Paramecium Secretory Granule Content: Quantitative Studies on In Vitro Expansion and Its Regulation by Calcium and pH

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ABSTRACT Ca^{2+}-dependent secretion in *Paramecium* involves the exocytic release of a paracrystalline secretory product, the trichocyst matrix, which undergoes a characteristic structural change from a highly condensed storage form (Stage I) to an extended needle-like structure (Stage III) during release. We studied trichocyst matrix expansion in vitro to examine factors regulating the state of secretory organelle content. A new method for the isolation of membrane-free, condensed (Stage I) trichocyst matrices is described. These highly purified, condensed matrices were used to develop a rapid quantitative, spectrophotometric assay for matrix expansion to examine factors regulating the Stage I to Stage III transition. Expansion from Stages I to III was elicited in vitro by addition of Ca^{2+} and we found that at neutral pH, expansion required a Ca^{2+} concentration slightly above 10^{-6} M. Previous studies indicate that calmodulin (CaM) antagonists inhibit matrix expansion in vivo. However, in vitro matrix expansion is normal even when trichocyst matrices are preincubated in CaM antagonists before stimulation. Thus, matrix components themselves are unlikely to be the site of CaM antagonist action in vivo. In vitro matrix expansion is also modulated by pH. Decreasing pH to 6.0 inhibits expansion, i.e., expansion requires higher Ca^{2+} concentration. Conversely, increasing pH to >7.0 promotes expansion, allowing it to occur at a lower Ca^{2+} concentration. The pH sensitivity of the Ca^{2+} binding sites of the matrix suggests that, in vivo, the interior of the trichocyst vesicle may be maintained at an acidic pH. Exposure of cells to acridine orange, a fluorescent amine that accumulates in acidic intracellular compartments, leads to its uptake and concentration within trichocysts. Thus intratrichocyst pH appears to be acidic in vivo and may serve as a regulatory or "safety" mechanism to inhibit premature expansion.

The involvement of the Ca^{2+}-dependent regulatory protein, calmodulin (CaM),[1] in stimulus-secretion coupling has been suggested in studies of various secretory systems. The precise role of CaM, however, has been difficult to assess. The ciliated protozoan, *Paramecium*, was recently used to investigate the role of CaM in exocytosis (10). In these cells, thousands of membrane-bounded secretory organelles known as trichocysts are positioned in the cell cortex at defined secretory sites. Release of the secretory product, the trichocyst matrix (tmx), follows stimulation and involves two separable Ca^{2+}-dependent steps: the fusion of trichocyst and plasma membranes to create the exocytic opening, and the expansion of the tmx from its highly condensed resting form (Stage I) to an elongated, needle-like secreted form (Stage III).

Earlier studies demonstrated that two structurally different CaM antagonists reversibly inhibit secretion (10). Ultrastructural examination of these cells revealed that a specific Ca^{2+}-dependent step in the release process, expansion of the tmx, is inhibited. We suggested that matrix expansion is blocked in vivo because CaM antagonists limit the access of Ca^{2+} to the matrix.

However, a possible mechanism for CaM antagonist action that had not been ruled out is the direct interaction of these agents with the tmx itself. In this study, we examined the mechanism of tmx expansion and its regulation. Matrix expansion was examined in vitro using preparations of isolated, membrane-free condensed trichocysts. We describe a novel

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[1] *Abbreviations used in this paper*: CaM, calmodulin; HM, homogenization medium; tmx, trichocyst matrix.

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 DECEMBER 1984 2193-2199

purification scheme for tmx that yields highly purified Stage I matrices; these matrices were free of subcellular particles or cell fragments. This purified tmx preparation was used to develop a rapid, quantitative, spectrophotometric assay for expansion based on the turbidity change that accompanies the Stage I-Stage III (condensed-expanded) transition. Using this assay, we have made the first accurate determination of the Ca^{2+} concentration necessary to induce expansion. We have demonstrated that matrix components per se are unaffected by CaM antagonists and can therefore be eliminated as a site of action for these agents in vivo. In addition, the effect of alterations in pH on Ca^{2+}-induced in vitro expansion were examined. We show that at low pH (~6.0) expansion requires higher Ca^{2+} concentration whereas at pH >7.0, expansion is facilitated (i.e., it occurs at a lower Ca^{2+} concentration). The possible role of pH in regulating expansion in vivo was examined using acridine orange, a fluorescent amine that accumulates in acidic intracellular compartments (18). We found that brief incubation of *Paramecium* in acridine orange led to its uptake and concentration within trichocysts. This suggests that, in vivo, intratrichocyst pH is acidic, and that a primary function of low intragranule pH may be to maintain secretory products in their storage form.

Results of in vivo (10) and in vitro studies on the effects of CaM antagonists, Ca^{2+}, and pH on the regulation of matrix expansion and release in *Paramecium* are synthesized into a working hypothesis. This model suggests that similar strategies for secretory product storage and release have been maintained through evolution from progenitor cells such as *Paramecium* to more specialized secretory cells in higher organisms.

MATERIALS AND METHODS

Culture Conditions: Cell cultures of *Paramecium tetraurelia*, wild type, were grown at 27°C in bacterized monoxenic (*Enterobacter aerogenes*) Cerophyle medium (31) (Cerophyl Laboratories, Inc., Kansas City, MO), and generally harvested at late log phase (3,000–4,000 cells/ml).

Purification of Stage I tmx: Purification of tmx was carried out using a modification of a procedure of Matt et al. (20). Late log phase cells (1 liter) were harvested, washed twice in homogenization medium (HM) (20 mM Tris, 100 mM KCl, 5 mM EGTA [pH 7.0]), and the washed pellet of cells was resuspended to 3 ml in HM. Cells were allowed to stand in HM at room temperature for 15 min, and then homogenized on ice (50 to 100 strokes) in a tight-fitting glass Dounce homogenizer. The homogenate was diluted to 6 ml with HM, and centrifuged for 5 min at 1,500 *g* in a Sorvall HB-4 rotor. The resulting supernatant was discarded and the pellet resuspended to 3 ml in HM. The homogenization and centrifugation steps were repeated, the supernatant again discarded, and the pellet resuspended to 1 ml in HM. This was layered on 24 ml of 70% Percoll, and centrifuged for 15 min at 30,000 *g* in a Sorvall SS-34 rotor (Dupont Co., Wilmington, DE). A band was formed 10–15 mm from the tube bottom that contained purified Stage I tmx. Tmx were collected and washed by diluting at least 10-fold in either HM or wash buffer (50 mM KH_2PO_4-K_2HPO_4, 100 mM KCl, 5 mM EGTA [pH 7.0]), and centrifuged for 18 min at 1,500 *g* in HB-4 rotor. The resulting pellet of washed Stage I tmx was resuspended to 1–2 ml in HM.

Calibration of Percoll gradients was done using density marker beads (Pharmacia Code No. 17-0459-01) (Pharmacia Fine Chemicals, Piscataway, NJ).

Electron Microscopy: Trichocysts were placed on Parlodian (Mallinckrodt Inc., St. Louis, MO) and carbon-coated copper grids, stained with 1–2% phosphotungstic acid, and examined in a JEOL 100CX electron microscope.

In Vitro Expansion Assay: Ca^{2+}/EGTA buffers used in these assays were prepared according to Portzehl et al. (27). Unless otherwise noted they contained 75 mM KCl, 25 mM HEPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 7.0. The pH 6.0 buffer contained 25 mM PIPES instead of HEPES.

40–50 μl of a Stage I tmx suspension was added to a cuvette that contained 720 μl of HM or Ca^{2+}/EGTA buffer of specified free Ca^{2+} (10^{-8} M to 10^{-3} M or pCa 8.0 to pCa 3.0; $pCa = -\log[Ca^{2+}]$) and pH. The absorbance at 320 nm (OD_{320}) was read in a Hitachi 110 dual beam spectrophotometer with buffer alone in the reference cuvette. OD_{320} readings were taken 30 s and 1 min after the addition of Stage I tmx to the cuvette. The concentration of Stage I tmx was adjusted to yield an OD_{320} of ~0.2 when added to 720 μl HM.

Acridine Orange Staining: *Paramecium* cells were incubated in acridine orange (10 μg/ml) for 60 s, washed twice with fresh Cerophyl medium, and resuspended in 250 μl of medium. Cells were observed and photographed using a Zeiss inverted microscope equipped with epifluorescence. Those cells observed to exhibit trichocyst fluorescence remained alive throughout the period of observation as evidenced by beating cilia and contractile vacuole activity.

RESULTS

Purified Stage I tmx

Stage I trichocysts isolated according to the method outlined are shown in the phase-contrast micrographs in Fig. 1. Fig. 1 *a* shows the contents of the trichocyst band recovered from the Percoll gradient after washing. It contained >95% free Stage I trichocysts. The few Stage III trichocysts which are evident expanded during the final wash and centrifugation. The trichocysts recovered from the gradient before washing were exclusively Stage I. There was little, if any, cortical contamination and no vesicles were visible.

Stage I trichocysts isolated in this manner were without their trichocyst membrane (Fig. 1 *c*) as reported for Stage I trichocysts isolated by other methods (2, 20). This permitted the direct examination of matrix function. The condensed matrix retained its highly ordered, paracrystalline appearance and its 7-nm periodicity (Fig. 1 *c*).

The density of Stage I tmx was calculated by comparing their banding height in the 70% Percoll gradient with that of density marker beads (Pharmacia Fine Chemicals) of known density. The tmx band spans a density range from 1.099 to 1.108 g/ml, placing Stage I tmx among the denser organelles such as lysosomes and mitochondria (25).

Ca^{2+} Effects on tmx Expansion

The Ca^{2+} sensitivity of condensed tmx was maintained. Stage I tmx preparations exposed to 10^{-8} M (pCa 8.0) Ca^{2+} remained largely condensed (Fig. 1 *a*), while increasing the Ca^{2+} concentration to $>10^{-6}$ M (Fig. 1 *b*) causes expansion of the tmx to Stage III.

To determine whether the inhibition of matrix expansion by CaM antagonists in vivo (10) is due to a direct effect of these agents on the matrix, we exposed tmx preparations to CaM antagonists (14, 19, 34), and monitored expansion in response to a series of Ca^{2+}/EGTA buffers calculated to yield free Ca^{2+} ranging from 10^{-8} M to 10^{-3} M (pCa 8.0 to pCa 3.0).

Information on size and shape of macromolecules can be gained from their light scattering properties (6). We exploited this phenomenon to monitor the structural transition from the compact, highly condensed Stage I matrix to the extended rod-shaped Stage III matrix. Matrix expansion led to a decrease in the optical density when the turbidity of a tmx suspension was monitored using a spectrophotometer, measuring at 320 nm (OD_{320}).

As described in Materials and Methods, a small volume (~40 μl) of a tmx suspension that contained ~95% Stage I matrices in 5 mM EGTA buffer with no added Ca^{2+} was added to a cuvette that contained 720 μl of a Ca^{2+}/EGTA buffer adjusted to a desired free Ca^{2+} concentration. The results of such a turbidity assay are shown in Fig. 2. The Ca^{2+}

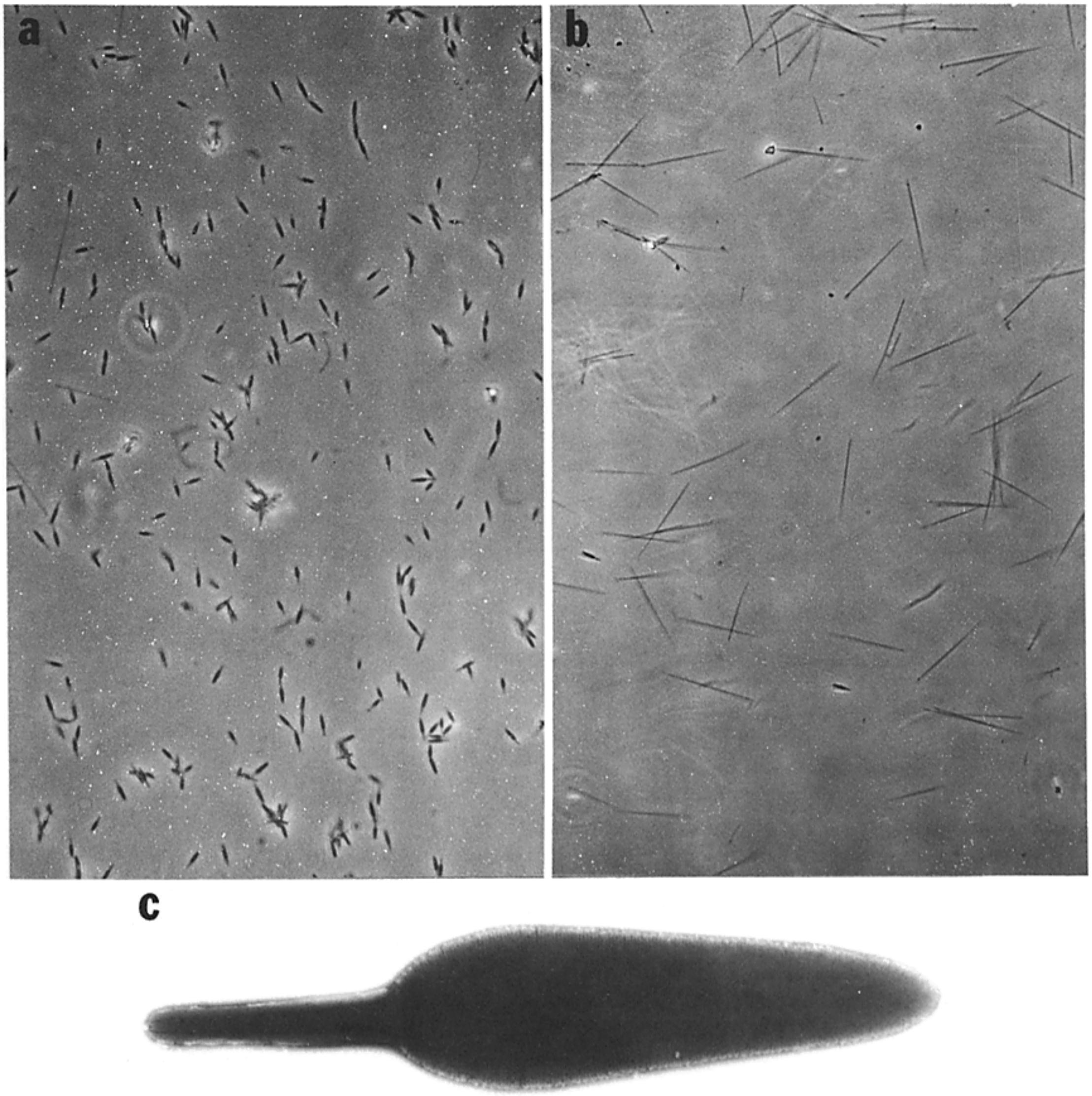

FIGURE 1 (*a*) Purified Stage I tmx collected from the Percoll gradient after wash. $Ca^{2+} = 10^{-8}$ M. (*b*) Ca^{2+} sensitivity of isolated tmx. Matrices exposed to 10^{-4} M Ca^{2+} expand to Stage III. (c) Negative stain image of isolated Stage I tmx. The periodic structure of the matrix is visible and the matrix has lost its membrane. (*a* and *b*) × 600. (c) × 26,000.

concentration is expressed as its pCa (pCa = $-\log[Ca^{2+}]$) equivalent. The pH of tmx suspension and Ca^{2+}/EGTA buffers in this experiment was 7.0. The OD_{320} of an unstimulated tmx suspension (5 mM EGTA buffer in cuvettes) (as indicated on the Y-axis in Fig. 2) was ~0.215. There was little change in the OD_{320} with increasing Ca^{2+} concentration (decreasing pCa) up to pCa 6.0. Below pCa 6.0, a rapid drop in OD_{320} occurred, which was essentially complete by pCa 5.5. Increasing Ca^{2+} concentration further had little effect. Light microscope examination of samples at different pCa's indicated that the drop in OD_{320} corresponded to tmx expansion. Preincubation of tmx in CaM antagonists at the indicated concentrations had no effect on matrix expansion, whether or not stimulation buffers contained CaM antagonists at the same concentration. Similar results were obtained when expansion was monitored by differential counts of Stage I versus Stage III tmx via phase-contrast microscopy: CaM antagonists did not affect matrix expansion in vitro.

A parameter that was found to influence Ca^{2+}-induced matrix expansion using this assay was pH. Fig. 3*a* is representative of an experiment showing Ca^{2+}-induced expansion as a function of pH. It is immediately obvious that the drop in OD_{320} corresponding to matrix expansion was shifted to the right or left, depending on pH of stimulation buffers. At pH 6.0, half-maximal expansion occurred at pCa 4.8 ($pCa_{1/2}$). Increasing the pH to 6.6–7.0 raised the $pCa_{1/2}$ to 5.5–5.7. At pH 7.4, $pCa_{1/2}$ was 6.2, and at pH 8.0, $pCa_{1/2}$ was 6.7 (Fig. 3*b*). Therefore, increasing pH allowed expansion to occur at lower Ca^{2+} concentration. This was the expected result since H^+ ions are presumably acting to displace or interfere with Ca^{2+} binding to sites on the tmx.

Some expansion appeared to occur at pH 8.0 even at low Ca^{2+} concentration (Fig. 3*a*). At pCa 7.0, OD_{320} is reduced from 0.195 to 0.174, a decrease of ~10%. A similar decrease in OD_{320} at pH 8.0 was also observed in another experiment at pCa 7.0. However, at lower Ca^{2+} concentration (pCa 8.0), OD_{320} at pH 8.0 was similar to that at pH 7.0 (data not shown), suggesting that an increase in pH alone is not suffi-

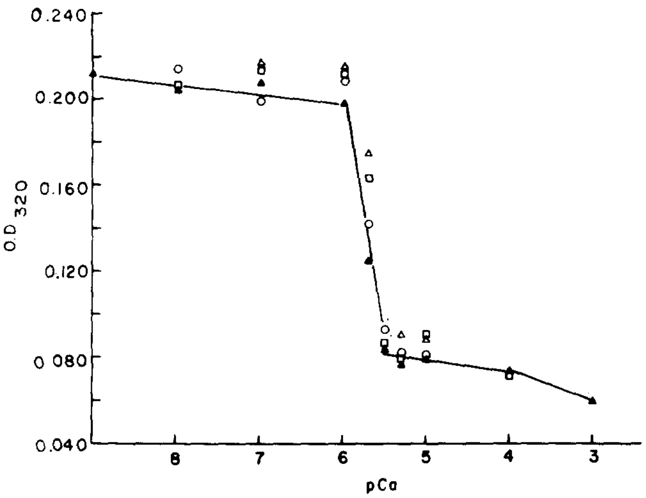

FIGURE 2 In vitro expansion of isolated tmx: effects of CaM antagonists (turbidity assay). Ca^{2+} concentration is expressed as its pCa equivalent (pCa = $-\log[Ca^{2+}]$). Control preparations (no drug addition) (▲) exhibit a sharp drop in OD_{320} corresponding to matrix expansion (Stage I to Stage III) below pCa 6.0. Preincubation of tmx in 30 μM trifluoperazine (△), 5 μM R24571 (○), or 40 μM N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (□) does not inhibit this expansion.

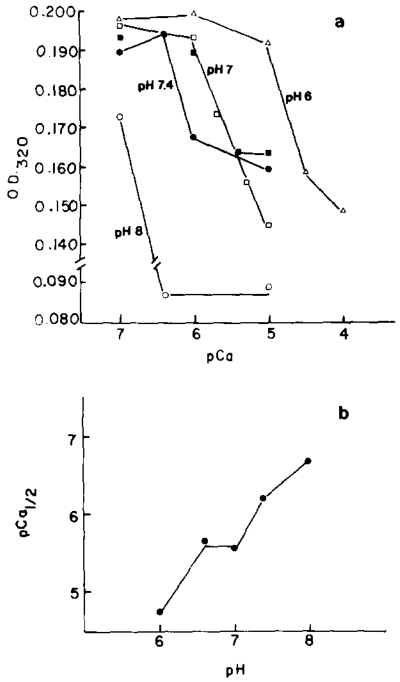

FIGURE 3 In vitro expansion of isolated tmx: effect of pH (turbidity assay). (*a*) In vitro expansion induced by Ca^{2+}/EGTA buffers of different pH: pH 8.0 (○); pH 7.4 (●); pH 7.0 (□); pH 6.6 (■); pH 6.0 (△). (*b*) $pCa_{1/2}$ for matrix expansion vs. pH. Data from three experiments.

cient to promote expansion, but instead it allows expansion at lower Ca^{2+}.

The effect of changes in pH at constant pCa is illustrated in Fig. 4. At pCa 7.0, raising the pH from 6.0 to 8.0 caused a slight drop in OD_{320}, indicating that a small fraction of the tmx undergo expansion at this low Ca^{2+} concentration at high pH. At pCa 6.0, alterations in pH between 6.0 and 7.0 did not promote expansion, but expansion occurred when pH was >7.0. At pCa 5.0, the change in OD_{320} was approximately linear with increasing pH. Therefore, at a subthreshold pCa (pCa 7.0), changing pH had little effect on expansion. At pCa 6.0, expansion occurred above a critical pH value, indicating a requirement for both pH and pCa to be in the correct range for expansion to occur. At pCa 5.0, Ca^{2+} did not appear to be limiting, and expansion occurred primarily as a function of pH.

The modulation of Ca^{2+}-induced in vitro expansion by changes in pH suggests that pH may play a role in vivo in regulating the state of the matrix. We examined this possibility by incubating *Paramecium* in acridine orange, a fluorescent amine that accumulates in acidic intracellular compartments (18). Figure 5*a* shows a phase-contrast micrograph of a cell after a 60-s exposure to acridine orange (10 μg/ml). One can see trichocysts docked beneath the plasma membrane along the cell periphery (arrows). Figure 5*b* shows the fluorescence image of the same cell. Elongate, fluorescent bodies within the cell cortex, lining the entire perimeter of the cell (arrows), are clearly visible. These correspond in shape, location, and number to trichocysts. This observation strongly suggests that in vivo, intratrichocyst pH is acidic.

In summary, in vitro studies of tmx indicate that: (*a*) at neutral pH, matrix expansion occurred at a Ca^{2+} concentration $>10^{-6}$ M; (*b*) matrix expansion in isolated Stage I tmx preparations was not inhibited by CaM antagonists; and (*c*) matrix expansion is dependent on pH as well as Ca^{2+}, showing inhibition by acidic pH and potentiation by alkaline pH. In addition, the fluorescent amine acridine orange accumulated within trichocysts in vivo, suggesting that pH may serve to modulate Ca^{2+}-induced expansion in vivo in a manner similar to that demonstrated in vitro.

DISCUSSION

Stage I tmx isolated according to the methods outlined in this report were devoid of their surrounding membranes (Fig. 2) yet maintained their characteristic paracrystalline appearance and exhibited the Ca^{2+}-dependent expansion reaction that normally accompanies release. We have examined this com-

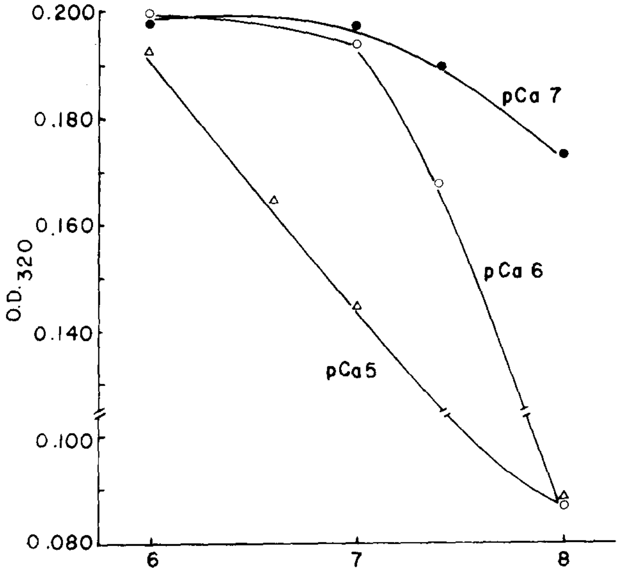

FIGURE 4 In vitro expansion of isolated tmx: effect of pH at constant pCa (turbidity assay).

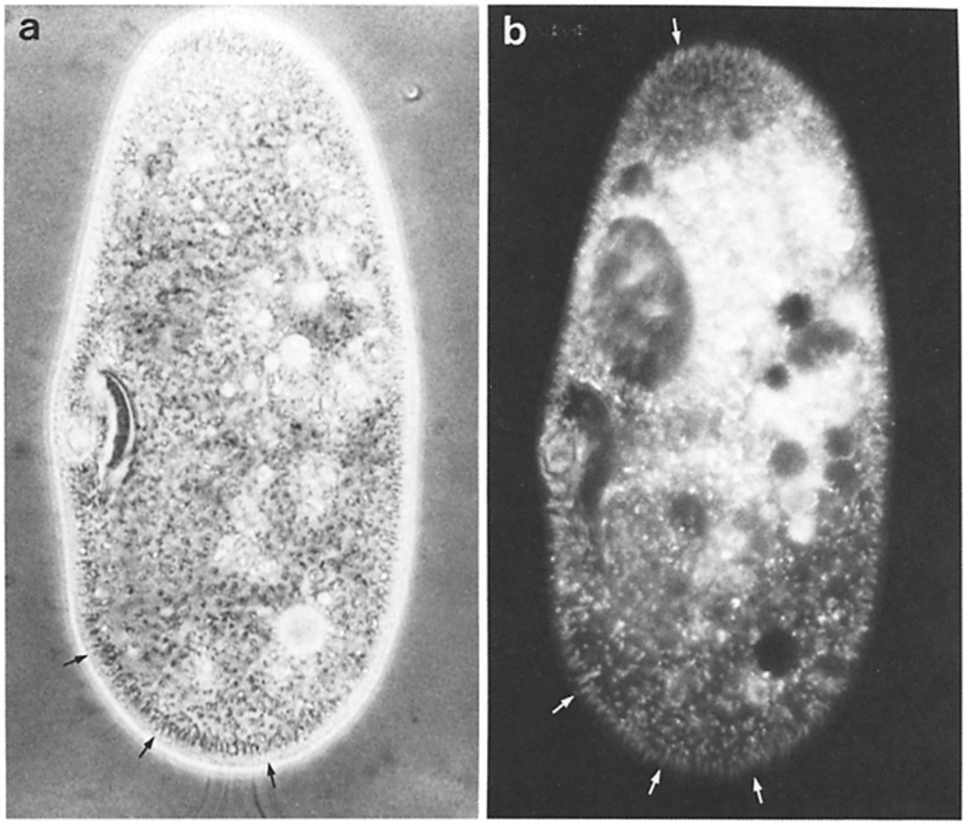

FIGURE 5 Acridine orange staining of *Paramecium*. (*a*) Phase-contrast micrograph of a *Paramecium* cell after incubation in acridine orange (10 μg/ml). One can see trichocysts docked beneath the plasma membranes along the cell periphery (arrows). (*b*) Fluorescent image of the same cell indicates that acridine orange accumulates in the trichocysts. The trichocysts are now more clearly visible as elongate fluorescent bodies within the cell cortex that line the entire perimeter of the cell (arrows).

ponent of the release reaction in isolation to determine if it is the site of CaM antagonist action in vivo. We found that CaM antagonists did not inhibit expansion of the isolated, functional secretory granule content. Therefore, inhibition of matrix expansion and secretion by CaM antagonists in vivo (10) was not due to a direct interaction of these agents with the tmx itself. Inhibition in vivo is due to an effect of CaM antagonists outside the matrix.

The influx of extracellular Ca^{2+} that follows stimulation in vivo is probably sensed initially by an intracellular Ca^{2+} receptor, most likely cytoplasmic CaM. The route of Ca^{2+} into the secretory vesicle seems to be via the cytoplasm since mutants incapable of membrane fusion (3, 5) exhibit matrix expansion when stimulated (11). This matrix expansion is also inhibitable by CaM antagonists (11). Although we consider it likely that the primary target of the CaM antagonists is cytoplasmic Ca^{2+}-CaM complexes (10), we cannot yet rule out an effect on other cellular targets, including Ca^{2+} and phospholipid-dependent protein kinase (protein kinase C) (22). Keeping this caveat in mind, we can discuss a potential role for Ca^{2+}-CaM complexes in initiating matrix expansion in vivo that is consistent with the data presented here. Cytoplasmic Ca^{2+}-CaM complexes that are formed after stimulation appear to act at the trichocyst membrane to initiate ionic changes that permit and/or activate matrix expansion. CaM antagonists do not block the influx of Ca^{2+} into the cytoplasm (23), but prevent the initiation at the trichocyst membrane of the critical changes leading to Ca^{2+} access to the trichocyst matrix. Our working hypothesis is depicted in Fig. 6.

The Effect of pH on Matrix Expansion

At low pH (6.0), matrix expansion required a higher Ca^{2+} concentration than at neutral pH (Fig. 3). Thus, tmx Ca^{2+}-binding sites are likely to resemble those of troponin C and CaM, which exhibit pH-modulated Ca^{2+} binding (7, 32).

The influence of pH on expansion might suggest that it is not Ca^{2+} alone that regulates this process in vivo. If pH within the trichocyst vesicle was maintained at a low value, expansion would not occur unless intratrichocyst Ca^{2+} became very high. This would provide an additional regulatory or "safety" mechanism; both pH and Ca^{2+} concentration would have to be in the correct range for expansion to occur. Preliminary evidence shown here (Fig. 5) indicates that the intratrichocyst pH is indeed acidic. Brief exposure of *Paramecium* to acridine orange leads to uptake and concentration of the dye within organelles corresponding in shape, location, and number to the trichocysts. Further work by Busch and Satir (8) has shown that *Paramecium* mutants lacking trichocysts also lack the elongate fluorescent bodies described here, and that proton ionophores eliminate the trichocyst fluorescence, indicating that the acridine orange distribution is dependent on an existing pH gradient across the trichocyst membrane.

Low intragranular pH may be a common feature of secretory granules. The pH of the interior of neurosecretory gran-

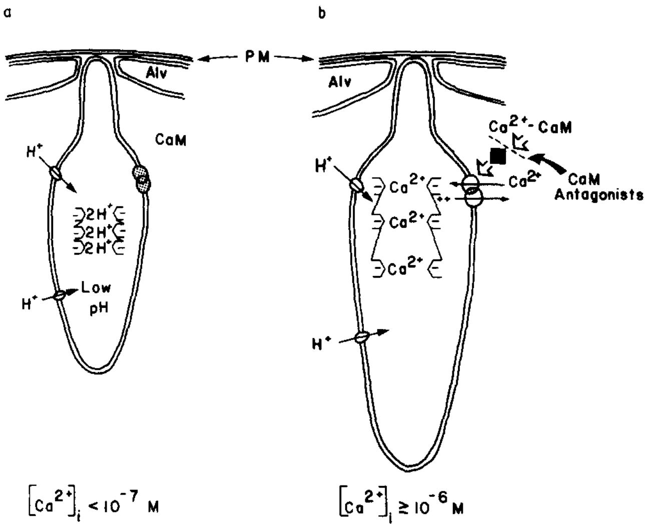

FIGURE 6 Model of regulation of secretion in *Paramecium*. (*a*) In the unstimulated condition, Ca_i^{2+} is low ($<10^{-7}$ M), CaM is largely Ca^{2+}-free, and the tmx is condensed (Stage I). The interior of the trichocyst vesicle may be maintained at a low pH by a proton pump in the trichocyst membrane. Within the acidic secretory granule, the interaction of H^+ with the matrix will help to maintain the condensed state, as demonstrated for matrix expansion in vitro. (*b*) After stimulation, Ca_i^{2+}, rises above 10^{-6} M and cytoplasmic Ca^{2+}-CaM complexes are formed. These complexes are thought to be the primary target for CaM antagonists, although other cellular targets cannot be ruled out. Data from previous studies (10) suggests that Ca^{2+}-CaM complexes act at the trichocyst membrane to control access of Ca^{2+} to the matrix. Whether CaM acts via direct binding to trichocyst membrane components, or via CaM-activated regulatory enzymes such as kinases or phosphatases, is not known (indicated by the black box). Transport of Ca^{2+} into the trichocyst may occur through a gate or channel in the membrane, or may be coupled to the outward movement of protons by an antiport mechanism. In this manner, stored energy in the form of a chemiosmotic gradient would be used to promote matrix expansion, while at the same time removing Ca^{2+} from the cytoplasm and thus terminating the signal for release. Ca^{2+} within the vesicle can then bind to sites on the tmx and lead to expansion. Membrane fusion must occur in a coordinated fashion with matrix expansion to allow release of secretory products to the extracellular space. (Alv, alveolar sacs; PM, plasma membrane).

ules from the posterior pituitary (29) and chromaffin granules (17, 26) has been determined to be acidic (pH 5.7–5.8). The low pH is maintained by a Mg^{2+}/ATP-driven proton pump ATPase in the chromaffin granule (4, 15, 26, 30) and is also Mg^{2+}- and ATP-dependent in neurosecretory granules of the posterior pituitary (29). The function of low intragranular pH is unclear; however, the observation reported here, that low pH inhibited matrix expansion, suggests that a primary function of low intragranular pH may be to preserve the integrity of storage complexes of granule contents. Indeed, the crystalline cores of a number of secretory granules are stabilized by low pH and solubilized by pH above 7.0 (9, 16, 28, 33). The observation of an analogous effect on the secretory product of *Paramecium* suggests that low intragranular pH may have arisen early in evolution as a means of maintaining secretory granule contents in a storage form.

After the stimulus-induced rise in cytoplasmic Ca^{2+} and the postulated Ca^{2+}/CaM complex formation, a coordinated mechanism can be envisioned which causes a rise in intravesicular pH as well as Ca^{2+} concentration (Fig. 6), bringing both parameters within the range where expansion can occur. We can speculate that low granule pH would establish a proton gradient across the vesicle membrane which could be used to promote Ca^{2+} accumulation within the vesicle. By invoking a Ca^{2+}/H^+ exchange mechanism or antiport, Ca^{2+} concentration and pH would rise concurrently, and optimal conditions for expansion would be achieved. Such an exchange mechanism for Ca^{2+} accumulation has been proposed for pancreatic β-cell insulin granules (13, 24) which have been shown to maintain a low intragranular pH (1).

Exocytosis includes the fusion of secretory organelle and plasma membranes as well as the resultant release of organelle content. This model aims to suggest that product release is not solely contingent on membrane fusion, but is in itself controlled by regulation of the state of granule content. Membrane fusion and expansion of content are coordinated but separable events and may have distinct regulatory mechanisms as suggested here. This is also suggested by images of exocytic fusion occurring in the absence of product release when tmx expansion is blocked (12). Similarly, membrane fusion is not solely contingent on matrix expansion, as evidenced by "pseudoexocytosis" in fusion-incompetent secretory mutants stimulated with ionophore A23187 (12, 21). Thus, while these two events appear to share a requirement for Ca^{2+}, they exhibit distinct requirements as well.

The clear parallels between trichocysts and other secretory granules with regard to the crystalline appearance of vesicle content, the effect of pH on granule content, and the Ca^{2+} requirement for release suggests that a large number of common features have been evolutionarily conserved, and additional parallels will become evident with further study.

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