Cortical Alveoli of *Paramecium*: a Vast Submembranous Calcium Storage Compartment

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Abstract. The plasma membrane of *Paramecium* is underlain by a continuous layer of membrane vesicles known as cortical alveoli, whose function was unknown but whose organization had suggested some resemblance with muscle sarcoplasmic reticulum. The occurrence of antimonate precipitates within the alveoli first indicated to us that they may indeed correspond to a vast calcium storage site. To analyze the possible involvement of this compartment in calcium sequestration more directly, we have developed a new fractionation method, involving a Percoll gradient, that allows rapid purification of the surface layer (cortex) of *Paramecium* in good yield and purity and in which the alveoli retain their in vivo topological orientation. This fraction pumped calcium very actively in a closed membrane compartment, with strict dependence on ATP and Mg^{2+}. The pumping activity was affected by anti-calmodulin drugs but no Triton-soluble calmodulin binding protein could be identified, using gel overlay procedures. The high affinity of the pump for calcium (K_m = 0.5 μM) suggests that it plays an important role in the normal physiological environment of the cytosol. This may be related to at least three calcium-regulated processes that take place in the immediate vicinity of alveoli: trichocyst exocytosis, ciliary beating and cytoskeletal elements dynamics during division.

THE cell membrane of *Paramecium*, as that of most ciliates (Allen, 1971; de Puytorac, 1984), is underlain by a vast network of membrane vesicles known as cortical alveoli (Fig. 1). The ultrastructural analysis of Allen (1971) in *Paramecium* and of Satir and Wissig (1982) in *Tetrahymena* has shown that the alveoli are in fact interconnected, making up a continuous network that is only interrupted at the points of emergence of cilia and those of implantation of secretory vesicles. The alveoli thus appear to constitute an insulating layer between the cell membrane and the cytoplasm. The biological function of this layer has, however, remained unknown. On the basis of their morphology, especially their close apposition to the plasma membrane, Allen and Eckert (1969) and later Satir and Wissig (1982) suggested that the alveoli constitute a calcium compartment that resembles muscle sarcoplasmic reticulum. Indeed, the cortex of *Paramecium* contains three major organellar systems which are known to be regulated by calcium: the several thousand cilia which are covered by the plasma membrane, the approximately thousand exocytotic membrane vesicles known as trichocysts, which are attached just beneath the plasma membrane and several cytoskeletal networks which underly the cell surface (see Fig. 1, *a* and *b*). The involvement of calcium has been best characterized in ciliary beating. Both orientation of the power stroke and frequency of beating are controlled by the concentration of calcium within the intraciliary space (Eckert and Brehm, 1979; Kung and Saimi, 1982; Bonini et al., 1989). The penetration of calcium within the ciliary lumen has been intensively studied and is known to depend on the presence of a number of ion channels in the ciliary membrane, the best studied being the fast voltage-sensitive calcium channel (see Ramanathan et al., 1988 and Machemer, 1988 *a*, *b* for reviews). The return to the very low intracellular calcium concentration ($<10^{-7}$ M) that characterizes resting cells (Kung and Saimi, 1982), after the surge due to activation of the ciliary channels, is thought to be dependent on a temperature-sensitive Ca pump (Browning and Nelson, 1976). However, the location of the corresponding ATPase and the indentification of the path taken by calcium ions have remained elusive in spite of the fact that several Ca-ATPases have been identified in cortex and cilia (Bilinski et al., 1981; Riddle et al., 1982; Andrivon et al., 1983; Doughty and Kaneshiro, 1983; Travis and Nelson, 1986; Levin et al., 1989). In addition to a Ca pump embedded in the ciliary membrane, the possible occurrence of a calcium sequestering device at the base of the cilium is conceivable (Schultz and Klumpp, 1988; Bonini et al., 1989). Trichocyst extrusion is also known to be strictly dependent on the presence of exogenous calcium (Plattner et al., 1985; Satir, 1989), and has recently been demonstrated to be associated with a calcium influx (Kerboeuf and Cohen, 1990), but an additional intracellular store may also be involved (see

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Adoutte, 1988 for review). Here again, it must be assumed that calcium concentration is tightly controlled in the vicinity of the organelles to prevent their erratic firing; a nearby membranous compartment could play this important role and could also deliver at least part of the calcium trigger upon proper stimulation. It is worth pointing out in this context that alveolar membranes are very tightly apposed to those of trichocysts (Plattner et al., 1973). Finally, calcium is known to play a role in the dynamics of some of the classical cytoskeletal fibers of higher eukaryotes (microtubules, microfilaments). Microtubules as well as several other distinct cytoskeletal networks (Allen, 1971; Cohen and Beisson, 1988) are abundant in the cortex and, in addition, one of its major nonactin filamentous network, the infraciliary lattice, has recently been shown to be contractile and to be made of one predominant calcium binding protein (Garreau de Loubresse et al., 1988). Several of these subcortical cytoskeletal networks undergo extensive disassembly followed by reassembly during the morphogenetic processes of binary fission (Iftode et al., 1989). Fine tuning of calcium concentration in the vicinity of the cortex is therefore probably also required to regulate this third category of cellular events.

In this paper, we describe a direct test of the calcium sequestering function of alveoli. This was made possible by the development of a fractionation method using Percoll gradients enabling the isolation, in good yield, of a very pure cortical fraction, devoid of cilia. Mechanical homogenization of paramecia under mild conditions yields large cortical fragments containing the alveoli still attached to the plasma membrane. Cilia, which would constitute a serious contaminant in an assay seeking to measure calcium fluxes in alveoli, are readily detached from cell bodies during the homogenization procedure. The remaining difficulty with *Paramecium* cortex fractionation is that cortical fragments tend to cosediment with a specialized portion of the cell surface, the "gullet" (oral apparatus), whose numerous cilia are not detached even under harsh deciliation and homogenization conditions. Previous work (Kéryer et al., 1990) has shown that an elaborate six-step sucrose gradient allows the separation of cortex fragments from gullets. Although excellent purity is obtained by this method, allowing qualitative work to be carried out, its low yield precludes extensive biochemical tests. The methods described in this paper circumvent this difficulty by providing excellent physical separation of deciliated cortex fragments from gullets and other contaminants. The procedure requires a relatively short time, allowing ion flux experiments to be carried out on the same day. Using the cortical fraction, we show that it has all the properties of an active calcium sequestering compartment.

Materials and Methods

Strains and Culture Conditions

The d4-2 stock of *Paramecium tetraurelia* was used as the wild-type reference strain throughout this study (Sonneborn, 1975). Mutant *tam8*, which forms morphologically normal trichocysts which never attach to the cell surface (Beisson and Rossignol, 1975; Lefort-Tran et al., 1981), was isolated in stock d4-2. Mutant *pawn d4-500* (Satow and Kung, 1980) totally lacks functional voltage-sensitive Ca^{2+} channels. It was isolated in stock 51 of *Paramecium tetraurelia*, a close relative of stock d4-2.

The cells were grown at 27°C in wheat grass powder medium (Pines International Co., Lawrence, KS) buffered with 0.75 g/liter Tris-HCl, 0.2 g/l NaH_2PO_4 and 0.75 g/l Na_2HPO_4 and bacterized with *Aerobacter aerogenes* the day before use. Before utilization, 0.5 μg/ml β-sitosterol was added.

Cortex Purification

Cells were harvested from early stationary phase cultures (2,500–5,000 cells/ml; 6–12 liters of culture), using first an International Equipment Co. (Needham Heights, MA) continuous flow centrifuge and, at the last steps, a GGT (Giovanni Giaccardo Torino, Torino, Italy) centrifuge equipped with pear-shaped oil-testing vessels. The pellets were washed twice in 20 mM Tris-Maleate buffer (pH 7.8), 3 mM EDTA (homogenization medium, HM;[1] according to Bilinski et al., 1981). All subsequent steps were carried out at 4°C. The cells were resuspended in two volumes of cold HM containing 0.25 M sucrose, in the presence of protease inhibitors (0.01 mg/ml leupeptin and 1 mM PMSF; both from Sigma Chemical Co., St. Louis, MO). They were transferred to a Potter homogenizer (reference no 3431-E15, size A. Thomas, Philadelphia, PA) equipped with a Teflon pestle (0.15 mm clearance), and left to stand on ice for 10–15 min until complete immobilization. They were then submitted to ~40 hand strokes; the extent of cell breakage was monitored regularly by phase contrast microscopy. Homogenization was terminated when ~95% of the cells had broken up, yielding cortex fragments very heterogeneous in size but mostly detached from gullets. After dilution in 30 vol of HM, the homogenate was centrifuged at 270 g_{max} for 5 min in a SS34 rotor (Sorvall Instruments, Div., Norwalk, CT). The crude pellets was resuspended in a total volume of 1–3 ml HM and aliquots of 150 μl were layered over 10-ml transparent tubes containing a 25% Percoll-250 mM sucrose solution in HM (12–24 tubes required). The tubes were spun at 27,500 rpm in a Beckman 40 fixed angle rotor (Beckman Instruments, Fullerton, CA) for 20 min (50,000 g_{av}) and the bands recovered by pipetting.

For Ca^{2+} uptake assays, each fraction was diluted in 5 volumes of HM (without sucrose and EDTA), well mixed and spun in a Sorvall SS34 rotor at 6,000 rpm for 10 min (4,300 g_{av}). The pellets were resuspended in HM sucrose without EDTA.

For gel electrophoresis, the pellets were directly resuspended in a small volume of HM without sucrose and stored at −20°C. Aliquots were dissolved in SDS sample buffer (Laemmli, 1970) before loading on gels.

For EM, the pellets were washed by centrifugation in HM without sucrose to eliminate the percoll beads and directly resuspended in the fixative.

Protein concentrations were measured according to Lowry et al. (1951) using BSA as a standard.

Gel Electrophoresis and Immunotransfer

The procedures were exactly those described in Kéryer et al. (1990). Anti-band 4 polyclonal antibody (see Kéryer et al., 1990 for nomenclature, preparation, and characterization) was raised against a high-molecular weight band excised from preparative SDS-PAGE gels of crude cortex preparations.

Ca^{2+} Uptake Assay

Purified cortices were resuspended at 0.5–1 mg/ml in the incubation medium containing: 250 mM sucrose, 20 mM Tris-maleate, pH 7.4, 5 mM $MgCl_2$, 1.5 mM Na_2-ATP, 0.5–1 μCi $^{45}Ca^{2+}$ and either 20 μM Ca^{2+} or 0.2 mM and $CaCl_2$ to yield the indicated free Ca^{2+} concentration. The mixture was incubated during the appropriate time at 27°C. The reaction was stopped by diluting the mixture with 4 ml of the washing solution containing 250 mM sucrose and 40 mM NaCl, followed by filtration through a Whatman GF/C glass fiber filter (Whatman Instruments, Maidstone, England). The filter was washed three times with the washing medium and counted for radioactivity in a scintillation spectrometer.

The effect of potential inhibitors was checked by preincubating cortices during 5 min in incubation medium lacking ATP and containing the inhibitor. ^{45}Ca uptake was then initiated by the addition of 1.5 mM ATP and stopped as described above.

The total Ca^{2+} concentrations were determined by atomic absorbtion and adjusted at the desired concentrations. The free Ca^{2+} concentration in the presence of EGTA was calculated using a computer program as described by Fabiato and Fabiato (1979) and Burgess et al. (1983).

1. *Abbreviation used in this paper*: HM, homogenization medium.

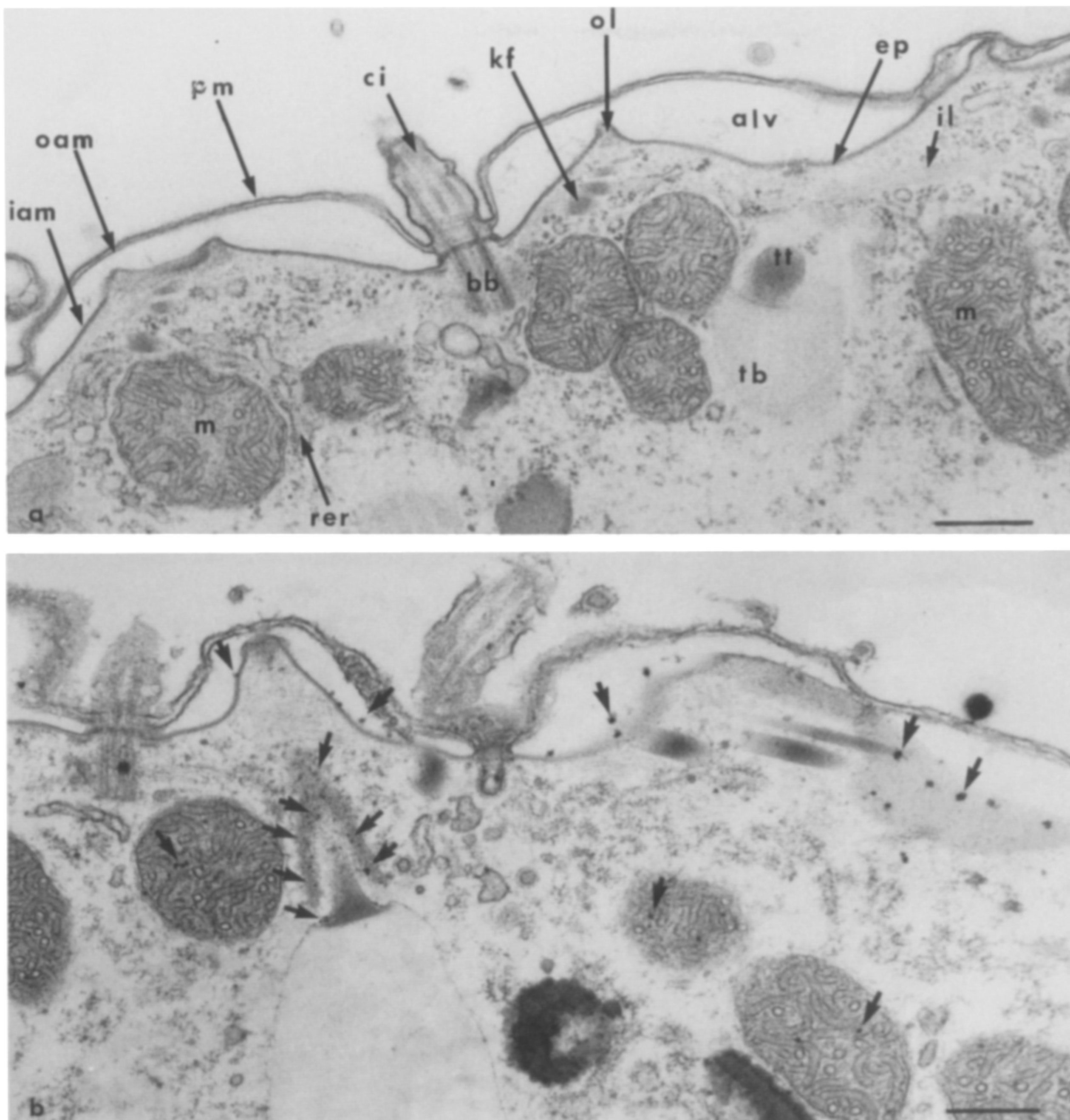

Figure 1. Ultrastructure of *Paramecium* cortex and sites of antimonate deposits. The surface of *Paramecium*, as observed in conventional EM sections (*a*), consists in a continuous plasma membrane (*pm*) subtended by a single layer of large, tightly apposed membrane vesicles, the alveoli (*alv*), which are interrupted only at the points of implantation of cilia (*ci*) through their basal bodies (*bb*) and trichocysts (*tt*, trichocyst tip; *tb*, trichocyst body). The alveoli are surrounded by an outer alveolar membrane (*oam*) in topological continuity with an inner alveolar membrane (*iam*). They are subtended continuously by a membrane skeleton, the epiplasm, (*ep*). The epiplasm itself is interrupted at the ridges of the alveoli giving way to filaments known as the outer lattice (*ol*). Deeper in the cytoplasm, the filaments of the infraciliary lattice (*il*) are found, running across the rough endoplasmic reticulum (*rer*) and the mitochondria (*m*). When cells are preincubated in antimonate (*b*) dense deposits are observed within the alveoli and trichocyst tips (*arrows*). Both cross sections (*central alveoli*) and more tangential ones (*rightmost alveolus*) show the deposits in alveoli preferentially located over the inner alveolar membrane. A few grains are also visible in mitochondria. Bar, 0.5 μM.

Electron Microscopy

(a) Standard Observations. Whole cells or cell fractions were fixed in 0.25% glutaraldehyde in 0.05 M Na Cacodylate, pH 7.4, 15 min at 4°C and postfixed in 2% OsO_4 in the same buffer for 1 h at room temperature. After washing in the buffer, the cells were preembedded in a fibrin clot, dehydrated in acetone, and embedded in Epon-Arldite. The sections were either observed unstained or after staining with uranyl acetate followed by lead citrate.

(b) Ca Precipitation Experiments. Three major antimonate and one oxalate precipitation techniques were tested, along with minor variations in each of them (Salisbury, 1982; Berruti et al., 1986; Mentré and Escaig,

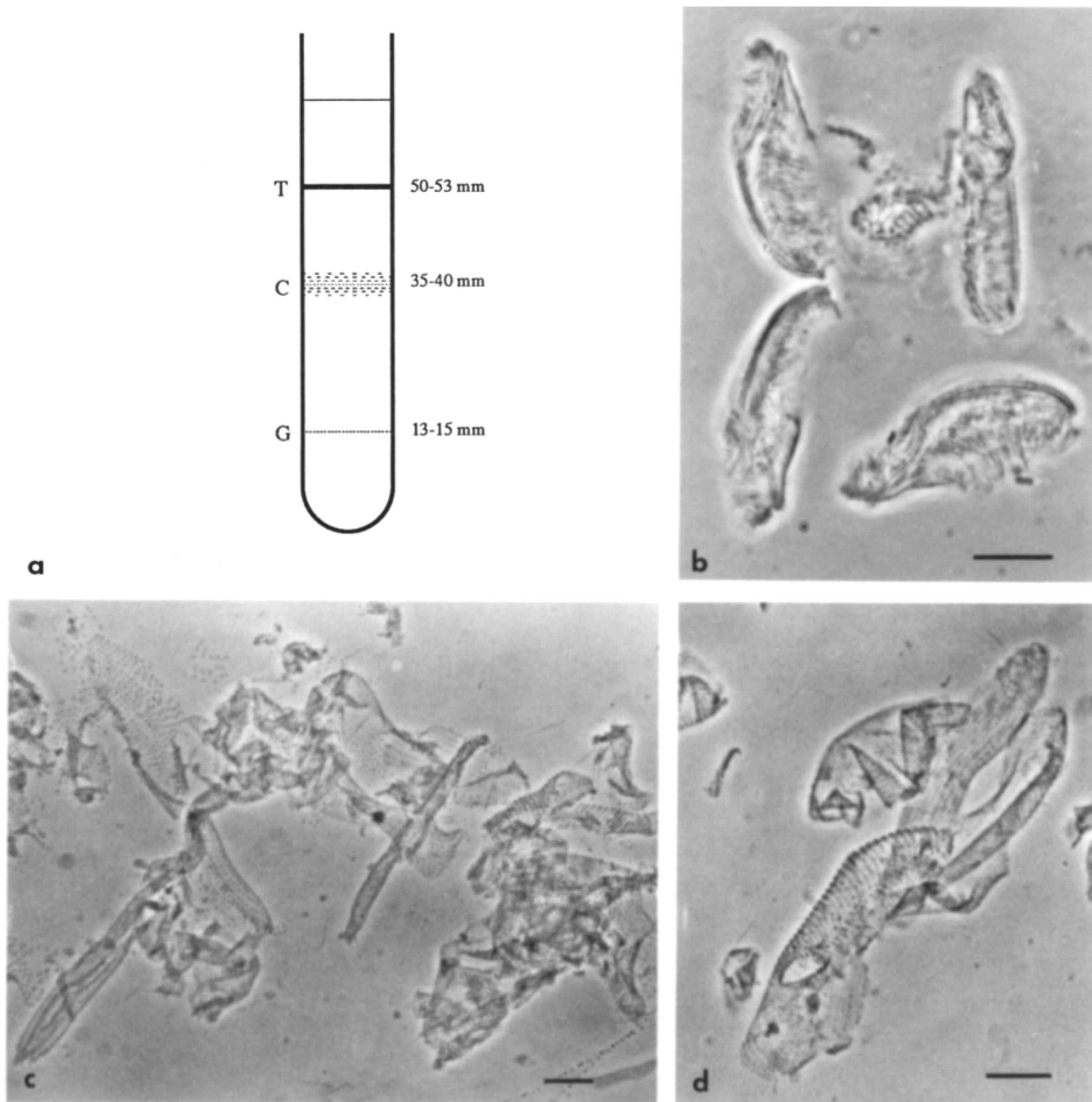

Figure 2. Phase-contrast images of Percoll gradient fractions. (*a*) The typical appearance of a 10-ml 25% Percoll gradient tube after centrifugation of a crude *Paramecium* brei, obtained as described in Materials and Methods. *T*, trichocyst band; *C*, cortex band; *G*, gullet band. Distances to the bottom of the tube are indicated in millimeters. (*b*) Phase-contrast image of a sample from the *G* (*gullets*) band. Five gullets are seen in the field with their typical comma-shaped appearance and the numerous cilia still attached to them. (*c* and *d*) Phase-contrast images of samples from the *C* (*cortex*) band. Cortex fragments of various sizes can be easily recognized because of the regular alignments of basal bodies they contain (*small dark dots*). The fraction is devoid of cilia and of gullets. Bars: (*b*) 10 μm; (*c* and *d*) 20 μm.

1988; Probst, 1986). The differences between these methods reside in the type of fixative used, the concentration of antimonate, the buffering conditions, and the order of addition of the reactants. Reproducible positive results were obtained using slight modifications of Salisbury's (1982) method. The cells were resuspended in a solution containing 2% K pyroantimonte, 20 mM sucrose, 20 mM glycine, adjusted to pH 7.4 and 2% OsO_4. After 1 h, the cells were washed in 0.05 phosphate buffer, pH 7.4, then processed as above.

Results

Antimonate Precipitates Suggested that Alveoli Contain Calcium

The first suggestion that alveoli may correspond to a calcium compartment was obtained using the pyroantimonate histochemical procedure which is widely assumed to reveal loosely bound, exchangeable calcium in the form of electron-dense deposits (reviewed by Wick and Hepler, 1982). Although a variety of published assays were used (see Materials and Methods), positive results were obtained only when the living cells were directly submitted to the antimonate solution in the presence of osmic acid. The other methods either degraded the cell surface (for example when some of the aldehydic fixatives were added together with antimonate) or prevented the formation of antimonate precipitates. Adding both the reactant and osmium simultaneously probably established a compromise between local precipitation, ultrastructural preservation, and prevention of exces-

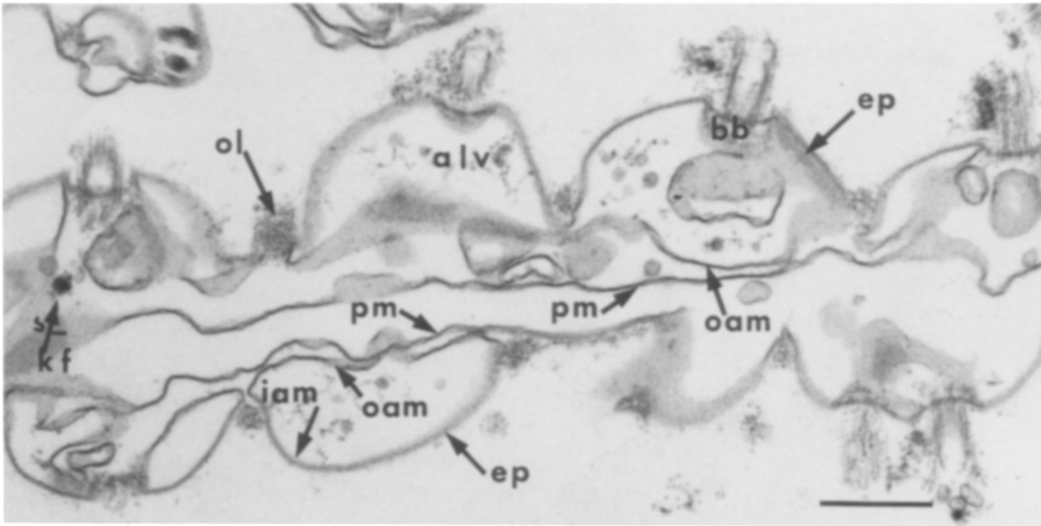

Figure 3. Ultrastructure of Percoll-purified cortex fragments. Two adjacent cortex strips in reverse orientation are seen. They contain many of the elements described in the in situ configuration (Fig. 1 *a*), in particular the succession of alveoli (*alv*) with their inner and outer membranes (*iam* and *oam*), together making up closed vesicles, located beneath the plasma membrane (*pm*) and subtended by the epiplasm (*ep*). Basal bodies (*bb*) and the outer lattice (*ol*) are conspicuous. Cilia and cytoplasmic organelles are absent. Bar, 0.5 μm.

sive calcium translocation (see Mentré and Halpern, 1987 for a detailed discussion of these points).

The deposits took the form of electron-dense grains of varying size (50–100 nm) that displayed a highly selective localization (Fig. 1 *b*). First, several organelles that could be assumed to contain calcium constantly yielded deposits, thereby providing an internal control for the method. This was clearly the case for the mitochondrial matrix as well as for the internal volume of trichocyst tips, in agreement with other techniques (Plattner and Fuchs, 1975; Fisher et al., 1976). Trichocyst tips were the most strongly staining compartment (see quantitation below), the labeling being concentrated along the outer sheath and also quite strongly at the junction between the tip and the body, along the inner lamellar sheath (see Bannister, 1972 for a definition of these terms). The crystalline portion of the trichocyst tip and body did not stain. Favorable sections showed that this trichocyst tip labeling occurred in "resting" organelles that is those that had not undergone membrane fusion and initiation of the extrusion process, thus indicating that their antimonate deposits were not simply due to direct access of the extracellular medium inside the organelle. Second, alveoli systematically displayed a significant number of grains; their density was smaller than in trichocyst tips and about to that in mitochondria. These grains were preferentially located towards the inner alveolar membrane and were seldom seen in the lumen. Sections which were tangential to this membrane therefore displayed the highest grain density. Third, the cytosol and a variety of other organelles were either fully negative (peroxisomes, a variety of membrane vesicles) or displayed a small number of grains of uncertain significance (cilia, Golgi apparatus). Antimonate deposits were counted over 30 independent sections and averaged per organelle or per square micron, yielding the following quantitative results: (*A*) 15 grains per trichocyst tip, 0 per trichocyst body, 2.5 per alveolus, 3 per mitochondrion; (*B*) 16 grains/μm^2 in trichocyst tips, 2 grains/μm^2 in the alveoli, 2.5 grains/μm^2 in mitochondria.

Percoll Allowed Improved Purification of Paramecium Cortex

To directly test the hypothesis of active calcium sequestration in the cortical alveoli of *Paramecium*, we searched for a method of cell fractionation that would yield highly purified cortical fragments less laboriously than with the six-step sucrose procedure of Kéryer et al. (1990). Percoll gradients were chosen because of the excellent purification of various forms of trichocysts of *Paramecium* from cortex fragments previously observed by Sperling et al. (1987). The formulation described in Materials and Methods was reached after a number of semiempirical tests in which the Percoll concentration, the run time, the centrifugal force, and the total volume were progressively adjusted, whereas the bands obtained were observed by phase-contrast microscopy. The aim was to obtain good separation of cortex fragments from gullets and other contaminants but also to try to have the cortex sediment as a relatively sharp band to facilitate its recovery from the gradient tubes. The compromise reached allowed the formation, through a short run, of a somewhat fluffy cortex band, 3–5 mm thick, located quite far from the nearest contaminants (10 and 25 mm for trichocysts and gullets, respectively) (Fig. 2 *a*). This pattern was extremely reproducible (>30 runs). Maximum protein concentration that could be loaded on a 10-ml gradient tube while maintaining good separation was 400 μg; the yield was typically 100 μg of cortex per tube. 12–24 tubes were routinely run simultaneously providing 1–2 mg (exceptionally up to 5 mg) of purified cortex in ~4–5 h.

A variety of tests established the purity of the fractions obtained through these gradients. First, light and electron microscopy showed the cortex fraction to be totally devoid of cilia, gullets, and cytoplasmic organelles, whereas the gullet band itself was devoid of independent cortex fragments but displayed small pieces of cortex remaining attached to the gullets (Fig. 2, *b–d* and Fig. 3). The cytology and ultrastructure of the cortex and gullets fractions were identical to those

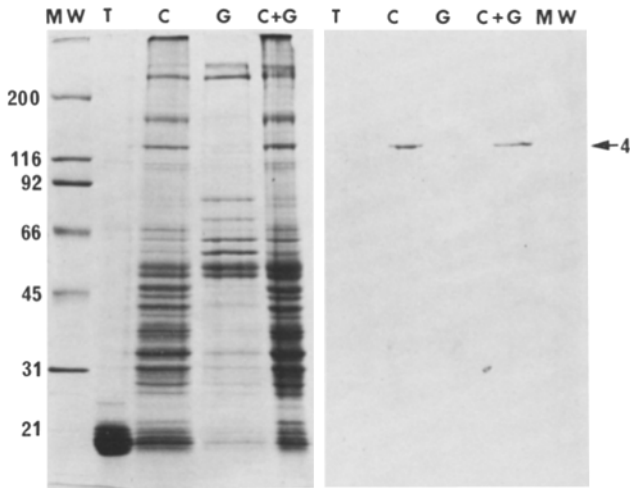

Figure 4. Electrophoretic pattern of Percoll gradient fractions. (*Left*) Coomassie blue-stained SDS-acrylamide gel (7.5-15% acrylamide gradient) displaying the trichocyst (*T*), cortex (*C*), and gullet (*G*) fractions obtained from a Percoll gradient and compared with the a crude fraction containing a mixture of cortices and gullets (*C+G*). (*Right*) Immunoblot of a gel identical to that seen on the left, treated with an anti-band 4 antibody. Positive reactions at the level of band 4 (*arrow*) are seen only in the cortex and mixed lanes, confirming the absence of this protein in the gullets fraction.

observed after sucrose gradient purification (Kéryer et al., 1990). In particular, the cortex fragments contained the plasma membrane, the alveoli, the epiplasm (a cytoskeletal fibrous sheath underlying the alveoli), the basal bodies and several additional minor cytoskeletal components (Fig. 3). The topology of the cortex appeared to be that existing in vivo, that is no process of formation of "inside-out" vesicles was detected in this fraction. This is easily understood considering the overall rigidity of *Paramecium's* pellicle, which is maintained by the continuous stiff layer of the epiplasm. Second, electrophoretic analysis of the proteins of the different fractions (Fig. 4) showed the cortex and the gullets to contain several distinct nonoverlapping "signature" bands, including some quantitatively major ones. Thus, at the level of sensitivity of some major Coomassie blue-stained bands, cross-contamination of cortex and gullets appeared to be minimal. The electrophoretic pattern of the Percoll-purified cortex was identical to that of the most highly purified cortex (Kéryer et al., 1990) obtained in sucrose gradients and is therefore not described in detail here. Finally, several polyclonal as well as mAbs specific to proteins belonging either to the cortex or to the gullets have been routinely tested on immunoblots of these two fractions and high specificity was constantly observed. An example of an immunoblot is given in Fig. 4 for "anti-band 4", an antibody specific to a cortical component (see Materials and Methods) described in detail in Kéryer et al. (1990). This antibody specifically decorates the "striated bands", a filamentous network attached to the cortical epiplasm and absent from the gullet. As can be seen in Fig. 4, the antibody labels a single band (band 4) very specifically in the cortex lane and is totally unreactive on the gullets lane.

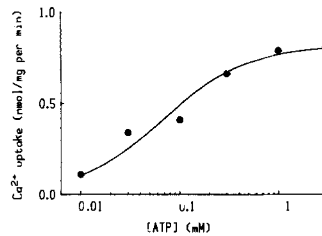

Figure 6. Dose response curve for the initial $^{45}Ca^{2+}$ uptake by purified cortex as a function of ATP concentration. The purified cortex was preincubated during 5 min at 27°C in the incubation medium containing 20 μM $^{45}Ca^{2+}$ and 10 mM $MgCl_2$. $^{45}Ca^{2+}$ uptake was initiated by the addition of the indicated concentrations of ATP and was stopped 3 min later. Each point is the mean of triplicate determinations in one experiment repeated twice. The curve was drawn according to the following parameters: K_m = 64 μM; V_{max} = 0.82 nmol/mg per min; and n_H = 1.

Purified Cortex Displayed ATP-dependent Calcium Pumping

A typical time course of $^{45}Ca^{2+}$ uptake in a cortex preparation is shown on Fig. 5. The figure shows that uptake, measured in the presence of 0.2 mM EGTA and 0.1 mM $CaCl_2$ (i.e., ~100 nM free Ca^{2+}), was negligible in the absence of added ATP (<0.1 nmol/mg protein), whereas it became quite sustained in the presence of 10 mM ATP, starting to decline without reaching a true plateau only after ~30–40 min. At 40 min, uptake had reached 2.6 nmol/mg protein.

Addition of 10 μM of the calcium ionophore A23187 induced the release of 97% of the accumulated $^{45}Ca^{2+}$ within 2 min (Figs. 5 and 7), indicating that Ca^{2+} uptake occurred in a closed membrane compartment and that virtually no unspecific superficial binding was recorded in the assay. The calcium uptake assay therefore corresponds to a pumping activity inside a vesicular compartment. This activity was further characterized as follows.

Fig. 5 shows that the presence of magnesium in the incubation buffer was absolutely required to observe Ca^{2+} uptake.

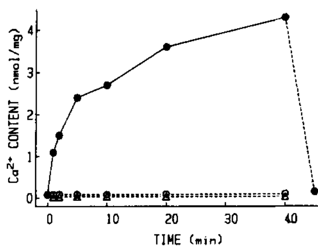

Figure 5. Kinetics of $^{45}Ca^{2+}$ accumulation in purified cortex. The purified cortex was incubated at 27°C for the indicated periods in a medium containing 0.2 mM EGTA and 0.1 mM $^{45}Ca^{2+}$ (~0.1 μM free Ca^{2+}), as described under Materials and Methods. Incubation medium contained either 10 mM ATP and 10 mM $MgCl_2$ (*filled circles*), or 10 mM ATP without $MgCl_2$ (*triangles*), or 10 mM $MgCl_2$ without ATP (*open circles*). Ca^{2+} ionophore A23187 was added at 10 mM after 40 min incubation. Each point is the mean of triplicate determinations in two different experiments.

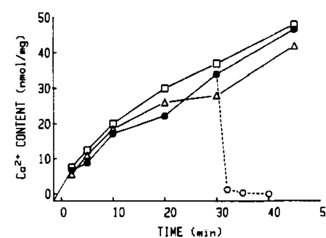

Figure 7. Kinetics of $^{45}Ca^{2+}$ accumulation into the cortex purified from wild-type, *tam* or *pawn* strains. The purified cortex was incubated as indicated in Materials and Methods in a medium containing: 20 μM Ca^{2+}, 5 mM $MgCl_2$, and 1.5 mM ATP. Data show the ATP-dependent $^{45}Ca^{2+}$ accumulation into cortex from wild-type (*squares*), *pawn* (*filled circles*) and *tam* (*triangles*) strains. A23187 was added at 10 μM after 30 min (*open circles*). Each point is the mean of triplicate determinations in one experiment.

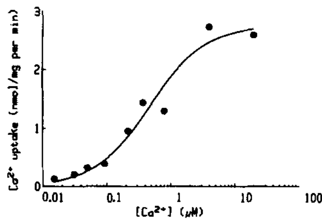

Figure 8. Dose-response curve for the initial $^{45}Ca^{2+}$ uptake rate by purified cortex as a function of Ca^{2+} concentration. Purified cortex was preincubated for 5 min in the incubation medium as described in Materials and Methods. Medium contained 5 mM $MgCl_2$, 0.2 mM EGTA, and increasing concentrations of $CaCl_2$. The free Ca^{2+} concentrations were calculated as indicated under Materials and Methods. $^{45}Ca^{2+}$ uptake was initiated by addition of 1.5 mM ATP and stopped 3 min later. Each point is the mean of triplicate determinations in one experiments. The curve was drawn according to the following parameters: K_m = 0.52 μM; V_{max} = 2.7 nmol/mg per min and n_H = 1.

The initial Ca^{2+} uptake was measured at varying Mg^{2+} concentrations, in the presence of 1.5 mM ATP and 100 nM Ca^{2+}. No calcium uptake occurred when Mg^{2+} was omitted. In the presence of 1.5 mM Mg^{2+}, calcium uptake proceeded at 90% of the maximum which was reached at 3 mM Mg^{2+} and remained constant up to 10 mM Mg^{2+}.

The dose dependency of the pump activity as a function of the ATP concentration was determined in the presence of 10 mM Mg^{2+} and 20 μM Ca^{2+}. The data shown on Fig. 6 are consistent with a Michaelis-Menten type kinetics with K_m and V_{max} values of 48 μM and 0.8 nmol/mg per min, respectively (n = 2).

The initial $^{45}Ca^{2+}$ uptake rate, which is equivalent to the pump flux was also measured at different pH, between 6.6 and 8.0. The pump activity was maximal in the pH range of 7.0 to 7.4. At pH 6.6 and 8.0, the pump activity was only 56 and 45%, respectively of the maximum measured at pH 7.4.

The Trichocyst Compartment and the Calcium Channels Are Not Involved

Since cortex fragments still contain a small number of trichocysts attached to them and an ATPase activity has been detected by cytochemical procedures at the point of attachment of the organelles membrane to the plasma membrane (Plattner et al., 1977), it was important to evaluate whether this compartment was contributing to calcium uptake. We took advantage of the availability of mutants with unattached trichocysts, which, in addition to the total absence of trichocysts at the level of the cortex, are known to be devoid of the intramembranous particle arrays that are associated with the ATPase activity (Plattner et al., 1980). The cortex of the mutant strain *tam8* (Lefort-Tran et al., 1981) was purified in parallel with that of the wild-type strain, checked to be totally free of contaminating trichocysts and used in the uptake assay with the wild type serving as a control. Fig. 7 shows that the uptake in the mutant, measured in the presence of 20 μM free Ca^{2+}, was identical to that of the wild type. No major contribution of trichocysts therefore occurs in our assays.

Similarly, we wished to eliminate the remote possibility that some calcium uptake might be due to leakage through the voltage-sensitive calcium channels. These channels, which are concentrated in the ciliary membrane (Ogura and Takahashi, 1976; Dunlap, 1977), can function in purified ciliary membrane vesicles (Thiele and Schultz, 1981). Although cilia are absent from our cortex preparation, minor contamination by such vesicles is difficult to exclude completely. Again, we used a *pawn* mutant of *Paramecium*, *d4-500*, in which the activity of this class of channels has been totally abolished (Satow and Kung, 1980) and found (Fig. 7) that calcium uptake was not modified.

The Pump Had a K_m for Calcium Compatible with a Physiological Function

The ATP-dependent calcium uptake was also measured as a function of the free calcium concentration in a medium containing 1.5 mM ATP and 5 mM Mg^{2+}. The medium also contained 0.2 mM EGTA and increasing concentrations of $CaCl_2$ necessary to obtain the indicated free Ca^{2+} concentrations (Fig. 8). The results are consistent with a Michaelis–Menten-type kinetics. Non linear regression analysis allowed the calculation of a K_m and a V_{max} of 522 nM and 2.6 nmol/mg per min, respectively. Such a K_m value, close to 0.5 μM, strongly suggests that the pump may be active at concentrations of calcium, which are common in a cytosolic environment (Carafoli, 1987).

Calmodulin May Be Involved in the Pump Activity

A typical property of plasma membrane calcium ATPases is their stimulation by calmodulin (Penniston, 1983). We therefore tested both the effect of added exogeneous calmodulin on the uptake and that of calmodulin inhibitors. Addition of bovine calmodulin had no reproducible stimulatory effect on calcium uptake. The phenotiazine trifluoroperazin did inhibit calcium uptake but only when used at rather high concentrations (>10 μM; Fig. 9), which may be indicative of unspecific effects. The most striking results were obtained using calmidazolium: addition of 10^{-5} M resulted in >85% inhibition of Ca^{2+} uptake (Fig. 9).

Inhibitors of mitochondrial functions such as oligomycin, carbonyl cyanide *m*-chlorophenylhydrazone, or ruthenium red had no effect on the uptake (Table I). Other inhibitors such as the sulfhydride reactive agent *p*-chloromercuribenzoate, the trivalent cation La^{3+} and vanadate had a clear inhibitory effect (Table I).

To further explore the possible involvement of calmodulin-regulated proteins in Ca^{2+} sequestration, we attempted to identify calmodulin-binding proteins in the cortex using an overlay procedure. The ^{125}I-labeled calmodulin (Fraker and Speck, 1978) was overlaid directly on the gels after removal of detergent and renaturation. Pig brain fodrin provided an

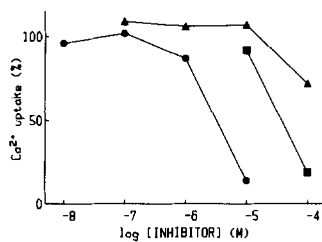

Figure 9. Effects of calmodulin inhibitors on the initial $^{45}Ca^{2+}$ uptake rate by purified cortex. Purified cortex was preincubated for 5 min in the incubation medium containing 20 μM $^{45}Ca^{2+}$ and the indicated concentrations of calmidazolium (*filled circles*), W7 (*triangles*), and trifluoroperazin (*squares*). $^{45}Ca^{2+}$ uptake was initiated by addition of 1.5 mM ATP and stopped 3 min later. Data are expressed as percent of the uptake measured in the absence of inhibitor (13 nmol/mg per 3 min). Each point is the mean of triplicate determinations in one experiment.

Table I. Effect of Inhibitors on Initial $^{45}Ca^{2+}$ Uptake Rate by Purified Cortex

Inhibitor	Calcium uptake
	%
None	100
p-Chloromercuribenzoate *(0.1 μM)*	106
p-Chloromercuribenzoate *(1 μM)*	5
Carbonyl cyanide *m*-chlorophenylhydrazone	118
Oligomycin *(10 μM)*	84
Ruthenium red *(10 μM)*	97
Vanadate *(10 μM)*	75
Vanadate *(100 μM)*	25
Vanadate *(1 mM)*	1
Lanthanum	10

Purified cortex was incubated during 3 min at 27°C in a medium containing 20 μM $^{45}Ca^{2+}$, 5 mM $MgCl_2$, and 1.5 mM ATP and the indicated inhibitor.

internal positive control; addition of EGTA and chlorpromazine abolished the labelings.

Although several bands were intensely labeled both in whole cells and in purified cortices lanes, none of the positive cortical bands was extractable by Triton X-100 treatment, i.e., Triton-treated cortices yielded the same labeled bands as untreated controls and the Triton-soluble supernatant yielded no positive bands (data not shown).

Discussion

Paramecium Alveoli Contain Calcium

Although Ca^{2+} precipitation methods have a number of pitfalls, there are several reasons to give some significance to the deposits obtained with potassium antimonate in the present study (see Wick and Hepler, 1982 and Mentré and Halpern, 1987 for a critical appraisal of this method). First, the deposits are observed only in organelles that are known or strongly suspected through completely independent physiological data to contain calcium or to involve calcium in their functioning: mitochondria, trichocysts, and cilia. Second, although no electron microprobe analysis was performed in the present study, the bulk of the available literature as well as the fact that the deposits are prevented by EGTA and increased by the calcium ionophore A 23187 (data not shown) all suggest that calcium is the major if not the exclusive ion visualized. Finally, x-ray microanalysis by Schmitz et al. (1985) and preliminary data obtained by analytical ionic microscopy (Stelly et al., manuscript in preparation) indicate a high calcium concentration in the pellicle of *Paramecium* as observed in situ.

The pyroantimonate technique does not allow visualization of calcium at a concentration $<10^{-6}$ M. In addition, strongly chelated calcium is probably not available for the formation of complexes with antimonate. It is thus assumed that the fraction of calcium visualized by this technique corresponds neither to the free ions nor to the very tightly bound ones but to the loosely bound ions susceptible of undergoing exchange. In several other biological systems, this fraction is presently thought to occur in vivo in the form of complexes with high-capacity low-affinity binding proteins (Carafoli, 1987) such as calsequestrin in muscle sarcoplasmic reticulum. An important physiological role in a number of processes of regulated calcium release has recently been assigned to it. It is tempting to speculate that an analogous situation is occurring in *Paramecium* alveoli. The immediate prediction is that calcium-binding proteins should be present in the lumen of the alveoli. So far, only the localization of calmodulin has been investigated in *Paramecium* cortex (Maihle et al., 1981; Klumpp et al., 1983; Momayezi et al., 1986) and although it is clearly associated with a number of cytoskeletal and membranous structures, it was not seen within the alveoli in spite of the use of immunolocalization on EM sections (Momayezi et al., 1986). We are presently attempting to identify other calcium-binding proteins by a variety of direct biochemical and immunocytochemical approaches. So far, tests using "Stains all", a dye that strongly reacts with calsequestrin in SDS-PAGE gels (Campbell et al., 1983) have yielded negative results and radioactive Ca^{2+} overlays have not yielded bands at the known molecular weight of metazoans calsequestrin.

That antimonate deposits were mostly located over the inner alveolar membrane suggests that the putative Ca^{2+}-binding proteins may be tightly associated with it and/or that the Ca^{2+} present in the lumen of the alveoli is more readily extractable.

Physiological Significance of Calcium Pumping into Alveoli

Because the major closed membrane compartment present in our fraction are alveoli and because the calcium fluxes are rapidly and completely abolished by A23187, we consider that all the calcium retention activity measured in our assays corresponds to sequestration inside the alveoli. This obviously fits quite well with the calcium localization experiments discussed above. The only other closed membrane compartment that might have contributed to calcium pumping is trichocysts which are membrane bound exocytotic vesicles attached to the cortex. We have shown, however, through the use of mutants devoid of trichocysts that pumping is not related to the presence of these exocytotic vesicles in the fraction. In addition, the use of a mutant completely defective in the voltage-sensitive ciliary calcium channel confirmed, as was expected, that this channel is not involved in the fluxes that we were measuring. It should be recalled here that the topology of the membranes in the isolated cortex fraction corresponds to that existing in vivo. Thus, the measured fluxes reflect, in principle, the pumping of external (i.e., cytosolic) calcium into an intracellular membrane compartment (i.e., topologically equivalent to the endoplasmic or sarcoplasmic reticulum).

The two major observations reported in this paper are that calcium is actively sequestered inside the alveoli by a process that is ATP and Mg^{2+} dependent and that this sequestration activity can occur at sufficiently low calcium concentration to be physiologically significant in vivo. We therefore suggest that the alveolar membrane contains a calcium ATPase that is activated when the cytosolic calcium concentration reaches relatively high levels (i.e., $>10^{-6}$ M) and therefore that the cortical alveoli of *Paramecium* have an important role in regulating calcium homeostasis in the vicinity of the cortex. This work therefore has gone on step towards proving the homology between the alveoli and the sarcoplasmic reticulum of muscle cells and, in general, with specialized calcium-storing compartments. A rather striking analogy with the recently identified endoplasmic reticulum

network of ascidian eggs, which makes up a vast Ca compartment lying just beneath the plasma membrane, can also be noted (Gualtieri and Sardet, 1989).

Physiologic or experimental conditions under which alveoli might function in selectively releasing sequestered calcium have not been approached in this study. Attempts at inducing calcium release by the messenger inositol(1,4,5)-triphosphate, which is involved in a wide variety of cell functions by releasing Ca^{2+} from internal pools (Berridge and Irvine, 1988) have yielded erratic and globally negative results. Other factors that initiate fast Ca^{2+} release via the Ca^{2+}-induced Ca release system or via a change in membrane potential have not been studied yet. As indicated in the introduction, fine regulation of calcium concentration in the cortex is potentially important for at least three physiological processes: ciliary beating, exocytosis, and cytoskeletal fibers dynamics. Some buffering function with respect to the external medium for this fresh water class of organism subject to rapid and important variations in its ionic environment is also conceivable. It is tempting to suggest that the presence of a vast membranous compartment capable of pumping and storing calcium implies a role of this compartment in the above functions. Direct proof of the involvement of the cortical alveoli in the regulation of these various physiological processes must, however, await further experimentation. The recent isolation of a class of *Paramecium* mutants which appear to be defective in calcium extrusion or sequestration processes (Evans et al., 1987; Evans and Nelson, 1989) should provide a powerful tool to test our suggestion.

Relationship of the Calcium-pumping Activity to Previously Described ATPases in Paramecium

Several Ca-ATPases activities have been previously described in relation to *Paramecium* surface structures.

First, a Ca^{2+}-stimulated ATPase firmly bound to the ciliary membrane has been recently characterized by Travis and Nelson (1986) and is possible identical to that characterized in ciliary membranes by Doughty and Kaneshiro (1983, 1985). A second potent Ca^{2+}-ATPase, distinct from the tightly bound one, is recovered as a soluble enzyme in the "ciliary supernatant fraction" after deciliation induced by a Ca^{2+}-shock (Riddle et al., 1982). After purification, two major bands at 68 and 53 kD are obtained on SDS gels. Their peptide maps are quite similar. The substrate and ionic optima of the enzyme have been characterized in detail and antibodies raised to the purified enzyme (Levin et al., 1989). This activity may be identical to the "deciliation supernatant ATPase" of Doughty and Kaneshiro (1983) and to the ATPase loosely bound to ciliary membranes of Andrivon et al. (1983). The function of these ATPases has not been definitely established yet. Ca^{2+}-ATPase activities more specifically associated with the "somatic membranes" (i.e., cortical, nonciliary) have been described by Noguchi et al. (1979). Bilinski et al. (1981) and Doughty and Kaneshiro (1983, 1985). The comprehensive work of the last authors clearly establishes the distinctness of the pellicle activities from the ciliary, soluble or intracellular organelles bound ones. In summary, a number of Ca^{2+}-ATPases (identified as bands on Triton gels) are specifically associated with a cortical fraction containing trichocysts. Finally, a Ca^{2+}-ATPase activity has recently been characterized by Wright and van Houten (1990) in a cortex fraction identical to the one used in the present work. This ATPase has several enzymatic properties that resemble those of the pumping activity described in this paper (rate of inhibition by divalent cations, sensitivity to calmidazolium) but is distinctly different in its vanadate sensitivity. The authors conclude that it probably corresponds to a plasma membrane and not to an alveolar pump.

Plattner et al. (1977, 1980) have provided an interesting clue to the possible function of at least some of these cortical ATPases. They demonstrated cytochemically that a Ca^{2+}-ATPase activity was associated with the exocytosis site and was strictly correlated with the presence of the rosette of intramembranous particles and/or connecting material. Since in the present work we have shown that mutants with unattached trichocysts, and therefore lacking the rosette, have identical Ca^{2+} sequestration activity to wild type, it appears that this ATPase is not the one analyzed in our experiments.

The question of calmodulin dependent of the Ca^{2+} ATPase analyzed in the present work remains unsettled. Inhibitor studies are suggestive, specially since calmidazolium, which displayed a clear inhibitory effect on Ca^{2+} uptake, is considered as one of the most specific anticalmodulin drug (Van Belle, 1981; Gietzen et al., 1981). However, attempts at direct identification of a Triton X-100-soluble band displaying affinity for calmodulin in gel overlay experiments have yielded negative results. Several major calmodulin-binding proteins are indeed present in the cortex fraction but none of these is soluble in Triton. Thus, either a calmodulin-dependent ATPase is indeed present in the cortex but it remains tightly linked to cytoskeletal components or the ATPase does not interact with calmodulin. A similar situation has recently been described by Levin et al. (1989) for the "deciliation supernatant ATPase" of *Paramecium* in that the purified enzyme is sensitive to anticalmodulin drugs, whereas it does not contain a calmodulin subunit. In any case, the several Triton-insoluble bands interacting with calmodulin in the cortex preparations strongly suggest that there are a number of major calmodulin-dependent substrates in this fraction.

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