried gels were exposed to Kodak X-OMAT film at −70 °C. The relative molecular weights of labeled proteins were estimated by comparison with the relative mobilities of Sigma prestained molecular weight markers run simultaneously on each gel.

Calmodulin overlay. Bovine brain calmodulin (Sigma) was biotinylated by the method of Billingsley et al. (1985). Bovine calmodulin (1.2 mg · ml^{-1} in distilled water was concentrated and resuspended three times using a Centricon-10 with 10000 molecular weight cut-off, replacing the water with 0.1 mol · l^{-1} phosphate buffer; pH 7.4). The concentration of calmodulin was brought to 1.2 mg · ml^{-1}. Biotinyl-Σ-aminocaproic acid *N*-hydroxysuccinimide ester (Calbiochem) was dissolved in *N*, *N*-dimethylformamide at 3.2 mg per 100 µl and was added to the calmodulin solution for a final concentration of 1 mmol · l^{-1}. This yields a molar ratio of calmodulin to biotin of 1:14. This solution was stirred on ice for 2 h. To remove the residual biotin, the solution was spun five times in the Centricon-10 to replace the buffer with 0.1 mol · l^{-1} phosphate buffer. The biotinylated calmodulin was stored frozen (−20 °C).

Paramecium pellicles were prepared as above. Human red blood cell membranes were prepared as in Garrahan and Rega (1978). Proteins from pellicle or red blood cell membranes were separated on 8% standard Laemmli (1970) gel for Fig. 3 and on an acid gel (see above) for Figs. 1, 2. Gels were transferred to nitrocellulose by the method of Towbin et al. (1979) as described in Van Houten et al. (1991), followed by incubation in blocking solution: Buffer A [50 mmol · l^{-1} TRIS-HCl (pH 7.4), 150 mmol · l^{-1} NaCl, 1 mmol · l^{-1} $CaCl_2$] contains 5% (w/v) non-fat dry milk (Billingsley et al. 1985). The blots were washed for 20 min with buffer A. The blots were incubated with 25–30 µg biotinylated calmodulin for 1 h in 2.5 ml blocking solution containing either 1 mmol · l^{-1} $CaCl_2$ or 5 mmol · l^{-1} EGTA for control studies. Following incubation, the blots were washed 3 times for 20 min in buffer A. For specific incubations, 1 mmol · l^{-1} $CaCl_2$ was included in all buffers and washes, while for control incubations 5 mmol · l^{-1} EGTA was used in all solutions. After washing, the blots were reacted with avidin-alkaline phosphatase (14 µl of a 1 mg · ml^{-1} stock solution) dissolved in 7 ml blocking solution for 30 min, followed by three 20-min washes. For color development alkaline phosphatase-nitro blue tetrazolium chloride (200 µl at 50 mg · ml^{-1} in 50% dimethylformamide) and 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt (100 µl at 50 mg · ml^{-1} in 50% dimethylformamide) were dissolved in 30 ml of 100 mmol · l^{-1} TRIS-HCl, pH 9.5, 100 mmol · l^{-1} NaCl, 50 mmol · l^{-1} $MgCl_2$. Blots were rinsed in distilled water and air dried.

Chemoresponse assay. This consists of a T-maze assay of chemoresponse as described in detail in Van Houten et al. (1982). The index of chemokinesis (I_{che}) indicates attraction if >0.5, repulsion if <0.5, and neutral response at 0.5.

Mutant stocks and wild type. Paramecium tetraurelia (51–S), sensitive to killer, were grown in a wheat grass culture medium supplemented with 1 mg · l^{-1} stigmasterol, and 0.67 g · l^{-1} proteose peptone, with sodium phosphate and TRIS-HCl buffering [see Sas-

ner and Van Houten (1989) for more details]. The medium was inoculated with *Klebsiella pneumoniae* 24–48 h before the addition of *Paramecium*. Cells are cultured at 25 °C and harvested in late log phase. Mutants were derived from stock 51-S and were provided courtesy of C. Kung, T. Evans, and D. Nelson. [The characterizations of the mutants are described in Evans et al. (1987); Preston et al. (1991).]

Reagents and inhibitors. All reagents were from Sigma, except Triton X-100, biotinyl-Σ-aminocaproic acid, *N*-hydroxysuccinimide ester, and calmidazolium which were phosphoenzyme intermediates (PEI) from Calbiochem. ^{45}Ca and ^{32}P-ATP were from Amersham.

Results

The phosphoenzyme intermediate

Ion pumps of the P-type transiently form PEIs with base- or hydroxylamine-labile acyl bonds that distinguish them from kinase substrates [review: Carafoli (1991)]. At any time about 10% of the molecules can be isolated with label from ^{32}P-ATP (Schatzman 1983). Dyneins, although they are ATPases, will not form this intermediate. We have found that a PEI formed in *Paramecium* pellicle runs on acid gels at approximately 133 kDa in molecular mass [Fig. 1; Wright and Van Houten (1990)], within the size range expected for a Ca^{2+}-calmodulin-regulated plasma membrane Ca^{2+} pump (Carafoli 1991). Data in Fig. 1 confirm as previously shown that the PEI is hydroxylamine-sensitive and Ca^{2+}-dependent [Fig. 1; Wright and Van Houten (1990)].

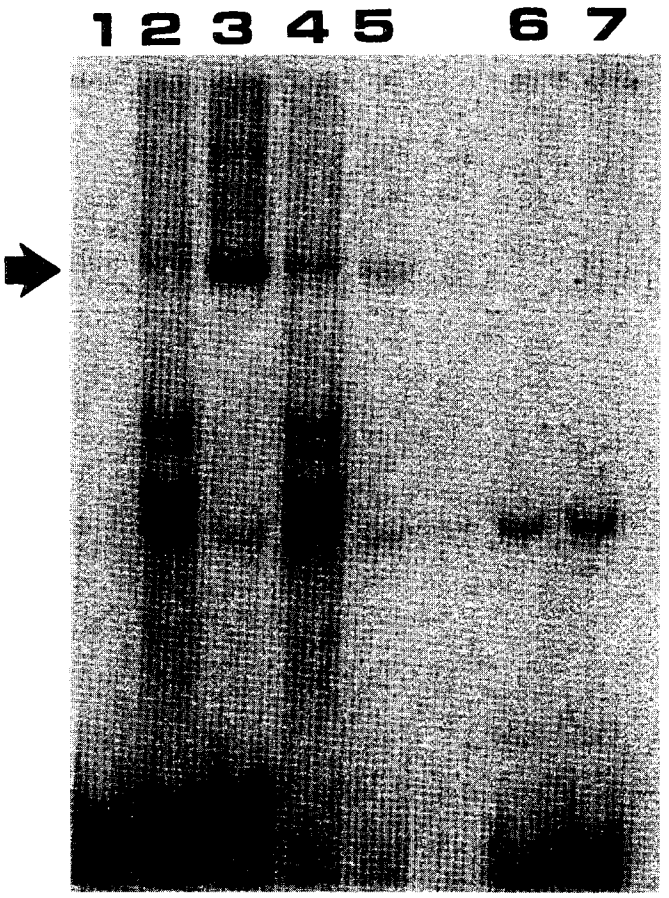

Fig. 1. Phosphoenzyme intermediate is greatly reduced in cilia. Acid gel of the ^{32}P-ATP-labeled PEIs from pellicles and cilia. The same amount of protein is loaded onto each lane. See Materials and methods for details. Lanes 1–5: from pellicle; lanes 6–7 from cilia. *Lane 1:* labelling in no Ca^{2+} or Mg^{2+}; *Lane 2:* labeling in Mg^{2+}; *Lane 3:* labeling in 1 mmol · l^{-1} Ca^{2+}; *Lane 4:* labeling in Mg^{2+} followed by hydroxylamine treatment; *Lane 5:* labeling in Ca^{2+} followed by hydroxylamine treatment; *Lane 6:* cilia labeled without Ca^{2+}; *Lane 7:* cilia labeled with Ca^{2+}. See Wright and Van Houten (1990) for more details. Note that little PEI can be found in the lanes with cilia proteins (Lanes 6 and 7)

An important finding is that very little if any of the PEI is found in cilia. In Fig. 1, lanes 1 and 2 show that PEI formation is dependent upon Ca^{2+}. Lane 5 confirms the hydroxyl-amine lability of the PEI. The proteins for lanes 1–5 come from pellicles while those for lanes 6 and 7 are derived from cilia. There is little formation of PEI with or without Ca^{2+} in cilia. A similar experiment was performed using a low concentration of Triton X-100 to ensure access of reagents to the interior of the cilia with similar results (data not shown).

A protein that runs with the same mobility as the PEI binds calmodulin. As shown in Fig. 2, both red blood cell membranes and *Paramecium* pellicle PEI formation is dependent upon Ca^{2+} (Fig. 2A, lanes 1 vs 3 and 2 vs 4, respectively). Companion lanes cut from the same gel used for Fig. 2A were blotted and developed with biotin-labeled calmodulin and avidin-alkaline phosphatase (Fig. 2B). Biotin-labeled calmodulin binds to a tight band corresponding to the slowest migrating part of the PEI band. This location was determined by superimposing the autoradiogram from the PEI analysis and the blot, which was possible since they were from the same gel. However, photographic reproduction does not allow us to reproduce exactly the blot and autoradiogram sizes and the reader must examine the molecular weight markers to line up the PEIs with the calmodulin-bound bands.

The binding of the biotinylated calmodulin to the PEI is Ca^{2+}-dependent (Fig. 3). *Paramecium* pellicle and red blood cell membrane proteins were blotted from a Laemmli gel onto nitrocellulose and developed with biotinylated calmodulin. Figure 3A shows total protein; Fig. 3B shows the binding of calmodulin to bands of the right size ranges for red blood cell and *Paramecium* PEI in the presence of Ca^{2+}. Figure 3C demonstrates the lack of calmodulin binding to the red blood cells and *Paramecium* PEIs in the absence of Ca^{2+}.

The Ca^{2+}-ATPase enzyme activity

A Ca^{2+}-ATPase activity is associated with the pellicle preparation (Wright and Van Houten 1990). It has the characteristics of a plasma membrane pump activity: K_m for Ca^{2+} is 95 nmol · l^{-1}; Ca^{2+} stimulates activity and 3 mmol · l^{-1} Mg^{2+} is required for optimal activity; ATP is required, and no other nucleotide will substitute; vanadate, calmidazolium, and mellitin, but not azide or oligomycin inhibit activity. This activity is not from dynein of contaminating cilia as evidenced by different pH optima and nucleotide specificity and is not due to con-

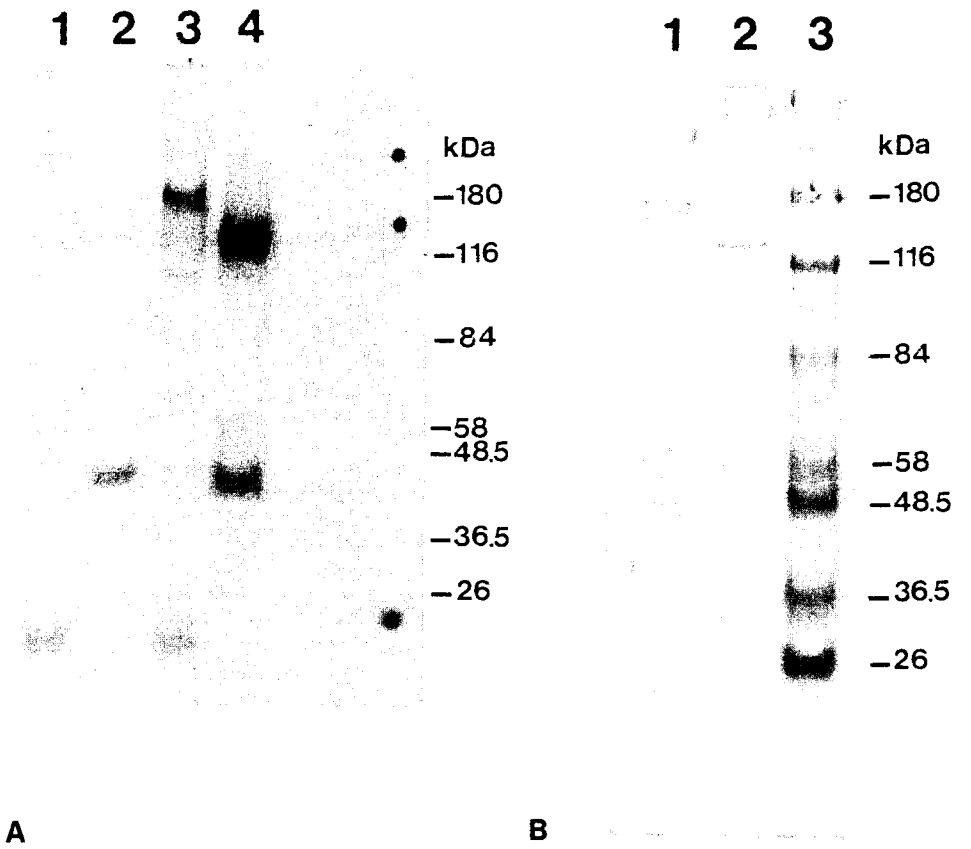

Fig. 2A, B. Phosphoenzyme intermediate binds biotinylated calmodulin. Pellicle proteins were labeled with ^{32}P–ATP and run on acid gels as described in Materials and methods. Two sets of lanes were run for autoradiography and electroblotting. The blot was blocked, incubated with biotinylated calmodulin, and developed with avidin-alkaline phosphatase. Red blood cell membranes are run as controls for a plasma membrane Ca^{2+}-ATPase. **A** Autoradiogram *Lane 1:* red blood cell membrane protein labeling in the absence of Ca^{2+}; *Lane 2: Paramecium* pellicle proteins labeled in the absence of Ca^{2+}; *Lane 3:* red blood cell membrane proteins labeled in 25 μmol · l^{-1} free Ca^{2+}; *Lane 4: Paramecium* pellicle proteins labeled in the presence of Ca^{2+}. Note the phosphoenzyme intermediates in lanes 3 and 4. **B** Blot *Lane 1:* red blood cell membrane proteins bound with biotinylated calmodulin in the presence of Ca^{2+}; *Lane 2: Paramecium* pellicle proteins bound with biotinylated calmodulin in the presence of Ca^{2+}; *Lane 3:* molecular weight markers. The PEIs and calmodulin binding proteins are superimposable in the original blot and autoradiogram

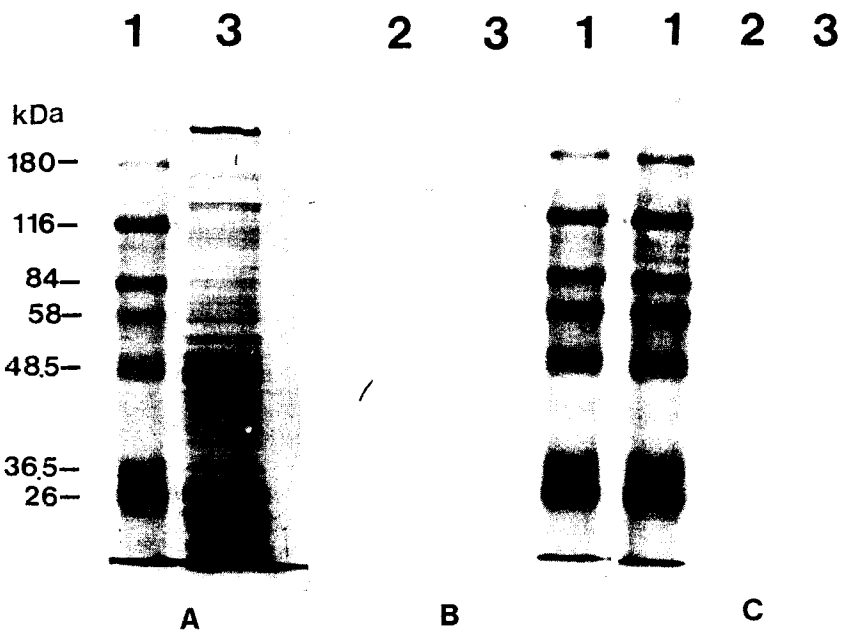

Fig. 3A–C. Calcium dependence of calmodulin binding to the phosphoenzyme intermediate. *Lanes 1:* prestained molecular weight markers; *Lanes 2:* red blood cell membranes with Ca^{2+}-ATPase as positive control (Mmass = 138 kDa); *Lanes 3: Paramecium* pellicles with putative pump band at 133 kDa. **A** Blot stained for total protein with amido black; **B** blot incubated with biotinylated *Paramecium* calmodulin in 1 mmol · l^{-1} Ca^{2+} and color developed with avidin-alkaline phosphatase; **C** blot incubated with biotinylated calmodulin in EGTA, and color developed as in **B.** The gel here is a standard Laemmli gel (Laemmli 1970) and not an acid gel as in Wright and Van Houten (1990) or in Fig. 1 and 2

taminating ER or mitochondria as shown through the use of inhibitors (Wright 1990; Wright and Van Houten 1990).

The most feasible sources of Ca^{2+}-ATPase activities in the pellicle are the plasma membrane pump and the alveolar sacs, which are membrane-bound organelles underlying the cell surface and apparently sequestering Ca^{2+} (Stelly et al. 1991). In order to determine the potential contribution of the putative alveolar Ca^{2+}-pump to the total Ca^{2+}-ATPase activity, we have compared the effects of several pharmacological agents on the Ca^{2+}-ATPase activity and the ^{45}Ca uptake into pellicles considering this as a measure of alveolar uptake. The Ca^{2+}-ATPase and alveolar uptake can be distinguished on the

Table 1. Ca^{2+} uptake and Ca^{2+}-ATPase activity in *Paramecium* pellicles

Treatment	% Control Ca^{2+} uptake	% Ca^{2+}-ATPase activity
A		
2 μmol · l^{-1} Mellitin	91.4 ± 18.0	20.1 ± 18.0
1 μmol · l^{-1} Calmidazolium	96.3 ± 7.7	nd
0.5 μmol · l^{-1} Calmidazolium	nd	50.7 ± 8.3
B		
None	100	100
2 μmol · l^{-1} Vanadate	98.6 ± 1.3	52.6 ± 3.1
20 μmol · l^{-1} Vanadate	59.4 ± 9.6	nd
25 nmol · l^{-1} Thapsigargin	nd	105.0
50 nmol · l^{-1} Thapsigargin	25.3 ± 9.4	89.4 ± 13.7
100 nmol · l^{-1} Thapsigargin	19.3 ± 8.2	101.2 ± 13.6

Numbers are given as a percent of uptake, which achieves equilibrium within 120 s. The data are averages of 3–4 experiments done in duplicate (with the exception of the 25 nmol · l^{-1} thapsigargin experiment in **B** which was done once) with standard deviation shown. [Methods are given in Wright and Van Houten (1990)]

Table 2. Mellitin treatment and calmodulin activation

Experiment #	% Activity		% Increase With added CM
	No addition	Added CM	
A Control activity			
1	100	99	0
2	100	120	20
	100	102	2
B Mellitin pretreatment			
1	44	66	50
2	34	69	100
3	53	64	22
4	18	47	155

Data are averages of duplicates. The control activity in nmoles · mg^{-1} · min^{-1} did not vary significantly from experiment to experiment. 100 μg of pellicles were incubated in a volume of 0.5 ml with 4 μmol · l^{-1} mellitin in 5 μmol · l^{-1} Ca^{2+}, 25 mmol · l^{-1} KCl, Mops-TRIS buffer at pH 7.0 for 10 min, followed by pelleting and resuspension in Mops-TRIS buffer for ATPase assay. Control activity omitted mellitin pretreatment

Table 3. Chemoresponse of calmodulin mutants

	Response to:			
	K-acetate	Na-acetate	K-lactate	Na-lactate
Normal	0.84 ± 0.07	0.82 ± 0.11	0.83 ± 0.06	0.73 ± 0.04
fna	0.79 ± 0.11	0.51 ± 0.08[a]	0.87 ± 0.09	0.48 ± 0.03*[a]
fnaP	0.77 ± 0.02	0.55 ± 0.02[a]	nd	nd

Data are averages of three or more T-maze assays ± one standard deviation. Solutions are buffers (see Methods) with 5 mmol · l^{-1} of the attractant salt in test solution and 5 mmol · l^{-1} of control salt in control solution. * 90 min instead of the standard 30 min for the assay.

[a] Statistically significantly different from comparable K-acetate or K-lactate value by either Mann-Whitney U or *t*-tests

basis of their sensitivities to inhibitors. First, calmidazolium and mellitin, both calmodulin antagonists, are less effective in inhibiting the alveolar uptake than in inhibiting the Ca^{2+}-ATPase activity (Table 1A). Second, thapsigargin, an inhibitor of ER Ca^{2+} pumps (Thastrup et al. 1990), reduces alveolar uptake but has relatively little effect on the Ca^{2+}-ATPase activity (Table 1B). Third, vanadate, a general inhibitor of ATPases including Ca^{2+} pumps, is shown as a control (Table 1B). Even the amount of vanadate for half-maximal inhibition of the ATPase and alveolar uptake activities differ. Based on the three inhibitor studies, we surmise that the majority of the Ca^{2+}-ATPase activity is from the plasma membrane and distinct from the alveolar uptake mechanism.

Ca-ATPase, calmodulin, and chemoresponse

By process of elimination in studies like those above, the major pellicle Ca^{2+}-ATPase appears to be primarily from the plasma membrane. This Ca^{2+}-ATPase should, if similar to most plasma membrane pumps, be stimulated by calmodulin (Carafoli 1991). However, the addition of calmodulin to pellicle preparations produces only slight and variable increases in activity (Table 2). After attempts at stripping off any endogenous calmodulin with mellitin, added calmodulin restores 22–150% of the control activity (Table 2).

To further address calmodulin regulation of the Ca^{2+}-ATPase and its potential role in chemoresponse, we utilized two calmodulin mutants *fnaP* (also called *cam3*) and *fna* (also called *cam11*) (Preston et al. 1991). These mutants are very different in phenotype. Each has a different single amino acid change in calmodulin but different changes in Ca^{2+}-dependent conductances. *fnaP* has both an increased Ca^{2+}-dependent Na^+ conductance and a decreased Ca^{2+}-dependent K^+ conductance; *fna* has an increased Ca^{2+}-dependent K^+ conductance and decreased Ca^{2+}-dependent Na^+ conductance (Preston et al. 1991). The cells have fairly normal phenotypes in 5 mmol · l^{-1} K^+ solution but show their distinctive "paranoiac" *(fnaP)* and "fast" *(fna)* phenotypes in Na^+ solutions without K^+ (Saimi and Kung 1987; Preston et al. 1991). These conditional mutants allow tests of the mutant calmodulin in Ca^{2+} pumping and chemoresponse, albeit indirect.

The mutants show chemoresponse behavior within a normal range when assayed in K^+ buffers of the stimuli acetate and lactate (Table 3). When in Na^+ buffers, the mutant phenotypes are more evident and the mutants are not attracted to acetate or lactate, two simuli believed to work through signal transduction pathways that couple to the Ca^{2+} pump (Van Houten 1990; Van Houten et al. 1991a). To ensure that the attraction of the mutants was not missed due to a difference in response time-course, we repeated the behavioral assays allowing 90 min instead of 30 min for distribution and found the same results (Table 3).

Table 4. Calmodulin mutant Ca^{2+} efflux and Ca^{2+}-ATPase activities

Strain	$^{45}Ca^{2+}$-Efflux activity		Ca^{2+}-ATPase activity V_{max}
	in KCl	in NaCl	
fnaP	0.26 ± 0.10^{a}	0.50 ± 0.26*	57.2 ± 39.8
Normal	0.19 ± 0.06	0.20 ± 0.05	31.1 ± 9.3
fna	0.37 ± 0.04*	0.13 ± 0.01	118.5 ± 16.3
Normalized Data			
fnaP	1.4	2.5	1.8
Normal	1.0	1.0	1.0
fna	2.0	0.7	3.8

Efflux: cells are loaded with $^{45}Ca^{2+}$ to equilibrium and the flux of Ca^{2+} out of the cells is measured in a filtration assay (Wright 1990; Wright et al. 1992). The cells are in either a KCl or NaCl buffer. Data are from averages of three experiments done in duplicate and normalized to the normal cells' value. Units are nmol · mg^{-1} · min^{-1}.
Ca^{2+}-ATPase activity: data are averages of 4–11 experiments done in duplicate ± one standard deviation. The units are nmol · mg^{-1} · min^{-1}.
Data are normalized to the normal cells' values for ease of comparison.
* Statistically significantly different from normal values at $P = 0.05$ level by t-test.
[a] Statistically significantly different from efflux of same mutant in NaCl buffer at the $P = 0.02$ level

The efflux of $^{45}Ca^{2+}$ from cells loaded to equilibrium is shown in Table 4 and is also normalized to that for wild type cells to assist comparison. In K^+ buffers, *fnaP* cells show an efflux similar to that of normal cells. However, in Na^+ buffers, the mutant's efflux rate almost doubles compared to that in K^+. The opposite is observed with *fna* cells in K^+ and Na^+ buffers. The rate of normal cell efflux is not affected by the monovalent cations, K^+ or Na^+, of the incubation buffers, but the mutants show different rates of efflux depending on the external monovalent cation.

The mutants both show significant Ca^{2+}-ATPase activity in pellicle preparations (Table 4): *fnaP* shows Ca^{2+}-ATPase activities somewhat higher than wild type but within the normal range, and *fna* Ca^{2+}-ATPase activity is sharply higher. Mellitin inhibits the mutant and normal Ca^{2+}-ATPase activity with about the same ED_{50} as with activites from normal cells (data not shown).

Discussion

A plasma membrane pump is of interest by virtue of its potential role in generating a hyperpolarizing conductance in one of *Paramecium*'s chemosensory signal transduction pathways (Preston and Van Houten 1987). Therefore, we have examined the calmodulin binding and regulation of the PEI and Ca^{2+}-ATPase activities that are associated with Ca^{2+}-pumps in the *Paramecium* pellicles, i.e. surface membrane preparations.

The phosphoenzyme intermediate

The PEI described here and in Wright and Van Houten (1990) is clearly a Ca^{2+}-pump from the pellicle preparation. It is Ca^{2+}-dependent in its formation, hydroxylamine labile [Fig. 1; Wright and Van Houten (1990)] and Ca^{2}-dependent in its binding to calmodulin (Figs. 2, 3). The band bound by calmodulin is sharper than the PEI autoradiograph band due to the lack of focus of the isotope band or to one or more additional PEIs that do not bind calmodulin under these conditions. The proteins are denatured on the blot and some may thus lose calmodulin interaction by denaturation or by proteolytic degradation during the pellicle preparation. Alternatively, additional PEIs could be derived from contaminating ER membranes or the alveolar sacs, the membrane-bound organelles underlying the surface membrane and comprising part of the pellicle preparation. ER was previously ruled out as the source of a PEI from pellicle, on the basis of its lower molecular weight and lack of enhanced labeling with La^{3+}, although it is impossible to be absolutely certain (Wright and Van Houten 1990). However, the alveolar sacs could contribute a PEI to the pellicle preparation because the alveolar sacs sequester Ca^{2+} and thus must have some transport mechanism, most likely a pump since $^{45}Ca^{2+}$ uptake into the pellicle is ATP-dependent (Wright and Van Houten 1990; Stelly et al. 1991). This uptake activity associated with the sacs can account for only 10% of the total Ca^{2+}-ATPase enzyme activity of the pellicle (Wright and Van Houten 1990), but alveoli nonetheless could contribute a PEI. Additonally, the alveolar PEI might be less strongly bound by calmodulin and thus not bind biotinylated calmodulin on blots; we have found that alveolar Ca^{2+} uptake is less sensitive to calmodulin inhibitors than the Ca^{2+}-ATPase activity (see next section). The alveoli are likened to ER (Satir and Wissig 1982; Stelly et al. 1991) and the decreased sensitivity of uptake to calmodulin inhibitors would be consistent with other ER pumps that typically are smaller in molecular weight than plasma membrane pumps because they lack the calmodulin-binding domain (Carafoli 1991). Also, in keeping with ER character of the alveolar transport is the inhibition of uptake by thapsigargin (see next section), an inhibitor of ER Ca^{2+}-pumps [Thastrup et al. (1990); Table 1B]. In contrast, thapsigargin has little, if any, effect on

what we believe is primarily a plasma membrane Ca^{2+}-ATPase activity.

In view of these results, the simplest interpretation is that there is a plasma membrane calmodulin-regulated Ca^{2+}-pump and possibly an additional pump protein(s) that does not bind calmodulin. This second form may be from the alveolar sacs or be the result of modification of the plasma membrane protein rendering it unable to interact with calmodulin on blots.

The cilia have been the focus of attention in several previous Ca^{2+}-ATPase studies (Wright and Van Houten 1990). The expectation is that there will be some mechanism, perhaps a Ca^{2+}-pump, to rapidly remove the Ca^{2+} that accumulates in the ciliary compartment during the opening of voltage-gated Ca^{2+} channels during the action potential. However, the Ca^{2+}-ATPase activity described here and by Wright and Van Houten (1990) is typical of a Ca^{2+}-ATPase pump and is highly enriched in the cell body pellicles and not in the cilia. Perhaps the Ca^{2+}-ATPase activity of the pellicle is associated with the stubs of cilia that are part of the pellicle preparation, but the Ca^{2+}-ATPase activities reported for ciliary preparations do not appear to qualify as pump activities. Affinities and/or nucleotide selectivity differ from those of other known Ca^{2+}-pumps and the Ca^{2+}-ATPase described here [see Wright and Van Houten (1990) for discussion].

Ca^{2+}-ATPase activity

There is a major Ca^{2+}-ATPase activity associated with the *Paramecium* pellicle. The most likely contributions for this activity come from the plasma membrane and the alveolar sacs, not ER or mitochondria (Wright and Van Houten 1990). The alveolar sacs must have a Ca^{2+} uptake activity and this is likely to be an ER-like Ca^{2+} pump. The uptake into the alveoli is less sensitive to calmodulin inhibitors and vanadate than the Ca^{2+}-ATPase activity of the pellicle [Table 1; Wright and Van Houten (1990)]. Thapsigargin, an inhibitor of ER Ca^{2+} pumps (Thastrup et al. 1990), selectively inhibits alveolar uptake while not significantly affecting the Ca^{2+}-ATPase (Table 1).

One demonstration of calmodulin regulation would be the stimulation of Ca^{2+}-ATPase activity with added calmodulin. In the pellicle preparation, broken-cell system, several different calmodulin antagonists can completely inhibit Ca^{2+}-ATPase activity [Fig. 1 shows partial inhibitory concentrations; see Wright and Van Houten (1990) for more information]. Results of the addition of calmodulin vary from no stimulation to a 22% increase in Ca^{2+}-ATPase activity. We hoped to reduce the variability of these results by first stripping calmodulin from the Ca^{2+}-ATPase with mellitin before the addition of calmodulin; this produced a consistent increase in activity with added calmodulin, but again the results were variable. These studies were not continued because a definitive interpretation of results is not possible. For example, one could argue that the added calmodulin was not acting by binding to the Ca^{2+}-ATPase to directly activate the enzyme but was merely binding to and removing any remaining mellitin which is known to inhibit the Ca^{2+}-ATPase of *Paramecium* (Wright and Van Houten 1990). Moreover, it is possible for Ca^{2+}-ATPase activity to be inhibited but not activated by calmodulin. The Ca^{2+}-ATPase can be activated during preparation by limited proteolysis and acidic phospholipids (Garrahan 1986), leading to an enzyme that can be inhibited but not further activated.

Previously we estimated, on the basis of Ca^{2+} uptake, that the alveolar pump could contribute only up to 10% of the Ca^{2+}-ATPase activity of the pellicle (Wright and Van Houten 1990). Therefore, the combined studies suggest that the alveolar pump cannot significantly contribute to the Ca^{2+}-ATPase measured in the pellicles. Also, since there is one major PEI that binds calmodulin and one major Ca^{2+}-ATPase activity that is not associated with the alveoli, it is most likely that the PEI and activity are the same plasma membrane Ca^{2+} pump.

Calmodulin mutants and chemoresponse

The Ca^{2+} pump would generate the current that hyperpolarizes the cell in some attractant stimuli (Preston and Van Houten 1987; Van Houten 1990, 1991). The alveolar pump, which would only sequester Ca_i^{2+}, cannot account for this hyperpolarizing current, but a plasma membrane pump could. As described above, the pellicle Ca^{2+}-ATPase activity measured is likely to be from a calmodulin-regulated plasma membrane Ca^{2+} pump. Unfortunately, it is not possible to make the direct correlation of increased Ca^{2+}-ATPase activity with chemostimulation because it would require an increase in activity of $<1\%$ to account for the hyperpolarizing current in attractant stimuli (Preston and Van Houten 1987; M.V. Wright, unpublished observations) and this increase is within the error of the assay procedure; therefore, an indirect correlation using calmodulin mutants is necessary. The mutants used were *fnaP* and *fna*, with different mutant alleles of the calmodulin gene and very different phenotypes. *fnaP* has both an increased Ca^{2+}-dependent Na^+ conductance and a decreased Ca^{2+}-dependent K^+ conductance; *fna* has an increased Ca^{2+}-dependent K^+ conductance and decreased Ca^{2+}-dependent Na^+ conductance (Preston et al. 1991). The cells have fairly normal phenotypes in 5 mmol $\cdot l^{-1}$ K^+ solutions, but show their distinctive "paranoiac" *(fnaP)* and "fast" *(fna)* phenotypes in Na^+ solutions without K^+ (Saimi and Kung 1987; Preston et al. 1991).

Both mutants show significant Ca^{2+}-ATPase activity in pellicle preparations (Table 4) implying that there is basal pump activity but providing no information about calmodulin regulation. Because the mutants display normal or mutant behavioral phenotypes in K^+ and Na^+ buffers, respectively, we have made use of these conditions to examine chemoresponse behavior and whole-cell $^{45}Ca^{2+}$ efflux as a measure of Ca^{2+} pump activity. The simplest level on which to interpret these results with *fnaP* and *fna* is to correlate these known alterations in calmodulin with the alterations in what we believe to be

a measure of pump activity, i.e. the whole-cell $^{45}Ca^{2+}$ efflux and T-maze assays of chemoresponse. The conditions under which the cells display their *fna* or *fnaP* behavioral phenotypes are also conditions under which they show both defective chemoresponse and altered $^{45}Ca^{2+}$ efflux (Tables 3 and 4).

The interpretation of results with the mutants must be made carefully because multiple pleiotropic effects could be involved in the reduced chemoresponse or altered $^{45}Ca^{2+}$ efflux. For example, the conductances (Ca^{2+}-dependent K^+ or Na^+ conductances) that have been shown to be directly regulated by calmodulin and altered in the calmodulin mutants (Saimi and Ling 1990; Preston et al. 1991) could lead indirectly to altered chemoresponse and efflux. There are two arguments against this: first, previously using electrophysiology we examined the conductance and searched for channel activity that might be necessary for generating the hyperpolarizing current of chemoresponse and found no direct involvement of the Ca^{2+}-dependent K^+ or Na^+ conductances (Preston and Van Houten 1987). Second, we have examined other mutants with alterations in the same major conductance that is increased in *fnaP*, but the gene mutated in these mutants is not for calmodulin. One such "paranoiac" mutant (d4-90) with an increased Ca^{2+}-dependent Na^+ conductance and phenotype similar to *fnaP* (Van Houten et al. 1977) shows normal chemoresponse in both K^+ and Na^+ solutions (Van Houten 1978). Third, we have produced partial phenocopies of the *fna* mutant. *fna* has an extremely hyperpolarized membrane potential and shows extremely fast swimming in Na^+ solutions. The treatment of normal cells with IBMX mimics these effects by increasing cyclic nucleotide levels inside the cell and thus inducing extremely fast swimming (Bonini et al. 1987; J. Van Houten and M.V. Wright, unpublished observations), but cells show normal chemoresponse despite the IBMX-induced fast swimming (Van Houten and Preston 1987). Additionally, there is a mutant with abbreviated action potentials from an altered Ca^{2+}-activated K^+ conductance, the same conductance altered in both *fna* and *fnaP*. This mutant, *TEA*^{A}, shows normal attractant-induced hyperpolarization (Preston and Van Houten 1987). Therefore, the chemoresponse defects in *fnaP* and *fna* in Na^+ (K^+-free) solutions could be argued to be directly attributable to their mutations in calmodulin.

In the red blood cell Ca^{2+}-ATPase system, calmodulin with mutations in the *C*-terminal but not *N*-terminal Ca^{2+}-binding domains show defects in activating the enzyme (Bzdega and Kosk-Kosicka 1992). Among the *Paramecium* calmodulin mutants, *fnaP* has an amino acid substitution in the *C*-terminal region while *fna*'s substitution affects the *N*-terminal region. However, since both of these mutants show Na^+-dependent alterations in Ca^{2+} efflux and chemoresponse, there may be differences between the red blood cell and *Paramecium* Ca^{2+} pump calmodulin-binding domains, or there may be conformation changes specific to the *Paramecium* calmodulin amino acid substitutions that differ from those arising from the site-directed changes in red blood cell calmodulin.

In conclusion the weight of this correlative evidence indicates that the major Ca^{2+}-ATPase activity of the pellicle must come from the plasma membrane. The PEI that binds calmodulin in overlays also most likely originates in the plasma membrane. Thus, without more probes, these correlations are the best evidence that these two entities (activity and PEI) are one and the same and are from the plasma membrane and not from other membrane-bound organelles. Currently, members of a family of pump genes are being cloned and will provide some of the probes that are necessary for further clarification. Similarly from correlative evidence from calmodulin mutants, calmodulin is implicated in both Ca^{2+} pumping and chemosensory transduction.

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