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Characterization of a putative Ca^{2+}-transporting Ca^{2+}-ATPase in the pellicles of *Paramecium tetraurelia*

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In *Paramecium*, no Ca^{2+}-ATPases with the properties of Ca^{2+} pumps have been identified. Here we report a pellicle associated Ca^{2+}-ATPase activity and a corresponding phosphoprotein intermediate characteristic of a pump. The Ca^{2+}-ATPase activity requires 3 mM Mg for optimal Ca^{2+} stimulation (K_{Ca} = 90 nM) and is specific for ATP as substrate (K_m = 75 μM). Vanadate and calmidazolium inhibit Ca^{2+}-stimulated activity with an EC_{50} of about 2 μM and 0.5 μM, respectively. Likewise, 10 μM trifluoperazine inhibits 80% of Ca^{2+}-ATPase activity, but bovine calmodulin fails to stimulate. The Ca^{2+}-ATPase is not inhibited by sodium azide (10 mM), oligomycin (10 μg/ml) or ouabain (0.2 mM). Incubation of pellicles with [γ-^{32}P]ATP specifically labels a 133 kDa protein in a Ca^{2+}-dependent, hydroxylamine-sensitive manner, and the level of phosphorylation is increased by 100 μM La^{3+}. Phosphorylation of an endoplasmic reticulum-enriched fraction labels a Ca^{2+}-dependent protein different from the pellicle protein, being lower in molecular mass and unaffected by La^{3+}. Ca^{2+} uptake by the alveolar sacs, integral components of the pellicle membrane complex, is poorly coupled to Ca^{2+}-stimulated ATP hydrolysis (Ca^{2+} transported/ATP hydrolysed < 0.2) and is much less sensitive to vanadate inhibition (EC_{50} approx. 20 μM) compared to the total Ca^{2+}-ATPase activity. Therefore, the majority of the Ca^{2+}-ATPase activity is likely to be plasma membrane associated.

Introduction

In *Paramecium*, as in other cell types, internal Ca^{2+} is maintained at low resting levels and increases in Ca_i^{2+} affect many cell functions [1–3]. Perhaps the best documented effect of Ca_i^{2+} fluctuations in *Paramecium* is on ciliary activity [3]. The rise in intraciliary Ca^{2+} is in response to depolarizing stimuli and occurs via voltage-sensitive Ca^{2+} channels located exclusively on the cilia [4]. The influx of Ca^{2+} raises the resting Ca^{2+} level from 10^{-8} to over 10^{-6} M in the ciliary compartment [5], evoking a directional change in ciliary beat [6,7]. The duration of ciliary reversal is very transient, suggesting that Ca^{2+} levels likewise remain elevated for only a short time. In contrast to the mechanism of Ca^{2+} influx, the mechanism(s) for reducing intracellular Ca^{2+} are poorly understood [3]. Browning and Nelson [8] have shown that Ca^{2+} is continuously expelled from the cells (even when they are not excited) by a temperature-dependent mechanism. Although they could not demonstrate a decrease in Ca^{2+} efflux with the use of metabolic inhibitors, the results suggest that an ATP-driven pump may be involved. The endeavor to identify the elusive Ca^{2+} efflux mechanism has uncovered a number of Ca^{2+}-stimulated ATPase activities in ciliary membranes and the pellicles (the cell body membrane system consisting of the plasma membrane and the tightly apposed membrane bound alveolar sacs) [9–17], none of which display properties common to known Ca^{2+}-pumping ATPases of other cell types (e.g., high Ca^{2+} affinity, nucleotide specificity, selective inhibitor sensitivity) [18]. In this paper we report the characteristics of a (Ca^{2+} + Mg^{2+})-dependent ATPase and an associated Ca^{2+}-dependent phosphoprotein located, in the pellicle membranes. The properties of this ATPase are different from any other Ca^{2+}-ATPase activity reported to date on *Paramecium*. The Ca^{2+}-ATPase's characteristics that implicate a role in Ca^{2+} transport, and its location within the pellicle membrane system will be discussed.

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Materials and Methods

Cell culture. Paramecium tetraurelia (51-s), sensitive to killer, were grown in a culture medium consisting of wheat grass extract supplemented with 1 mg/l stigmasterol, and 0.67 g/l proteose peptone, with $NaPO_4$ and Tris-HCl to buffer pH [19]. The medium was inoculated with *Klebsiela pneumoniae* 24 h before the addition of *Paramecium*, which were cultured at 25°C and harvested in late log phase.

Isolation of pellicle membranes. Pellicles were isolated by methods modified from Belinski et al. [15], and from Doughty and Kaneshiro [9]. Typically cells from 3 l of culture medium were harvested at room temperature by centrifugation at $350 \times g$ for 1 min in oil testing centrifuge tubes using an IEC model HN-II centrifuge. The cells were rinsed twice in ice-cold Dryl's solution [20] to promote trichocyst discharge and remove contaminating bacteria. The fluffy layer, consisting primarily of discharged trichocysts [21], was discarded after each centrifugation. The rinsed cells were resuspended in 15 ml Dryl's solution and placed on ice. To deciliate [21], the cells were mixed with an equal volume of STEN buffer (0.15 M sucrose, 20 mM Tris-HCl, 2 mM EDTA, 6 mM NaCl (pH 7.5)) and left on ice for 10 min. Subsequently, concentrated KCl and $CaCl_2$ were added to a final concentration of 30 mM and 10 mM, respectively. Deciliation usually was complete within 10 min as monitored with a phase contrast microscope. The deciliated cells were collected by centrifugation and rinsed twice in ice-cold HM buffer (20 mM Tris-HCl, 1 mM EDTA, 25 mM KCl, 50 mM sucrose (pH 7.5)). The final cell pellet was homogenized in HM buffer containing proteinase inhibitors (1 mM phenylmethylsulphanyl fluoride, 1 μg/ml pepstatin and 2.2 μg/ml leupeptin) referred to as HMI buffer. The cells were disrupted in a Potter-Elvehjem homogenizer by hand until no intact cells in samples examined by phase microscopy were observed. The homogenate was centrifuged at $1240 \times g$ in a Beckman JA-17 rotor for 5 min. The supernatant was discarded and the pellet resuspended in HMI buffer by vigorously vortexing for 30 s. The cycle of centrifugation and resuspension was repeated until the supernatant became clear, usually four more times. This process helped to remove mitochondria and internal membranes adhering to the pellicles [19]. The final washed pellet was resuspended in HMI and mixed 1 : 1 with 2.1 M sucrose dissolved in HMI. The pellicles were purified further by centrifugation at $1950 \times g$ for 3 h at 4°C and in a Beckman JA-17 rotor through a discontinuous 1.72/2.1 M sucrose gradient dissolved in HMI. The pellicles were collected from the 1.72/2.1 interface, diluted 1 : 1 with storage buffer (20 mM 3-[*N*-morpholino]propanesulfonic acid (Mops)-Tris (pH 7.0), 25 mM KCl, 50 mM sucrose, 0.1 mM EDTA) and centrifuged for 10 min at $6750 \times g$. The pellet was resuspended in an appropriate volume of storage buffer to yield a concentration of 1–2 mg/ml of protein. The membranes were stored at −20°C and in small aliquots until use and the activity remained stable for at least two weeks.

Isolation of an endoplasmic reticulum (ER) enriched fraction. The first supernatant from the homogenate used in the isolation of pellicles was collected and vortexed vigorously for 2 min. The fraction was then centrifuged at $4960 \times g$ for 10 min. The supernatant was collected and centrifuged at $19\,800 \times g$ for 20 min. The resulting pellet was suspended in pellicle storage buffer. Most of the ER marker enzyme, glucose-6-phosphatase, sedimented in this fraction (see Other assays); activity was enriched 6-fold over the homogenate and 34-fold over the isolated pellicles (average of three preparations). Ca^{2+}-ATPase activity (see below) was assayed in the presence of 10 mM sodium azide to inhibit mitochondrial ATPases.

ATPase assay. (Ca^{2+} + Mg^{2+})-dependent ATPase activity was assayed in a medium which typically contained, in a total volume of 0.5 ml, 20 mM Mops-Tris (pH 7.0), 25 mM KCl, 3 mM $MgCl_2$, 1 mM Tris-EGTA, 15–30 μg membrane protein, 2 mM Na- or Tris-ATP (Sigma) and $CaCl_2$ to achieve the desired free Ca^{2+} concentration. Free Ca^{2+} in the assay was calculated by the method of Pershadsingh and McDonald [22] with the aid of a computer program [23]. The assay was initiated after a 5 min preincubation at 30°C and by the addition of ATP and continued for 30–50 min depending on the activity of the membranes. The velocity of the Ca^{2+}-ATPase reaction was linear up to at least 60 min. The reactions were stopped by the addition of 0.5 ml 10% trichloroacetic acid (TCA) and placed on ice. The P_i liberated by the ATPase was measured spectrophotometrically by the method of Sanui [24]. The net Ca^{2+}-stimulated ATPase activity was calculated by subtracting the basal rate of ATP hydrolysis in the presence of Mg^{2+} from the total rate in the presence of Mg^{2+} plus Ca^{2+}.

Ca^{2+} uptake. Ca^{2+} uptake into isolated pellicles was measured at 30°C in a medium which contained in a total volume of 0.5 ml, 25–50 μg pellicle protein, 20 mM Mops-Tris (pH 7.0), 25 mM KCl, 5 mM Tris-oxalate, 3 mM $MgCl_2$, 10 mM sodium azide, 0.3 mM dinitrophenol, 2 mM Na-ATP, 0.4 mM EGTA and enough Ca^{2+} to yield 0.5 μM free Ca^{2+} concentration (with 10 μCi/ml $^{45}Ca^{2+}$). Pellicle membrane was preincubated in the medium for 1 min before uptake was initiated with the addition of ATP. Uptake was quenched at the indicated times by the addition of 0.5 ml of stop buffer (10 mM Mops-Tris (pH 7.0), 4 mM EGTA, 10 mM $MgCl_2$, 150 mM sucrose). The mixture was rapidly filtered through Millipore filters (HAWP, 0.45 μm) and rinsed with 8 ml of wash buffer (10 mM Mops-Tris (pH 7.0), 10 mM $MgCl_2$, 150 mM sucrose).

The dried filters were counted with 4 ml of scintillation fluid in a Beckman LS 6000 scintillation counter. Non-specific Ca^{2+} uptake was determined in samples in which ATP was not added and was subracted from the experimental values.

Phosphoenzyme intermediate analysis. In order to identify the Ca^{2+}-stimulated ATPase, pellicle membranes, ER-enriched fractions or cilia (isolated by the method of Adoutte et al. [21]) were incubated with [γ-^{32}P]ATP under conditions favorable for the formation of a stable phosphorylated enzyme intermediate, as modified from Wuytack et al. [25]. The incubation was performed in microcentrifuge tubes at 0 °C for 30 s. The reaction mixture contained, in a total volume of 0.2 ml, 150 μg protein, 20 mM Mops-Tris (pH 7.0), 25 mM KCl, 25 μM EDTA, 5 μM ATP with 15 μCi/nM [γ-^{32}P]ATP (Amersham) and 1 mM EGTA, 1 mM EGTA plus 3 mM $MgCl_2$, 50 μM Ca^{2+}, or 50 μM Ca^{2+} plus 100 μM La^{3+} as indicated in the legends to the figures. The reaction was started by the addition of ATP to the suspension and terminated with the addition of 0.5 ml ice-cold 10% trichloroacetic acid (TCA) containing 1 mM ATP and 50 mM H_3PO_4. After 10 min on ice the TCA-precipitated proteins were pelleted by centrifugation for 4 min in a Beckman microfuge. The pellets were washed three times by resuspending them in 0.5 ml of the TCA solution followed by centrifugation. The pellets were finally rinsed with 1 ml ice-cold water.

If the labeled proteins were to be tested for hydroxylamine sensitivity the water-rinsed TCA precipitates were suspended in 0.5 ml of either a control solution containing 0.2 M NaOAc plus 150 mM Tris-HCl (pH 6.0) or 0.2 M NaOAc plus 150 mM hydroxylamine (pH 6.0) for 10 min at room temperature [25]. The protein was then precipitated with TCA, pelleted, rinsed with water, and subjected to $NaDodSO_4$ polyacrylamide gel electrophoresis (PAGE) by a technique modified from Weber and Osborn [26].

The pellets were dissolved in 25 μl of sample buffer which contained 50 mM NaH_2PO_4 (pH 6.3), 10% glycerol, 2% $NaDodSO_4$, 5% β-mercaptoethanol and 10 μg/ml pyronin Y [27] and incubated at room temperature for 10 min. Subsequently, 20 μl samples were applied to a 5% polyacrylamide slab gel (10 $cm^2 \times 1.5$ mm) which was buffered with 100 mM NaH_2PO_4 (pH 6.3) and contained 0.1% $NaDodSO_4$. The electrode buffer also contained 100 mM NaH_2PO_4 and 0.1% $NaDodSO_4$ [27]. The gels were run at 20 mA for the first 15 min and then at 100 mA until the dye front had just migrated from the gel. The gels were soaked in a solution of 5% glycerol, 7% acetic acid, and 5% methanol for 15 min and then dried over-night between sheets of cellophane. Autoradiography was performed by exposing the dried gels to Kodak X-OMAT film at −70 °C. The apparent molecular weight of labeled proteins was estimated by comparison with the relative mobilities of Sigma prestained molecular weight standards run simultaneously on each gel.

Other assays. Paramecium plasma membranes do not appear to contain the typical enzymes routinely used for marker enzyme analysis of plasma membranes from multicellular organisms [13]. Therefore, we used the alternative method of morphological analysis [28] by phase microscopy to monitor qualitatively the enrichment or depletion of the pellicle membranes in the different fractions. The marker enzyme assays glucose-6-phosphatase [29] and succinate dehydrogenase [30] were performed at 30 °C using published methods. Protein concentration was determined using Pierce BCA protein assay reagents with bovine serum albumin as the standard.

Results

Isolation of pellicle membranes

Microscopic examination of the pellicle preparations showed sheets of pellicles often with the characteristic pattern typical of the cell surface (Fig. 1). Microscopy and $NaDodSO_4$ PAGE analysis of the pellicles (data not shown) also showed that some trichocyst contamination was unavoidable (Fig. 1, arrow) even though the cells were rinsed in ice-cold Dryl's solution and the discharged trichocysts removed. Isolated trichocysts had no Ca^{2+}-stimulated ATPase activity when assayed under standard conditions, however, and were not a particular concern (data not shown).

An examination of marker enzymes for ER (glucose-6-phosphatase) and mitochondria (succinate dehydrogenase) as well as (Ca^{2+} + Mg^{2+})-ATPase activity (assayed with 3 mM Mg^{2+} and 0.5 μM Ca^{2+}) in various fractions from the purification scheme reveals that the wash cycles and the sucrose gradient were successful in diminishing the content of ER and mitochondria about 5-fold and 1.6-fold in the final pellicle fraction while

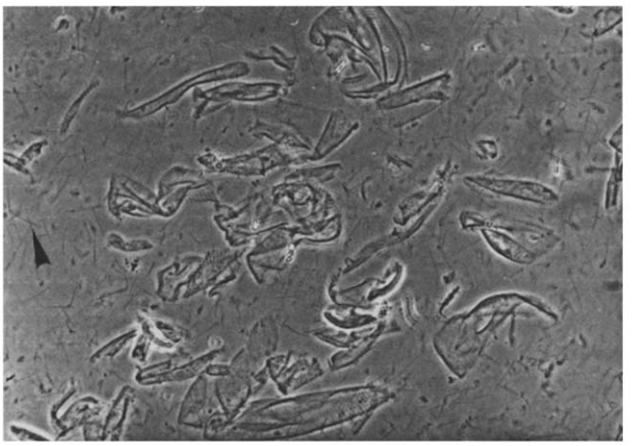

Fig. 1. Phase contrast micrograph of isolated pellicle membranes, magnification 360×. Arrow points to contaminating trichocysts.

TABLE I

Distribution of high affinity (Ca^{2+} + Mg^{2+})-ATPase, glucose-6-phosphatase, and succinate dehydrogenase in various fractions from Paramecium

Data for Ca^{2+}-ATPase and glucose-6-phosphatase are the means from three preparations ±S.E., and for succinate dehydrogenase from two preparations. Specific activity is in units of nmol/mg per min.

Fraction	Ca^{2+}-ATPase [a]		Glucose-6-phosphatase		Succinate dehydrogenase	
	spec. act.	rel. act.	spec. act.	rel. act.	spec. act.	rel. act.
Homogenate	10.0±1.2	1.0	32.9±4.8	1.0	0.17	1.0
Supernatant	17.2±1.7	1.73	56.9±8.8	1.73	0.15	0.84
First pellet	9.3±0.4	0.93	14.2±2.2	0.43	0.19	1.02
Final pellet	13.2±1.0	1.32	6.7±1.1	0.21	0.11	0.62
Pellicles	26.3±6.0	2.64	7.6±1.7	0.23	0.11	0.62

[a] Net Ca^{2+}-ATPase activity was assayed in the standard medium with 0.5 μM free Ca^{2+} and 10 mM sodium azide.

enriching for the Ca^{2+}-ATPase activity 2.6-fold over the homogenate (Table I).

The Ca^{2+}-ATPase activity measured coordinately with the marker enzymes for mitochondria and ER was assayed in the presence of 10 mM sodium azide, and therefore we assume that mitochondrial ATPases were inhibited and do not account for the Ca^{2+}-ATPase activity associated with the various fractions, or the isolated pellicles (see below). While it concerned us that the pellicle associated Ca^{2+}-ATPase activity may be of ER origin, this does not appear to be the case, based on the observation that as the pellicle-containing pellet is subsequently washed, its glucose-6-phosphatase activity decreases and the specific activity of a Ca^{2+}-ATPase increases (Table I). Therefore, at least two Ca^{2+}-ATPases appear to be present, one associated with the ER enriched fraction, and another with the pellicles.

To assess quantitatively the potential for ER-associated Ca^{2+}-ATPase contamination of the pellicles we subfractionated the ER enriched supernatant and obtained a preparation with a 6-fold enrichment in glucose-6-phosphatase activity relative to the homogenate and 34-fold higher activity compared to the corresponding pellicle fraction (average of three preparations). As expected the ER fraction possessed high Mg^{2+}-dependent Ca^{2+}-stimulated ATPase activity, averaging 54 nmol/mg per min. (Ca^{2+}-ATPase activity was assayed with 10 mM sodium azide to inhibit mitochondrial ATPases.) Based on the 34-fold difference in the amount of glucose-6-phosphatase activity in the ER-enriched fraction and the pellicle fraction, we calculate that the average contribution of ER-associated Ca^{2+}-ATPase activity to the pellicle Ca^{2+}-ATPase activity is 3.9% (n = 3), and in any one preparation the contamination is never more than 10%.

Ca^{2+} dependence

The pellicle membranes contain a basal Mg^{2+}-dependent ATPase activity and an additional activity stimulated by low concentrations of Ca^{2+}. When assayed with 3 mM Mg^{2+}, the activity has an apparent K_m of 90 nM for Ca^{2+} and a V_{max} of approx. 24 nmol/mg per min (Fig. 2) and the basal rate of ATP hydrolysis was stimulated 50–100% in various preparations by an optimal concentration of Ca^{2+}. Saturation of the Ca^{2+}-stimulated activity occurred consistently below 0.5 μM free Ca^{2+}, and no further stimulation was observed up to 10 μM Ca^{2+} (data not shown).

Mg^{2+} dependence

The Mg^{2+} dependent component of the (Ca^{2+} + Mg^{2+})-ATPase activity increased steadily as Mg^{2+} was added to the assay over the range of 0–3 mM Mg^{2+}. At 3 mM, maximum Mg^{2+}-dependent activity was observed and remained constant up to 8 mM Mg^{2+} (data not shown). The stimulation of the (Ca^{2+} + Mg^{2+})-ATPase activity by an optimal concentration of Ca^{2+} (1 μM) was dependent upon the concentration of Mg^{2+} in the assay, but not in a simple relationship (Fig. 3). In the absence of exogenous Mg^{2+} some Ca^{2+} stimulation of ATPase activity occurred, and as low concentrations

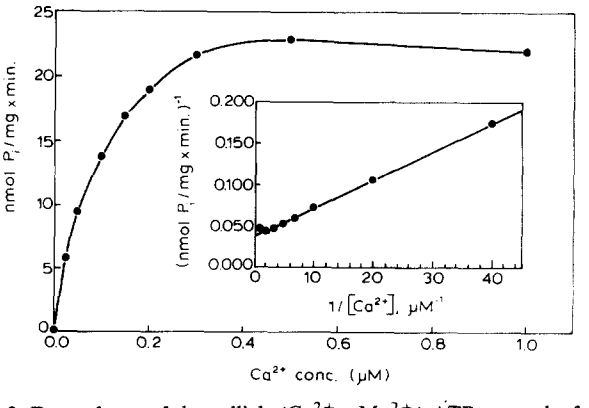

Fig. 2. Dependence of the pellicle (Ca^{2+} + Mg^{2+})-ATPase on the free Ca^{2+} concentration. Net Ca^{2+} activation of the (Ca^{2+} + Mg^{2+})-ATPase was assayed in the standard medium containing 1 mM EGTA plus an appropriate amount of Ca^{2+} to achieve the desired free concentration. The data points represent the means of four or five experiments conducted in duplicate. Inset: double-reciprocal plot; equation for the least-squares line is $y = 0.0377 + 0.0034(x)$.

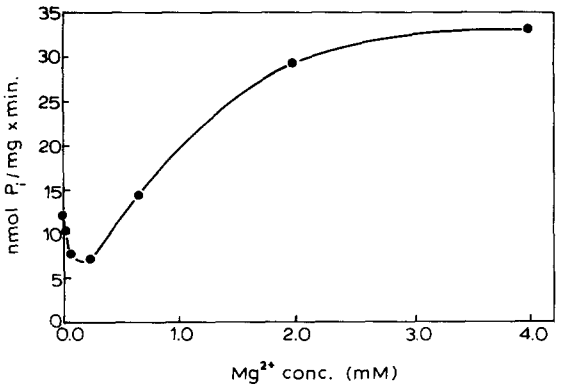

Fig. 3. Dependence of the pellicle ($Ca^{2+} + Mg^{2+}$)-ATPase on the concentration of total added Mg^{2+}. Net stimulation of the ($Ca^{2+} + Mg^{2+}$)-ATPase by 0.5 μM free Ca^{2+} was determined in the standard medium with variable total Mg^{2+} added to the assay. The data points are means from a representative experiment performed in duplicate. Similar results were obtained in two other experiments.

of Mg^{2+} were added to the assay (13–215 μM), the Ca^{2+}-stimulated component of the ($Ca^{2+} + Mg^{2+}$)-ATPase activity decreased. Upon addition of still higher concentrations of Mg^{2+}, the Ca^{2+}-stimulated rate of ATP hydrolysis eventually increased to about 200% of the rate in the absence of exogenous Mg^{2+}; maximum Ca^{2+} activation of the ATPase occurred with about 3 mM Mg^{2+} present in the assay.

pH dependence

The Mg^{2+}-dependent and the Ca^{2+}-stimulated component of the total ATPase activity exhibited entirely different pH dependencies. The Mg^{2+}-dependent activity slowly increased over the entire range of pH 6–8, showing no defined optimum (data not shown). On the other hand, the Ca^{2+}-stimulated activity had a distinct pH optimum between pH 6.9–7.0, while decreasing to 43% of maximal at pH 6 and 20% of maximal at pH 8 (Fig. 4).

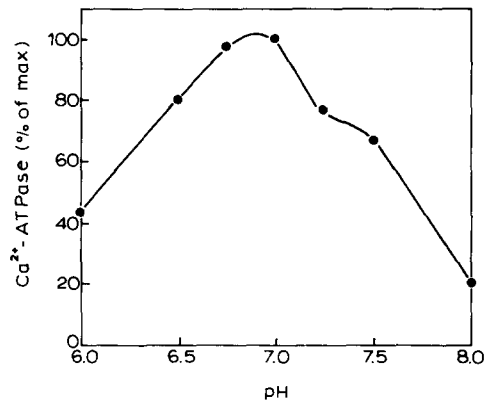

Fig. 4. Dependence of the pellicle ($Ca^{2+} + Mg^{2+}$)-ATPase on pH. Ca^{2+}-ATPase activity was assayed as in Materials and Methods except, Tris-maleate (50 mM) was adjusted to the indicated pH; EGTA was omitted from the Ca^{2+}-stimulated, Mg^{2+}-dependent conditions and total Ca^{2+} was 10 μM. EGTA (1 mM) was present to determine the basal Mg^{2+}-dependent rate. Data points are the mean of three experiments conducted in duplicate.

Substrate specificity and ATP kinetics

In the presence of 3 mM Mg^{2+} the Ca^{2+}-stimulated ATPase (1 μM free Ca^{2+}) exhibited strong selectivity for ATP as its substrate, hydrolysing GTP, CTP, UTP and ITP at less than 10% the rate of ATP (7.4%, 8.8%, 1.8% and 3.9%, respectively; mean of three experiments in duplicate). However, if no Mg^{2+} were added to the reaction medium the Ca^{2+}-ATPase activity which remained showed no specificity for ATP over the other nucleotides tested. In fact, GTP and CTP were hydrolysed at over 200% the rate of ATP. Similarly, the basal, Mg^{2+}-dependent activity was nonspecific in selection of nucleoside triphosphates for hydrolysis (data not shown).

A study of the concentration dependence of the Ca^{2+}-ATPase for ATP with 3 mM Mg^{2+} present reveals an apparent K_m of 75 μM when the data are transformed to a Lineweaver-Burk plot (data not shown). Considering the large and variable Mg^{2+}-dependent ATPase activity present in different membrane preparations, 2 mM ATP was routinely used in the assays to insure that substrate was depleted by less than 10% in any particular experiment.

Effect of various agents and inhibitors

We examined the effect of several agents on the pellicle Ca^{2+}-ATPase to allow comparisons with other well characterized Ca^{2+}-transporting activities (Table II). The inclusion of 25 mM NaCl or KCl in the assay medium enhanced the rate of Ca^{2+}-stimulated ATP hydrolysis by about 30%. LiCl (25 mM) did not substitute for Na^+ or K^+ in this stimulatory effect. Ouabain, a specific inhibitor of the Na^+/K^+-ATPase, had no effect on the Ca^{2+}-ATPase activity under standard assay conditions (25 mM KCl and 2 mM Na_2ATP).

Two commonly used mitochondrial ATPase inhibitors were tested to assess the possibility that the Ca^{2+}-

TABLE II

Effect of various agents on the pellicle ($Ca^{2+} + Mg^{2+}$)-ATPase

Net Ca^{2+}-ATPase activity was assayed in the standard medium with 0.5 μM free Ca^{2+} ± the indicated agent. When various salts were assayed Tris-ATP was used and KCl was omitted from the system unless it was being tested. Data are the means from three or four experiments performed in duplicate ± S.E.

Addition	% Control activity
Control	100
25 mM NaCl	128 ±10.2
25 mM KCl	133 ±15.8
25 mM LiCl	96 ±24.5
200 μM oubain	98.5 ± 3.3
10 mM sodium azide	106.8 ± 4.7
10 μg/ml oligomycin	176.1 ± 16.3
2 μM vanadate	41.4 ± 3.9
0.5 μM calmidazolium	51.0 ± 7.9
10 μM trifluoperazine	21.4 ± 2.3

ATPase could be of mitochondrial origin. Sodium azide (10 mM) had no effect on the Ca^{2+}-ATPase activity, while oligomycin, a highly specific mitochondrial ATPase inhibitor, actually stimulated the Ca^{2+}-dependent rate of ATP hydrolysis by about 76%. The magnitude of stimulation varied widely between experiments. Although the effect of oligomycin is unusual, we believe the data on both mitochondrial inhibitors, taken together, indicate that the Ca^{2+}-ATPase is not mitochondrial in origin.

Vanadate inhibits the activity of Ca^{2+}-transporting ATPases, as well as other ion transporting ATPases and dynein ATPases [31]. The efficacy of inhibition, however, is modulated by the concentration of Mg^{2+} and K^+ present in the assay [32]. In our system, using 3 mM Mg^{2+} and 25 mM K^+, vanadate completely inhibited the Ca^{2+}-ATPase with an EC_{50} of about 2 μM, a value similar to that reported for the erythrocyte plasma membrane Ca^{2+}-ATPase [32].

The majority of plasma membrane associated Ca^{2+} pumps, although not all, are calmodulin regulated [18]. Calmidazolium, reported to be a relatively specific calmodulin antagonist [33], inhibited the Ca^{2+}-stimulated activity with an EC_{50} of 0.5 μM. This concentration is below the range at which nonspecific inhibition is reported for calmidazolium in other systems [33], consistent with a role for calmodulin regulation of the pellicle Ca^{2+}-ATPase. Likewise, 10 μM trifluoperazine inhibited approx. 80% of the Ca^{2+}-stimulated ATPase activity. However, direct addition of bovine brain calmodulin to the assay system had no effect on the ATPase activity as measured under standard conditions.

Phosphoenzyme intermediate formation

If the Ca^{2+}-ATPase activity of the pellicle were in whole or part the activity of a Ca^{2+} pump it should be possible to identify its phosphoprotein intermediate [34]. Although Ca^{2+} and Mg^{2+} are required for the complete Ca^{2+}-ATPase reaction cycle the initial phosphorylation step requires only Ca^{2+}. Therefore, we ommitted Mg^{2+} from the Ca^{2+}-stimulated phosphorylation conditions because it promotes high background kinase activity in the pellicles. Pellicle membranes were incubated with [γ-^{32}P]ATP and 50 μM Ca^{2+}, or Mg^{2+}, or no divalent cation at 0 °C for 30 s followed by TCA precipitation of proteins, their separation by pH 6.3 NaDodSO$_4$-PAGE,

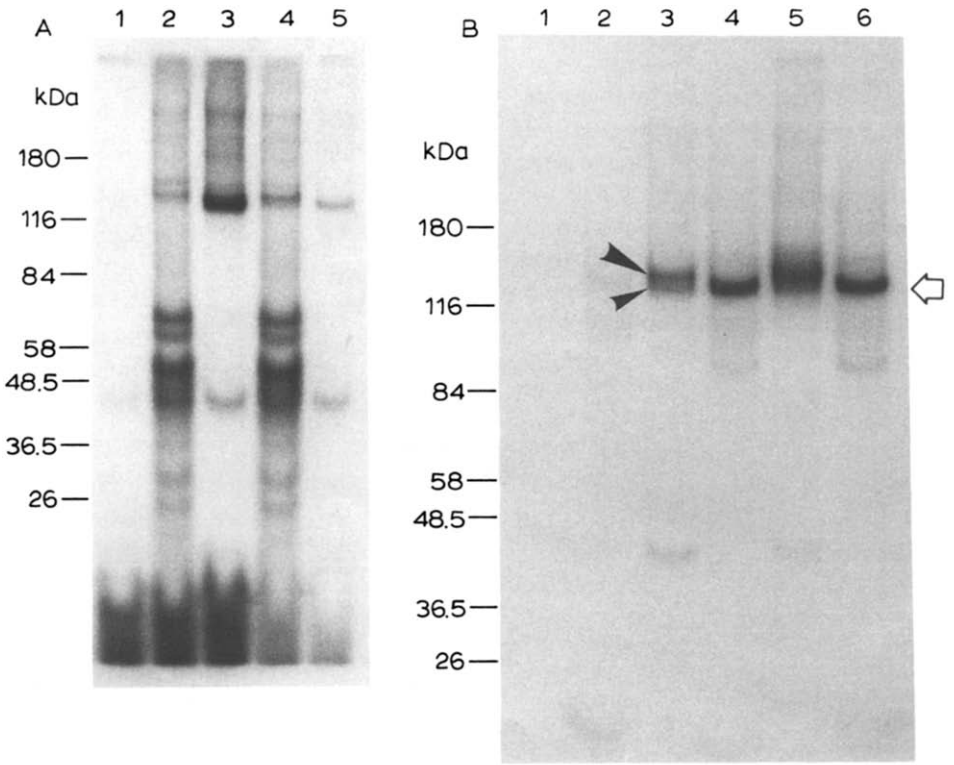

Fig. 5. Analysis of phosphoenzyme intermediate formation in isolated pellicles and an ER-enriched fraction. (A) Pellicles were incubated with [γ-^{32}P]ATP, as described in Materials and Methods, with the indicated additions to the medium: 1 mM EGTA, lane 1; 3 mM Mg^{2+}, lanes 2 and 4; 50 μM Ca^{2+}, lanes 3 and 5. TCA-precipitated proteins were then treated with a control solution, lanes 1–3, or 150 mM hydroxylamine (pH 6), lanes 4 and 5, for 10 min at room temperature, before subjecting to SDS-PAGE. (B) Pellicles, lanes 1, 3, and 5 or an ER-enriched fraction, lanes 2, 4, and 6 were incubated with [γ-^{32}P]ATP as in Materials and Methods with the indicated additions to the medium, TCA-precipitated, and then subjected to SDS-PAGE. Additions: 1 mM EGTA, lanes 1 and 2; 50 μM Ca^{2+}, lanes 3 and 4; 50 μM Ca^{2+} plus 100 μM La^{3+}, lanes 5 and 6. Large arrow: predominent pellicle-associated Ca^{2+}-dependent phosphoprotein; smaller arrow: minor pellicle-associated band, often unresolved; open arrow: ER-associated Ca^{2+}-dependent phosphoprotein.

and autoradiography. Virtually no proteins were phosphorylated in the absence of added divalent cations (Fig. 5A, lane 1), while several proteins were labeled in the presence of 3 mM Mg^{2+} (lane 2); but as discussed below, these labeled proteins are most likely kinase substrates. In incubations with Ca^{2+} one prominent band of approx. 133 kDa was labeled as well as a few faint bands of higher and lower molecular mass (Fig. 5A, lane 3). In some gels of higher resolution a second band, whose labeling is Ca^{2+}-stimulated, appears just below the predominent 133 kDa band (Fig. 5B, lane 3, small arrow). These bands around 133 kDa are of the molecular mass expected of phosphenzyme intermediate candidates and were examined further.

The acyl bond of a phosphoenzyme intermediate is hydroxylamine or base labile [25]. Therefore, to distinguish between phosphoenzyme intermediates and protein kinase substrates, we examined the sensitivity of the ^{32}P labeling to hydroxylamine [25]. Pellicle membranes were incubated as above with the addition of either Mg^{2+} or Ca^{2+} in the medium. After TCA precipitation, the proteins were treated with 150 mM hydroxylamine and 0.2 M NaOAc, pH 6 (Fig. 5A, lanes 1–3), or a control solution 150 mM Tris-HCl and 0.2 M NaOAc, pH 6, (Fig. 5A lanes 4 and 5) for 10 min at room temperature, prior to analysis by PAGE. The protein labeling pattern under the Mg^{2+}- stimulated conditions was unchanged with hydroxylamine treatment (Fig. 5A, compare lanes 2 and 4), indicating that all labeled proteins were the result of kinase activity [25]. However, the 133 kDa protein which becomes phosphorylated in the presence of Ca^{2+} lost most of its label when exposed to hydroxylamine (Fig. 5A, compare lanes 3 and 5). These data suggest that the Ca^{2+}-specific, hydroxylamine-sensitive 133 kDa phosphoprotein is the phosphoenzyme intermediate of the (Ca^{2+} + Mg^{2+})-ATPase. Furthermore, the 133 kDa apparent molecular mass is comparable to that of calmodulin regulated, plasma membrane associated Ca^{2+} pumps [18].

Ca^{2+}-pumping ATPase activity has been identified in the ER of the related ciliate *Tetrahymena* [35]. Therefore, we have considered the possibility that the phosphoprotein we have tentatively identified as a pellicle associated Ca^{2+}-ATPase could be a minor contaminant from the ER. To assess this possibility, we compared the formation of phosphoprotein intermediates in the pellicle fraction (Fig. 5B, lanes 1, 3 and 5) with a fraction that contained a 34-fold greater content of the ER marker enzyme glucose-6-phosphatase (Fig. 5B, lanes 2, 4 and 6). The phosphorylations were carried out as above in the presence of Ca^{2+} (Fig. 5B, lanes 3 and 4) or Ca^{2+} plus 100 μM La^{3+} (Fig. 5B, lanes 5 and 6). La^{3+} can be used as an agent to distinguish between plasma membrane associated Ca^{2+}-ATPases and those of the sarcoplasmic reticulum or ER based on the observation that La^{3+} will stimulate the level of phosphorylated intermediate formed in plasma membrane-associated Ca^{2+} pumps but not in sarcoplasmic reticulum- or ER-derived pumps [25,36]. Under the Ca^{2+} plus La^{3+} conditions, the 133 kDa band in the pellicle fraction increases in intensity, (Fig. 5B, lanes 3 vs. 5, large arrow) while the slightly lower molecular mass band in the ER-enriched fraction remains the same (Fig. 5B, lanes 4 vs. 6, open arrow). The subtle difference in molecular mass and the distinct difference in response to La^{3+} between the pellicle- and ER-associated phosphoproteins clearly demonstrates that the major Ca^{2+}-dependent phosphoprotein found in pellicles is not due to ER contamination.

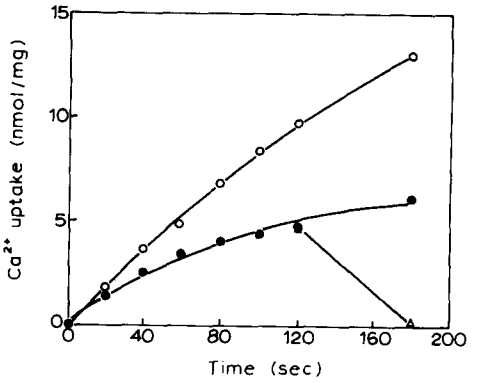

Fig. 6. Ca^{2+} uptake by pellicle membranes. Ca^{2+} uptake was measured as described in Materials and Methods. ●— — —●, standard medium; ○— — —○, standard medium containing 5 mM Tris-oxalate; △, 10 μM A23187 in 2.5 μl DMSO was added at $t = 120$ s. Data points are the mean of three experiments conducted in duplicate.

Ca^{2+} uptake

Pellicles are complex structures that are composed of a plasma membrane and a tightly apposed system of membranous sacs (alveoli) [37]. As prepared by our technique, the pellicles do not reseal to form compartments or artificial vesicles. Nevertheless, we have measured ATP-dependent Ca^{2+} uptake into the pellicle preparations (Fig. 6). The Ca^{2+} that is taken up is released by the Ca^{2+} ionophore A23187 indicating that the Ca^{2+} has been sequestered in a membranous compartment and is not externally bound. Therefore, the site of Ca^{2+} sequestration is likely to be the alveolar sacs. It should be noted that Stelly et al. [38] have previously reported Ca^{2+} uptake into the alveolar system. The rate of Ca^{2+} uptake is stimulated by oxalate to about 5 nmol/mg per min. Assuming a coupling ratio of 2 Ca^{2+} transported per ATP hydrolysed [39], less than 10% of the total measured Ca^{2+}-ATPase activity is required, on the average, to account for this rate of Ca^{2+} uptake.

To compare Ca^{2+} uptake and Ca^{2+}-ATPase activity we examined their vanadate sensitivities (Fig. 7).

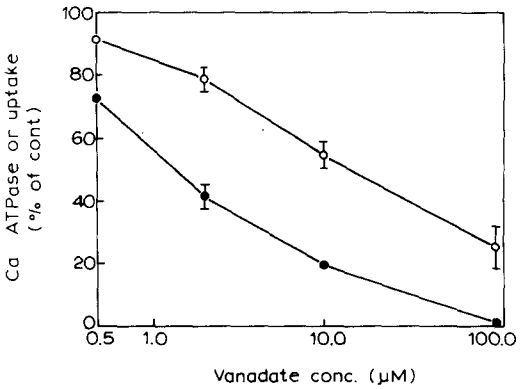

Fig. 7. Vanadate sensitivity of the pellicle Ca^{2+}-ATPase and Ca^{2+} uptake. Ca^{2+}-ATPase and Ca^{2+} uptake were assayed as described in Materials and Methods. Pellicles were preincubated in assay medium with and without vanadate and the reaction was started with the addition of ATP. Free Ca^{2+} was 0.5 μM in both systems. The rate of Ca^{2+} uptake was determined in the presence of 5 mM oxalate over the first min. All values are expressed as the percent activity remaining with the indicated concentration of vanadate compared to control activity. o — — — o, Ca^{2+} uptake; ● — — — ●, Ca^{2+}-ATPase. Data points are the mean of three experiments performed in duplicate ± S.E.

Vanadate inhibited Ca^{2+}-ATPase activity and Ca^{2+}-uptake with an EC_{50} of about 2 μM and 20 μM, respectively. Therefore, it appears that the Ca^{2+} uptake we observe is catalysed by an ATPase that is different from the major pellicle Ca^{2+}-ATPase activity we have characterized.

Discussion

Ca^{2+} pumps have been identified in isolated membranes of many other cell types by: the direct measurement of ATP-dependent Ca^{2+} transport [18]; measurement of the pump's Ca^{2+}-stimulated ATPase activity [18]; and specific Ca^{2+}-dependent phosphorylation of the pump intermediate [25,40]. In *Paramecium* the plasma membrane of the cell body is lined internally by a tightly applied system of membranous sacs (alveoli) [37]. Consequently, the isolated pellicles (plasma membrane plus alveoli) do not readily reseal or form artificial vesicles that can be used to measure Ca^{2+} transport by a plasma membrane Ca^{2+} pump, if one exists. Therefore, in the search for a pellicle Ca^{2+} pump we have relied upon the latter two techniques.

In this report we present the characteristics of a pellicle Ca^{2+}-ATPase activity and an associated Ca^{2+}-dependent phosphoprotein that are entirely consistent with the generalized properties of plasma membrane Ca^{2+} pumps as summarized by Rega and Garrahan [18]. These properties include: (1) high affinity for Ca^{2+}, (2) a requirement for exogenous Mg^{2+}, (3) specificity for ATP as substrate, (4) vanadate sensitivity, (5) potential calmodulin regulation and (6) a corresponding Ca^{2+}-dependent, hydroxylamine-labile phosphorylation of a pellicle protein of appropriate molecular mass. The combined weight of the data strongly suggests that we have identified a Ca^{2+}-pumping ATPase. However, considering the large body of research devoted to the identification of Ca^{2+}-pumping ATPases in *Paramecium*, it seems important to this current study to examine the previous ones.

Comparison with previous studies. Because of the role of Ca^{2+} in the regulation of ciliary beating, the cilia have been the primary focus of many studies for the identification of Ca^{2+}-homeostatic ATPases. The ciliary membrane associated Ca^{2+}-ATPase activities identified by numerous groups are of low Ca^{2+} affinity, and/or nonspecific in nucleotide selection [9,11,13,16,17]. Likewise, Noguchi et al. [14] and Belinski et al. [15] studied isolated pellicles, and they identified Ca^{2+}-ATPase activity that is of low Ca^{2+} affinity and nonspecific in nucleotide hydrolysis, clearly differing from the activity we describe here and other known Ca^{2+} pumps.

Perhaps the most significant difference in these studies compared to ours is the use of exogenous Mg^{2+} in our assay conditions. High-affinity Ca^{2+}-ATPase activities have been identified in the absence of exogenous Mg^{2+} in the plasma membranes of liver [41], corpus luteum [42], neutrophils [43] and the basolateral membranes of kidney [44]; and originally it was believed that the Ca^{2+}-ATPase activity represented the expression of a Ca^{2+} pump. However, the ATPases do not reflect the strict nucleotide requirement for ATP of Ca^{2+} transport [45,46,47], and plasma membrane Ca^{2+} transport in the same systems (and in most, if not all, other cell types studied) requires the addition of Mg^{2+} and is highly specific for ATP as an energy source. Thus, it has been suggested that Ca^{2+}-ATPase activities that are not characterized in the presence of exogenous Mg^{2+} may not represent Ca^{2+}-pumping activities [44,46].

To compare our results with the *Paramecium* studies cited above and with the studies on neutrophils [45] and kidney basolateral membranes [44] we examined the nucleotide specificity of the pellicle activity with and without 3 mM exogenous Mg^{2+}. Whereas the Ca^{2+}-stimulated activity was very selective for ATP in the presence of 3 mM Mg^{2+} (Table II), in the absence of exogenous Mg^{2+}, a pellicle-associated activity hydrolysed all of the nucleotides tested at a rate equal to or greater than ATP. For example, GTP was hydrolysed at a rate of 212% of ATP. Similarly, Doughty and Kaneshiro [17] observed a comparable rate of GTP hydrolysis (183% of ATP) in their pellicle preparation. Therefore, these data support the hypothesis that the most critical factor in the identification of this novel, ATP specific, Ca^{2+}-stimulated activity is the addition of Mg^{2+} to the assay.

ER and cilia as possible sources of activity. Travis and Nelson [48] have reported the existence of non-membrane bound Mg^{2+}-dependent, Ca^{2+}-activated ATPase activity in *Paramecium* cilia (axonemal dyneins) and

Muto and Nozowa [35] have identified a (Mg^{2+} + Ca^{2+})-ATPase activity in the ER of the ciliate *Tetrahymena*. Given the similarity in some of the properties of these ATPases with the pellicle (Ca^{2+} + Mg^{2+})-ATPase we felt it was important to determine whether the pellicle ATPase activity could be attributed to contamination by cilia or ER. As described in results, we have found that ER enriched fractions possess Mg^{2+}-dependent, high-affinity Ca^{2+}-stimulated ATPase activity, but based on the specific activity of the Ca^{2+}-ATPase and glucose-6-phosphatase only about 4% of the pellicle associated Ca^{2+}-ATPase activity could be due to endoplasmic reticulum contamination.

At the free Ca^{2+} concentrations used in our assays (< 1 μM) the predominent Ca^{2+}-stimulated ATPase active in cilia are dyneins [48]; but it was more difficult to quantitatively determine the level of contamination by dyneins because cilia do not possess a distinctive marker enzyme. However, we feel we can eliminate ciliary dyneins as a contaminant primarily for two reasons. First, dyneins from many systems, including *Paramecium* have a broad pH dependence and an optimal ATPase activity at pH 7.5 to 9.0 [49,50,51,52]. The pellicle Ca^{2+}-ATPase exhibits a marked pH optimum around 7.0 (Fig. 4), with the activity dropping to 20% of maximal at pH 8.0. Thus, if the pellicle Ca^{2+}-ATPase activity were due largely to contamination by ciliary dyneins we would expect to see a significantly greater activity at pH 7.5 and above. A second distingishing characteristic was substrate specificity. When pellicle was assayed in the absence of exogenous Mg^{2+}, a Ca^{2+}-ATPase hydrolysed GTP at a rate of 212% of ATP ($n = 3$), while cilia (assayed under identical conditions) hydrolysed GTP at 89% of ATP ($n = 2$).

Evidence for plasma membrane pump. If the pellicle-associated Ca^{2+}-ATPase is in fact a pump it should be possible to identify the protein by covalently labelling the phosphoenzyme intermediate with [γ-^{32}P]ATP [34]. To our knowledge, all Ca^{2+} pumps that have been isolated to date are proteins that range in apparent molecular mass from 100 to 150 kDa and form Ca^{2+}-dependent, hydroxylamine-labile, acyl phosphoprotein bonds during the reaction cycle [34], and no other proteins that mimic this set of characteristics have been identified. These observations have provided the means to identify Ca^{2+}-pumping ATPases in many complex cellular systems [25,36,40]. Phosphorylation of the pellicle membranes using established methods [25] has revealed the existence of a Ca^{2+}-dependent, hydroxylamine-labile phosphoprotein of approx. 133 kDa that we assume represents a Ca^{2+} pump.

The presence of a Ca^{2+}-dependent phosphoprotein in the pellicles, however, is only correlative evidence that the activity we have characterized is the biochemical expression of the Ca^{2+} pump (phosphoprotein). In other systems [46,53,54], it has been difficult or impossible to prove the link between Ca^{2+}-ATPase activity (or Ca^{2+} uptake) and the associated phosphoprotein without purification of the enzyme because Ca^{2+}-ATPase activity and phosphoenzyme formation must be assayed under different conditions, and specific agents that affect Ca^{2+}-pumping ATPase activities may not have a consistent or predictable effect on the steady-state level of the phosphoenzyme intermediate [46,55]. At this time, short of purifying the Ca^{2+}-ATPase to prove the relationship between the pellicle Ca^{2+}-ATPase activity and the associated phosphoprotein, we have attempted to rule out any alternative explanations for the observed association. We have shown above that the pellicle-associated Ca^{2+}-ATPase activity is not likely to be accounted for by contamination from ER or ciliary dyneins and therefore, the activity is likely to be of pellicle origin. Given this, the most plausible explanation for a mistaken association between the pellicle Ca^{2+}-ATPase and the phosphoprotein would be that the phosphoprotein is derived from a minor contaminating source that may have Ca^{2+}-pumping ATPase activity (i.e., ER or cilia). Comparison of phosphoenzyme formation in pellicles and an ER-enriched fraction shows that both fractions contain Ca^{2+}-dependent phosphoproteins of close but different molecular mass (Fig. 5B, lane 3 vs. 4). We also utilized La^{3+} to help discriminate between phosphoenzyme intermediates of the two fractions based on the level of phosphorylation. It has been shown that La^{3+} stimulates the level of phosphoenzyme intermediate in plasma membrane Ca^{2+} pumps of erythrocytes [56] and kidney [40] but it has no effect or a slight inhibitory effect on phosphoenzyme levels of ER- or sarcoplasmic reticulum-type Ca^{2+} pumps [36,40]. Consistent with these reports, we found that the ER-associated phosphorylated band did not increase in intensity (Fig. 5B, lanes 4 vs. 6), whereas the major pellicle band did increase when La^{3+} was present (Fig. 5B, lane 3 vs. 5). Thus, the data show a clear difference between the ER and major pellicle associated phosphointermediates, and suggest, by consistent analogy with the above studies, that the major pellicle phosphoenzyme intermediate is similar to plasma membrane-associated Ca^{2+} pumps.

Likewise, cilia were examined for Ca^{2+}-dependent phosphoenzyme intermediate formation. In two experiments, proteins comparable to those present in pellicle or ER were either absent or poorly labeled in isolated cilia (data not shown). The phosphoprotein that was identified may represent low levels of a ciliary Ca^{2+}-ATPase or it could be contamination from pellicles. In either case, the potential for minor contamination of the pellicles by cilia cannot account for the heavily labeled phosphoprotein in pellicles.

The most logical conclusion, at this time, is that the majority of the pellicle Ca^{2+}-ATPase activity is the biochemical expression of the major pellicle-associated, Ca^{2+}-dependent phosphoenzyme. However, the precise

location of the major Ca^{2+}-stimulated activity is uncertain. Stelly et al. [38] have measured ATP and Mg^{2+}-dependent Ca^{2+} uptake by pellicle membranes, presumably into the alveoli. We also have measured a similar Ca^{2+} uptake into pellicles, however, based on differential vanadate sensitivity (Fig. 7), it appears that about 10% of the total Ca^{2+}-ATPase activity may be derived from the alveolar uptake system and the remaining activity is from a separate enzyme. Thus, by a process of elimination, and by a consistent likeness of the Ca^{2+}-dependent ATPase activity and phosphoprotein to plasma membrane Ca^{2+} pumps of other cell types we tentatively conclude that the majority of the pellicle Ca^{2+}-ATPase activity is located in the plasma membrane.

Similar to our findings, intracellular Ca^{2+} pumps of ER [35,57] and sarcoplasmic reticulum [58,59] often are more resistant to vanadate inhibition than those of plasma membrane [32,60]. If the alveolar uptake system were analogous to other intracellular Ca^{2+} pumps, and our conclusions were correct, it should be possible to identify two different Ca^{2+}-ATPase moieties in the pellicles, one of ER type (alveolar) and one plasma membrane type. As noted in the phosphorylation studies, in some autoradiographs we can resolve two very closely spaced bands in the pellicle fraction (Fig. 5B, lane 3, small arrow) and the lower band co-migrates with the band labeled in the ER fraction (Fig. 5B, lane 4). At this time, however, we cannot determine whether the minor phosphoprotein band from pellicles represents a proteolysis product of the major band, an ER derived Ca^{2+} pump or an alveolar Ca^{2+} pump that is similar to the ER pump. Obviously, much work is needed to elucidate fully the mechanisms of Ca^{2+} homeostasis in *Paramecium*.

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