Stimulation and Inhibition of Secretion in *Paramecium*: Role of Divalent Cations

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ABSTRACT The effects of Ca^{2+} and Mg^{2+} on exocytosis in *Paramecium tetraurelia* cells were examined with light microscopy, freeze fracture (FEM) and transmission electron microscopy (TEM) of thin-sectioned embedded cells. Picric acid-Ca^{2+}-induced secretion in wild type (wt) cells was captured by "quick" fixation with OsO_4, and TEM demonstrated membrane fusion occurring before trichocyst matrix (tmx) expansion. Cells stimulated with picric acid in the presence of high extracellular Mg^{2+} showed very few sites of membrane fusion and no tmx expansion, suggesting that Ca^{2+} is required for both membrane fusion and tmx expansion. Further information was obtained by comparing secretory responses of wt cells with a temperature-sensitive secretory mutant, nd 9. These cells when grown at the permissive temperature (18°C) possess normal rosettes at the secretory site and secrete in response to picric acid-Ca^{2+}, but when grown at 27°C they lack rosettes and do not secrete (Beisson, J., M. Lefort-Tran, M. Pouphile, M. Rossignol, and B. Satir, 1976, *J. Cell Biol.*, 69:126–143). Quantitation of picric acid-Ca^{2+}-induced secretion revealed that: (*a*) the number of tmx secreted by wt and nd 9 cells was independent of their cultural growth phase, (*b*) wt cells secreted the same number of tmx when grown either at 18 or 27°C, and (c) nd 9 18°C cells secreted the same number of tmx as wt 18 or 27°C cells. Wild type and nd 9 cells had the same frequencies of occupied and unoccupied secretory sites as determined by quantitative analysis of freeze-fracture replicas. After stimulation with divalent cation ionophore A23187 and Ca^{2+}, wt cells showed a significant reduction in the frequency of occupied sites. FEM and TEM studies revealed that A23187-Ca^{2+} induced tmx expansion and normal fusion of the plasma and trichocyst membranes in wt and nd 9 18°C cells, but induced tmx expansion without concomitant membrane fusion in nd 9 27°C cells. The lack of membrane fusion in nd 9 27°C cells suggests that the molecules represented by rosette particles are required specifically for membrane fusion.

In many secretory systems Ca^{2+} is required for stimulus-secretion coupling (6). An extracellular stimulus induces a series of molecular changes that result in Ca^{2+} influx, fusion of the secretory organelle's membrane with the plasma membrane, and release of the secretory content to the external environment. However, the exact role of Ca^{2+} in bringing about the fusion of lipid bilayers has yet to be determined. The ciliated protozoan *Paramecium tetraurelia* offers many advantages for studying Ca^{2+}-dependent exocytosis. The cortex of the cell consists of rows of cortical units, which measure ~1 by 1.5 μm, each containing a potential site for exocytosis. The cell is ~150-μm long and 50 μm in diam, thus, there can be a large number of nearly simultaneous secretory events per cell. In addition, each site where exocytosis occurs can be easily identified in freeze-fracture electron microscopy (FEM). Freeze-fracture particle arrays marking the secretory site have been described for this and other cells (14, 22, 24, 26, 27, 34) and shown to be of essential importance using secretory mutants of *Paramecium* and *Tetrahymena* cells (4, 18).

The secretory organelle in *Paramecium* is the trichocyst, which contains a paracrystalline protein matrix. Upon fusion of plasma and trichocyst membranes, the trichocyst matrix expands and leaves the cell as a paracrystalline structure eight times its original length. Secretion is easily detected because the expanded trichocyst matrix is visible in the light microscope as a 20–40-μm long needle. We shall use here the designation "trichocyst" solely for the cytoplasmic, membrane-bounded secretory vesicle and "trichocyst matrix" (tmx) for the secreted content.

Secretion occurs very quickly in *Paramecium*, and therefore

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it is difficult to dissect the release response into particular stages such as Ca^{2+} influx, membrane fusion, and trichocyst matrix expansion. However, membrane fusion and tmx expansion are events that can be monitored ultrastructurally and to eventually understand the molecular interactions that regulate secretion, it is necessary to first distinguish and characterize conditions that stimulate or inhibit membrane fusion and tmx expansion.

Satir and Oberg (28) observed with light microscopy that Mg^{2+} inhibits secretion in *Paramecium*. The specific role of Mg^{2+} in inhibiting secretion is not clear because isolated trichocyst matrices undergo expansion in vitro upon addition of Ca^{2+} (5, 10). By substituting Mg^{2+} for Ca^{2+} and examining cells with transmission electron microscopy (TEM) and FEM, it can be tested whether Mg^{2+} inhibits secretion by inhibiting membrane fusion, tmx expansion, or both, thus allowing us to determine whether Ca^{2+} is required for both membrane fusion and tmx expansion in vivo.

Another approach to study the role of Ca^{2+} in secretion in *Paramecium* is to compare the responses of a secretory mutant, nd 9, with wt cells using different secretagogues. This temperature sensitive (ts) secretory mutant secretes and displays the usual secretory site particle array (rosette) when grown at 18°C (permissive temperature) but when grown at 27°C (nonpermissive temperature), lacks rosettes and is incapable of secretion (4). The response of this mutant to divalent cation ionophore and Ca^{2+} vs. Mg^{2+} provides further clarification of the specific role(s) of Ca^{2+} in membrane fusion and tmx expansion. In this paper, we show how these two approaches (effect of Mg^{2+} vs. Ca^{2+} and use of a secretory mutant) permit a preliminary dissection of the two closely linked events, membrane fusion and tmx expansion, which may help in understanding the underlying molecular processes.

MATERIALS AND METHODS

Cultures of *P. tetraurelia* were grown in bacterized (*Enterobacter aerogenes*) medium consisting of 0.25% Cerophyll and 0.05% Na_2HPO_4 (31). Wild type cells and a ts secretory mutant (nd 9) were cultured at either 18°C (permissive temperature) or 27°C (nonpermissive temperature). The temperature that follows the abbreviation "wt" or "nd 9" designates the temperature at which the cells are grown. All experiments were performed at room temperature (22°C).

Picric Acid-induced Secretion (Light Microscopy): Cells were harvested in early log phase (500 cells/ml), mid-log phase (2,000 cells/ml), and stationary phase (5,000 cells/ml), filtered through cheesecloth to remove bacteria, and spun at 1,000 rpm for 2 min in an IEC clinical centrifuge (International Equipment Co., Needham Heights, MA). Cells were then washed in buffer (5 mM Tris HCl, 1 mM KCl, 0.1 mM EDTA, pH 7.4) containing either 5–15 mM $CaCl_2$ or 5–15 mM $MgCl_2$, and resuspended in this buffer at a concentration of ~10,000 cells/ml. 5 μl of this cell suspension was placed on a glass slide and 10 μl of aqueous saturated picric acid (PA) added. In addition, wild type cells at stationary phase were tested directly in growth medium as a control for loss of tmx due to centrifugation and rinsing.

The released tmx were counted on phase-contrast micrographs taken at several different focal levels for each cell. Expanded trichocyst matrices were traced on each micrograph and the tracings from one cell were superimposed to prevent counting the same tmx more than once. No less than 12 cells were included in each experimental group.

Ionophore-induced Secretion (Light Microscopy): Cells are harvested, washed, and concentrated as described above, and stimulated to secrete by the divalent cation ionophore A23187 (Eli Lilly, Inc. or Calbiochem-Behring, Corp., American Hoechst Corp., La Jolla, CA) in the presence of either Ca^{2+} or Mg^{2+}. A 1-mM stock solution was prepared by dissolving A23187 in dimethyl sulfoxide (DMSO) (25); dilutions were made with Ca^{2+} or Mg^{2+} buffer. 5 μl of cell suspension was placed on a slide and 10 μl of 40 μM A23187 was added (final concentration of A23187 was 27 μM in 2.7% DMSO). This concentration was chosen to stop the cells from swimming, thus keeping the tmx within a small area surrounding the cell. Phase-contrast micrographs were used for quantitation of the number of released tmx as described.

40 μM A23187 and Mg^{2+}, in contrast with PA or 40 μM A23187 and Ca^{2+}, does not cause cells to stop swimming. Cells were fixed directly with OsO_4 after 2 min of exposure to A23187 and Mg^{2+}, and tmx were counted. Control cells exposed to DMSO (up to 4%) remained alive and swimming until fixation with OsO_4.

FEM: The frequency of particle arrays (rings, parentheses, and partial arrays) was determined for unstimulated wt 27°C and nd 9 18°C cells, which were rinsed in buffer containing 10 mM $MgCl_2$ (to prevent secretion) and fixed with 2.5% glutaraldehyde. The frequency of the arrays was determined in wt 27°C cells stimulated by ionophore (final concentration was 15 μM A23187, 1.5% DMSO as solvent) and 5 mM $CaCl_2$ for 3 min before fixation with 2.5% glutaraldehyde. Mutant cells (nd 9 27°C) either in Ca^{2+} buffer or exposed to A23187-Ca^{2+} were fixed as above. All cells were fixed with glutaraldehyde (2.5%) for 30 min, rinsed with buffer, transferred to 20% glycerol for at least 2 h, and frozen in supercooled liquid nitrogen (−205°C) or liquid propane (−185°C). Samples were fractured in the double-replica device of the Balzers freeze fracture apparatus (BAF 301; Balzers, Hudson, NH) and platinum-carbon replicas were made. Electron micrographs were taken of plasma membrane fracture faces (at least five different cells for each experiment) and the frequencies of rings, parentheses, and partial arrays at the secretory sites were determined.

TEM: Wild type cells in either Ca^{2+} or Mg^{2+} buffer were fixed with a mixture of PA and OsO_4 for 20–30 s, rinsed with 2% glutaraldehyde, then fixed with 2% glutaraldehyde for 30–60 min, followed by postfixation with 1% OsO_4 for 20–60 min. Cells exposed to 15 μM ionophore and 5 mM Ca^{2+} were "quick" fixed with OsO_4 for 20–30 s, followed by fixation with glutaraldehyde and postfixation with OsO_4. DMSO (ionophore solvent) precipitated upon addition of OsO_4; rinsing with glutaraldehyde removed the precipitate. Cells were examined under both conditions.

Samples were dehydrated in a series of increasing concentrations of ethanol (30–100%), stained en bloc with 2% uranyl acetate, and embedded in Epon. Blocks were thin sectioned with a Reichert microtome, stained with 1% uranyl acetate (aqueous) and lead citrate, and examined in either Siemens 101 or Jeol 100-CX electron microscopes.

RESULTS

Quantitation of Secretion

Fig. 1 illustrates the secretory response of three different cell types (wt 27°C; nd 9 27°C, nonpermissive; nd 9 18°C, permissive) under three different conditions. Stimulation by saturated PA in the presence of 15 mM $MgCl_2$ induced release of very few tmx (Fig. 1, *a–c*). However, in the presence of 15 mM $CaCl_2$, PA induced both wt 27°C and nd 9 18°C cells to release tmx (Fig. 1, *d* and *f*), in contrast with nd 9 27°C cells, which released very few, if any tmx (Fig. 1*e*). Addition of divalent cation ionophore A23187 in the presence of Ca^{2+} appears to cause all three cell types to release tmx (Fig. 1, *g–i*) as observed in a previous study (28). Comparison of the response is limited by this approach. The response was therefore quantitated to elucidate: (*a*) differences between wt cells and secretory mutants, and (*b*) differences between PA and A23187 as secretagogues.

Experiments were performed to determine (*a*) if the number of tmx released by *Paramecium* depends upon cultural growth phase, and (*b*) if secretion in wt cells is affected by the growth temperature (18 vs. 27°C). Table I shows that the number of tmx released by cells in different growth phases varies somewhat, but the variations are not consistent. The mean number of tmx released per cell for each cell type ranges from 271 to 280. Undisturbed wt cells tested directly from a culture flask release 393 ± 25 (n = 12) tmx per cell when stimulated with PA. This suggests that ~30% of trichocysts available for secretion are lost during centrifugation and rinsing.

Table I also shows that the number of tmx released by wt cells is independent of growth temperature and that the nd 9 mutant grown at the permissive temperature releases as many tmx as wt cells grown at 18 or 27°C. Since the number of released tmx is independent of the growth phase, late log or

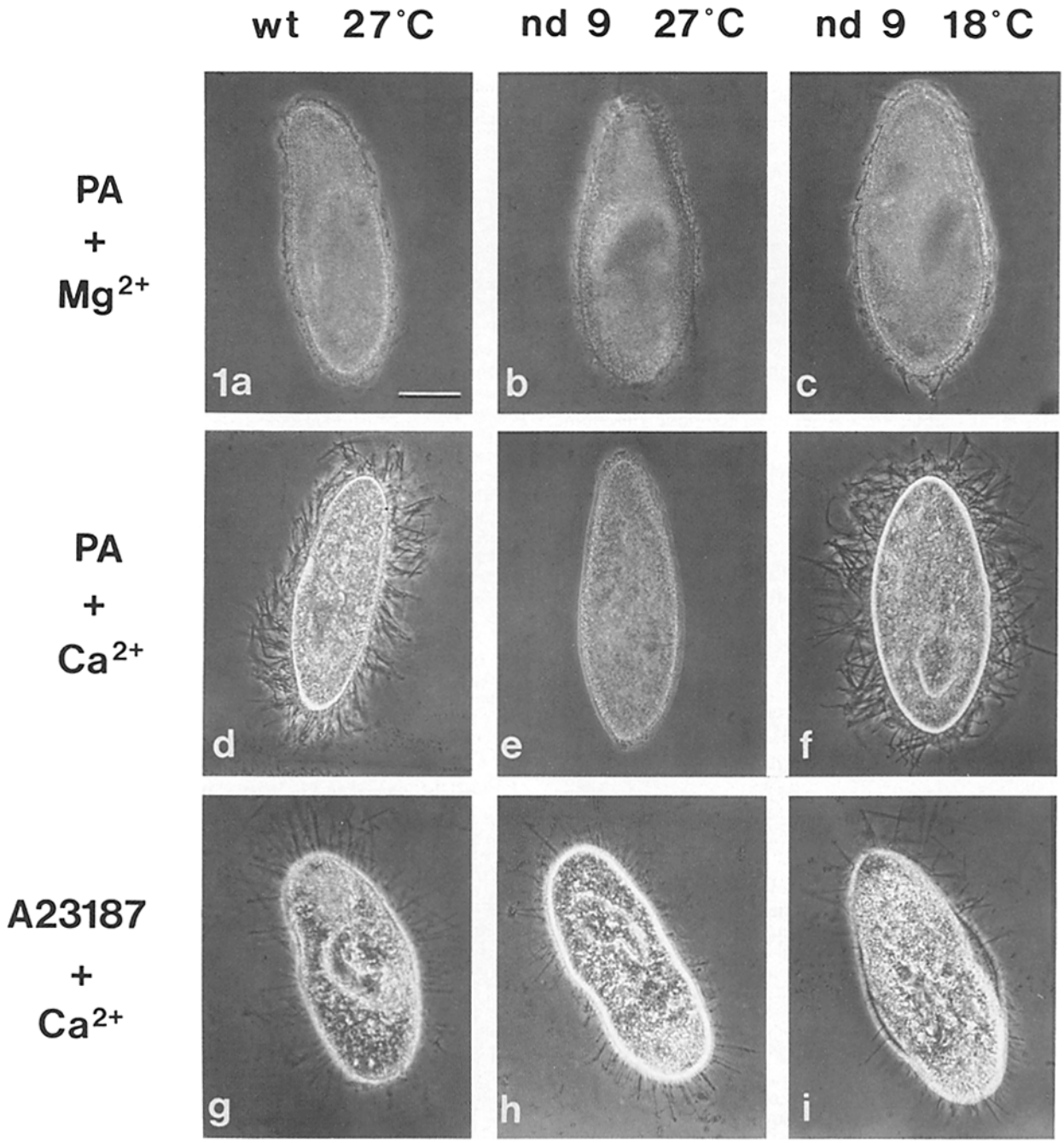

FIGURE 1 Secretory responses of wt and ts mutant cells are shown here in phase-contrast micrographs. The cell type is indicated at the head of each column and the conditions of stimulation to the left of each row. All three cell types in the presence of 15 mM $MgCl_2$ do not secrete in response to the standard secretagogue, saturated picric acid (*PA*) (*a*, *b*, *c*). When Ca^{2+} (15 mM) is substituted for Mg^{2+}, wt and nd 9 18°C (permissive) cells secrete in response to *PA* (*d*, *f*); nd 9 27°C (nonpermissive) cells do not secrete under identical conditions (e). The secretory product, expanded trichocyst matrices (tmx), appears as a halo of needles surrounding the cell. Ionophore A23187 (40 μM) in the presence of 15 mM $CaCl_2$ induces the release of secretory product from all three cell types (*g*, *h*, *i*). Bar, 25 μm; × 380.

early stationary phase cells were routinely used to obtain greater densities of cells. In addition, wt cells grow much faster at 27°C, so mutant cells grown at 18 or 27°C were compared directly to wt 27°C cells.

The observation that nd 9 27°C cells respond to A23187, but not to PA (Fig. 1*h* vs. 1*e*) suggests that PA and A23187 induce secretion via different mechanisms. The mutation in nd 9 cells interferes with one mechanism, but not the other. The responses of mutant and wt cells to the two secretagogues under different ionic conditions are compared in Table II.

As shown by light microscopy (Fig. 1, *g–i*), A23187 in the presence of Ca^{2+} induced a significant secretory response in all three cell types, while the ionophore solvent DMSO, in the presence of Ca^{2+}, did not induce significant secretion. Table II also confirms the previous report (28) that A23187-induced secretion was dependent upon extracellular Ca^{2+}

TABLE I

Secretion as a Function of Growth Phase and Temperature*

Cultural growth phase	500 cells/ml (early log)	2,000 cells/ml (mid-log)	5,000 cells/ml (stationary)	Average for all growth phases
wt 27°C	262 ± 20‡	284 ± 16‡	301 ± 26‡	280 ± 12‡
	$n = 16$§	$n = 12$	$n = 12$	$n = 40$
wt 18°C	303 ± 21‡	229 ± 19	298 ± 28‡	275 ± 14‡
	$n = 12$	$n = 13$	$n = 12$	$n = 37$
nd 9 18°C	302 ± 22‡	264 ± 15‡	235 ± 22	271 ± 13‡
	$n = 18$	$n = 12$	$n = 13$	$n \pm 43$

* Stimulated with saturated PA in the presence of 15 mM $CaCl_2$; expressed as mean number of tmx per cell ± SEM.
‡ These values are not significantly different.
§ n is the number of cells scored.

TABLE II

Secretion as a Function of Secretagogue and Cation*

	PA-Ca^{2+}	A23187‡-Ca^{2+}	A23187-Mg^{2+}	DMSO§-Ca^{2+}
nd 9 27°C	0.2 ± 0.1	127 ± 9	0.3 ± 0.2	1.2 ± 0.6
(nonpermissive)	$n = 12$	$n = 19$	$n = 35$	$n = 12$
nd 9 18°C	271 ± 13	201 ± 18	6.7 ± 1.9	5.4 ± 1.8
(permissive)	$n = 43$	$n = 12$	$n = 15$	$n = 12$
wt 27°C	280 ± 12	235 ± 18	3.6 ± 1.4	3.0 ± 0.6
	$n = 40$	$n = 12$	$n = 18$	$n = 14$

* Expressed as mean number of tmx per cell ± SEM.
‡ Final concentration: 27 μM A23187, 2.7% DMSO (solvent).
§ Final concentration: 2.7% DMSO.

because no significant secretion occurred when Mg^{2+} was substituted for Ca^{2+}. However, A23187-Ca^{2+} induced significantly less ($P < 0.05$) secretion in wt 27°C and nd 9 18°C cells than did PA-Ca^{2+}. These two cell types consistently secreted the same number of tmx under the same conditions. Although A23187-Ca^{2+} induced nd 9 27°C cells to release tmx (Fig. 1*h*), the response was significantly less ($P < 0.01$) than the response of nd 9 18°C or wt 27°C cells to A23187-Ca^{2+}. Cells stimulated with A23187-Ca^{2+} were next examined with FEM in order to obtain more information about quantitative differences in their secretory response.

Quantitation of Particle Arrays in Freeze Fracture

Trichocysts docked in place under the cell membrane are represented in FEM by rings of intramembrane particles (IMP); unoccupied sites (no docked trichocysts) are recognized by parentheses of IMP (4). Fig. 2 shows the P fracture face of the plasma membrane of a wt cell, and demonstrates the rings and parentheses that are associated with occupied and unoccupied secretory sites, respectively. The 9–11 larger particles in the center of each ring constitute the rosette; nd 9 27°C cells have rings, but incomplete or missing rosettes. Cells that have been stimulated to secrete are expected to show a decrease in the frequency of occupied sites (rings) and no immediate change in the frequency of unoccupied sites (parentheses).

Quantitation of particle arrays at the secretory site is summarized in Table III. Unstimulated wt and nd 9 cells grown at either temperature showed statistically the same frequency for each type of array. nd 9 27°C cells have 1–6 particles per rosette, while nd 9 18°C and wt cells have 9–11 particles, as shown earlier (4). A significant reduction in the frequency of rings was observed in wt cells stimulated with A23187 (15 μM) and Ca^{2+} (5 mM) for 3 min, indicating a decrease in the number of docked trichocysts after release of tmx. As expected, the frequency of parentheses (representing unoccupied secretory sites) remained unaffected by stimulation of exocytosis, but the frequency of partial arrays, representing sites where exocytosis recently occurred (20), increased significantly. Therefore, the FEM results confirm and extend the quantitative light microscopic results with regard to wt 27°C cells.

Glutaraldehyde and Ca^{2+} induce secretion in wt but not in nd 9 27°C cells (21). FEM of wt and nd 9 18°C cells fixed in the presence of 10 mM Mg^{2+} to inhibit glutaraldehyde-induced secretion suggest that Mg^{2+} inhibits membrane fusion because the frequency of partial arrays is very low (Table III). The frequency of rings in Mg^{2+}-inhibited cells was identical to the frequency of rings in the secretory mutant grown at the nonpermissive temperature. Assuming that all cells have the same number of cortical units, the quantitative differences in secretory response demonstrated in Table II cannot be due to differences in number of trichocysts available for secretion.

Freeze-fracture replicas of nd 9 27°C cells stimulated with A23187-Ca^{2+} appear quite different from those of wt or nd 9 18°C cells. The frequencies of particle arrays could not be determined and the appearance of the plasma membrane will be discussed in a later section. TEM observations of secretion in wt and mutant cells, presented next, will elucidate these differences and provide further evidence of the specific inhibitory effects of Mg^{2+}.

Electron Microscopy of PA-induced Secretion

Fig. 3*a* shows a resting trichocyst tip (*tt*) closely apposed to the plasma membrane (*pm*). The organelle is completely surrounded by a membrane (*tm*). A continuous system of alveolar sacs (subsurface cisterns, 29) lies under the plasma membrane penetrated by trichocysts and cilia. The trichocyst matrix is divided into body and tip, both consisting of densely packed material with transverse striations of 7 nm (body) and

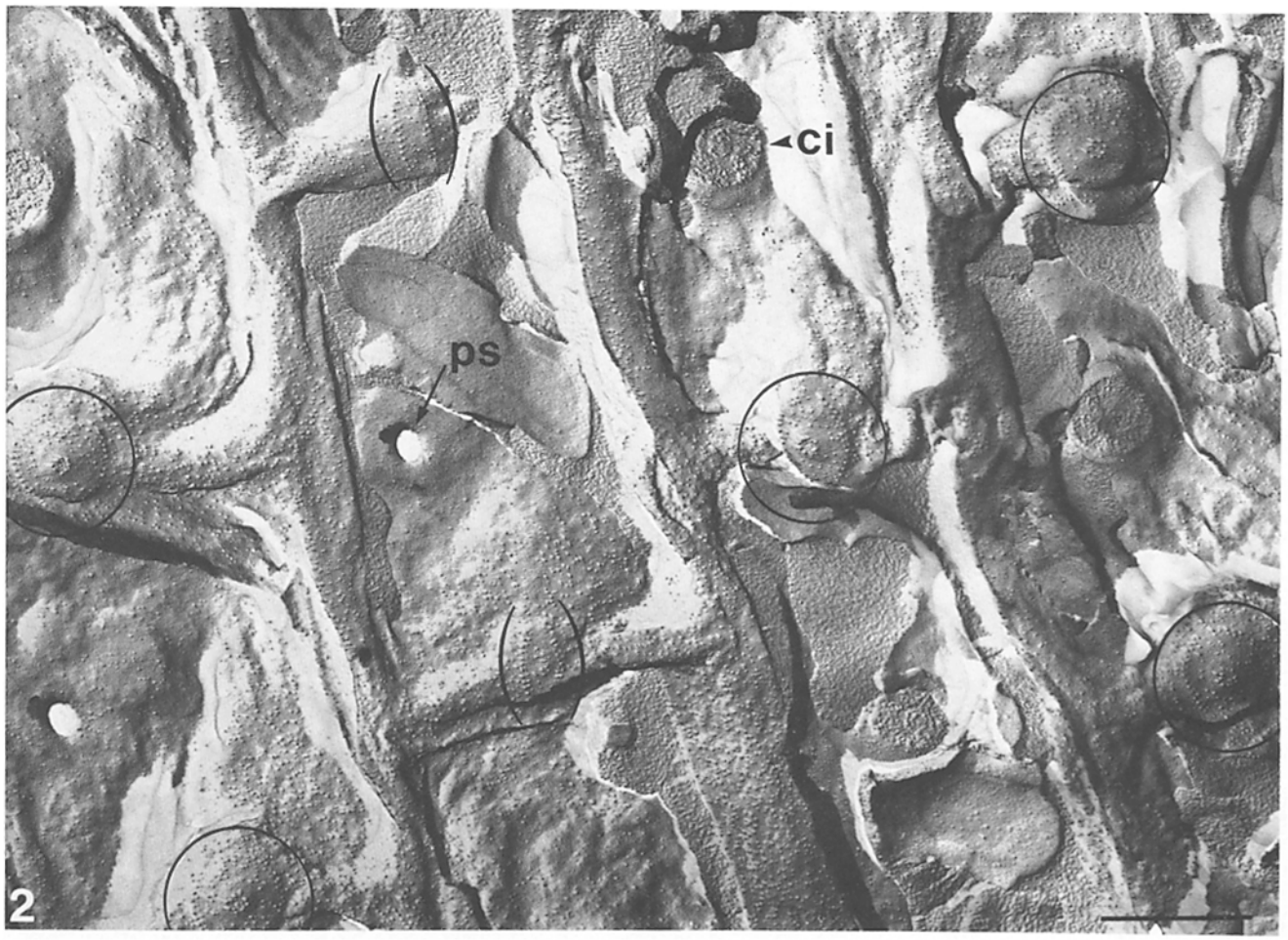

FIGURE 2 A portion of a freeze-fracture replica of a wt cell illustrates two distinct particle arrays found on the protoplasmic face of *Paramecium* plasma membranes. Both particle arrays, rings (circled), and parentheses designate secretory sites, but rings are characteristic of secretory sites with docked trichocysts, whereas parentheses mark unoccupied sites. This distinction permits quantitation of occupied and unoccupied sites in cells before and after secretion (see Table III). Rosettes of 9–11 large particles are located in the center of the rings. The secretory sites typically occur between cross-fractured cilia (*ci*) and each cilium is associated with an endocytic site (parasomal sac, *ps*). Bar, 0.5 μm; × 40,960.

TABLE III

Frequency of Particle Arrays at the Trichocyst Docking Site

	Rings	Parentheses	Partial arrays	Total sites
	%	%	%	
nd 9 27°C* (nonpermissive)	73	23	4	290
nd 9 18°C‡ (permissive)	75	19	6	52
wt 27°C‡	78	15	7	189
wt 27°C + A23187 and Ca^{2+}	4	20	64§	79

* Fixed in the presence of 10 mM $CaCl_2$.
‡ Fixed in the presence of 10 mM $MgCl_2$.
§ 9% early fusion sites and 3% crossfractured tmx were found in addition to the particle arrays.

15 nm (tip) periodicity (2, 13). The tip retains its 15-nm periodicity after expulsion from the cell, whereas the body has been observed in two states of expansion (2, 9, 13). In unstimulated cells, the trichocyst body is observed in the condensed state (Stage I) as in Fig. 3*a*. Fixation for electron microscopy may induce partial expansion to an electron-lucent state (Stage II), while the fully expanded state with 60-nm transverse striations (Stage III) is normally only observed outside the cell following secretion.

Fusion of the plasma and trichocyst membranes results in the formation of a narrow tubular connection between the extracellular environment and the content of the trichocyst (12). Fig. 3,*b–d* show an example from a wt cell induced to secrete with picric acid and Ca^{2+}. In this case, membrane fusion occurred before tmx expansion. Fig. 3*b* shows fusion (arrows) of the plasma and trichocyst membranes, forming a tubular opening 22 nm in diameter. (The diameter of a rosette is 75 nm.) Fig. 3*c* shows the fused membranes in a section serial to Fib. 3*b* and the outer opening of the tube is cut obliquely (arrow). Fig. 3*d* is a low-power micrograph of this

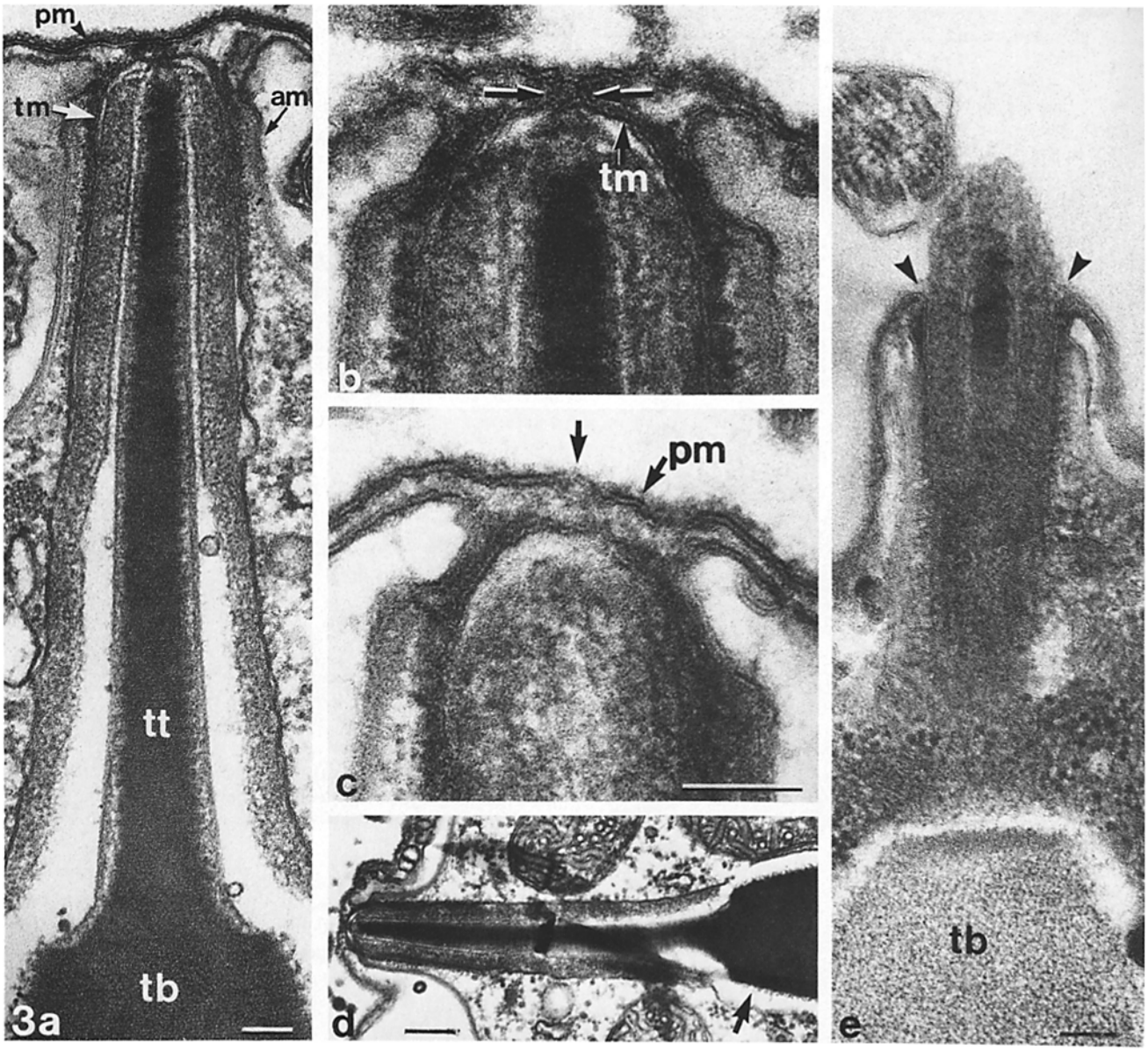

FIGURE 3 A trichocyst docked at the plasma membrane (*pm*) is shown in an unstimulated nd 9 18°C cell (*a*). Trichocysts interrupt the continuous alveolar sac system (alveolar membrane, *am*). The secretory organelle consists of a body (trichocyst body, *tb*) and tip (trichocyst tip, *tt*) and is surrounded by a membrane, (trichocyst membrane, *tm*). In a wt cell stimulated with PA and Ca^{2+}, quick fixation with OsO_4 captures an early image of fusion of the trichocyst and plasma membranes (arrows, *b*). In a serial section, the tubular opening resulting from membrane fusion is cut obliquely (arrow, *c*); the diameter of the tube is ~22 nm. At low magnification it can be seen that the body of this trichocyst is in Stage I, and has not yet undergone expansion (arrow, *d*). In a later stage of exocytosis the fused membranes of the tubular opening are pushed further apart (arrowheads, *e*) by the exiting (now Stage II) trichocyst matrix. (*a*) Bar, 0.1 μm; × 75,900; (*b* and *c*) Bar, 0.1 μm; × 181,500; (*d*) Bar, 0.25 μm; × 32,000; (*e*) Bar, 0.1 μm; × 88,000.

trichocyst demonstrating that the body is still condensed (Stage I, arrow), which suggests that membrane fusion may occur before tmx expansion in vivo. Fig. 3*e* shows a subsequent stage of secretion in which the tube formed by the fused membranes has widened (arrowheads) as the trichocyst tip is forced outward by the expanding trichocyst body.

Mg^{2+} inhibition of PA-induced secretion (Fig. 1, *a* and *c*) has been examined in greater detail with TEM. In general, cells fixed in the presence of Mg^{2+} and PA show docked trichocysts in Stage I (condensed matrix) without fusion of plasma and trichocyst membranes. This suggests that Mg^{2+} inhibits membrane fusion, in agreement with the FEM data just presented. Membrane fusion is observed only rarely under these conditions, but even then tmx expansion may be inhibited (Fig. 4). This is consistent with in vitro observations that high Mg^{2+} does not promote expansion of isolated trichocyst matrices (5). These TEM observations lead us to suggest that in vivo Ca^{2+} has roles in both membrane fusion and tmx expansion because Mg^{2+} does not substitute for Ca^{2+} in either process. In addition, the examples presented in Figs. 3 and 4 lead us to suggest that tmx expansion is not a prerequisite for membrane fusion to occur.

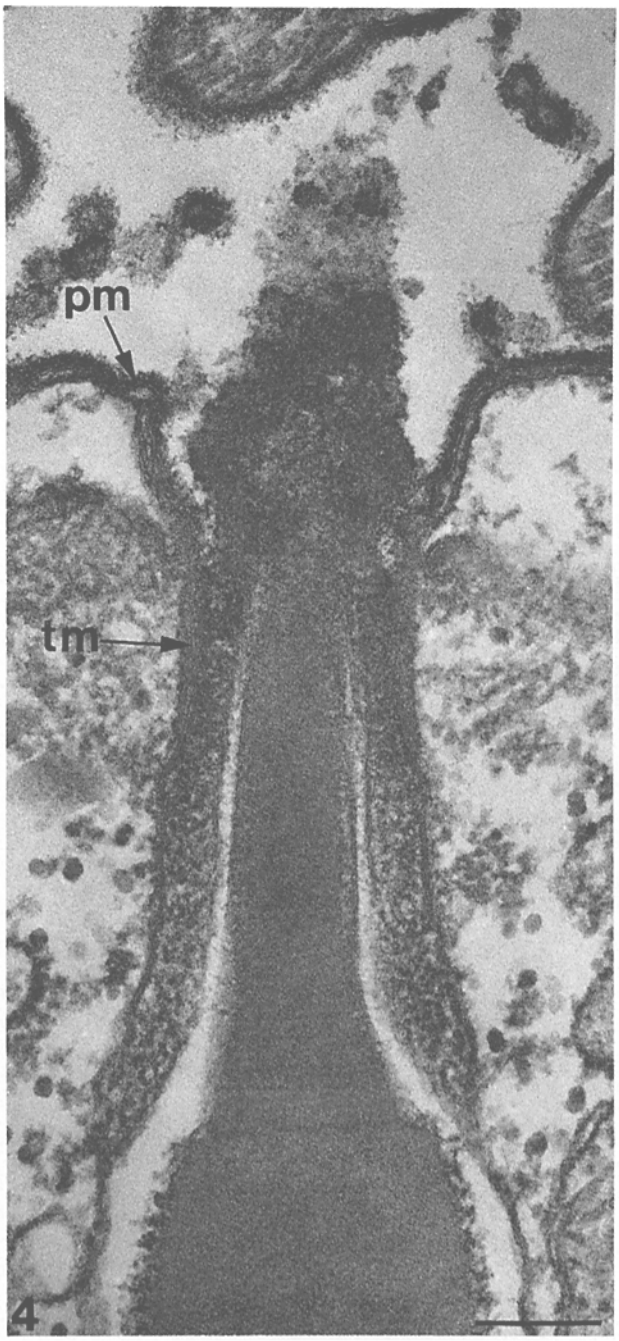

FIGURE 4 Wild type cells in the presence of 10 mM Mg^{2+}, quick fixed with OsO_4 following PA stimulation, occasionally exhibit fusion of plasma membrane (*pm*) and trichocyst membrane (*tm*), but without expansion of the trichocyst matrix. Bar, 0.1 μm; × 168,000.

Wild Type Cells Electron Microscopy of A23187-Ca^{2+}-induced Secretion

Ionophore A23187 (15 μM) and Ca^{2+} produced asynchronous discharge of tmx without killing the cells. Cells fixed shortly after exposure to A23187-Ca^{2+} exhibited several stages of exocytosis. In contrast, PA or high concentrations of A23187 (40 μM) in the presence of Ca^{2+} caused synchronous discharge of tmx and cell death. Fig. 5,*a* and *c* show two stages of normal exocytosis in wt cells exposed to 15 μM A23187 and Ca^{2+}. Fig. 5*a* shows a Stage III tmx captured as it is leaving the cell. The plasma and trichocyst membranes are continuous (open arrow) at the exocytic opening and the trichocyst membrane is being internalized (arrowheads) as earlier demonstrated (12). For comparison, Fig. 5*b* shows internalization of trichocyst membrane after PA-Ca^{2+} induced exocytosis.

Quick fixation of cells during A23187-Ca^{2+} induced exocytosis yields a DMSO-OsO_4 precipitate on the momentarily continuous plasma and trichocyst membranes (see Materials and Methods). This precipitate marks sites of exocytic events as in Fig. 5*c* (arrows). The absence of precipitate within the cytoplasm or alveolar sacs demonstrated the continued integrity of the plasma membrane during the exposure to A23187-Ca^{2+} and the subsequent release of tmx.

If these sites of recent exocytosis could be found in mutant cells stimulated with A23187-Ca^{2+}, then the released tmx observed with light microscopy were probably secreted via normal exocytosis (membrane fusion). nd 9 18°C cells do show sites of membrane fusion and recent exocytosis following exposure to A23187-Ca^{2+}, whereas these sites are not found in nd 9 27°C cells.

Mutant Cells Grown at the Nonpermissive Temperature

A common observation of nd 9 27°C cells treated with A23187 (15 μM) and Ca^{2+} (5 mM), is shown in Fig. 6*a*. The body of this trichocyst (not shown) has expanded to Stage II and the entire trichocyst is pushing upward, extending the plasma membrane. Fusion of trichocyst and plasma membranes has not taken place, as shown by others (17). Light microscopy does not reveal this aspect of the tmx discharge and without fixation for electron microscopy this tmx would proceed to Stage III and extend much further out of the cell (Fig. 1*h*).

Freeze-fracture images of nd 9 27°C cells exposed to A23187-Ca^{2+} differ considerably from those of wt cells under the same conditions. Fig. 6*b* shows an expanded tmx attempting to leave the cell. It stretches the cell membrane into a fingerlike projection. The rest of the cell surface no longer presents the usual cortical organization and even cilia cannot easily be recognized, perhaps due to the plasma membrane being pulled up by the expanding tmx.

Fig. 6*c* shows a rare observation of membrane fusion in an nd 9 27°C cell exposed to A23187-Ca^{2+}. The plasma membrane has fused with the trichocyst membrane and the content of the trichocyst is beginning to move out of the cell. It is not surprising to find this image occasionally because this mutant is "leaky" and may secrete 1–5 tmx in response to PA-Ca^{2+}. However, TEM and FEM studies of A23187-Ca^{2+}-induced secretion in nd 9 27°C cells suggest that in general these cells are incapable of membrane fusion at the secretory site, even when Ca^{2+} is made available intracellularly via A23187.

DISCUSSION

Dissection of the Roles for Ca^{2+}

The diagram in Fig. 7 depicts some of the Ca^{2+}-dependent events in trichocyst secretion and illustrates our beginning dissection of these events. Fig. 7*a* shows a docked trichocyst, as found in nd 9 and wt cells grown at 18°C and 27°C (see Fig. 3*a*). The trichocyst matrix is in Stage I and plasma and trichocyst membranes are not fused since exocytosis has not been triggered.

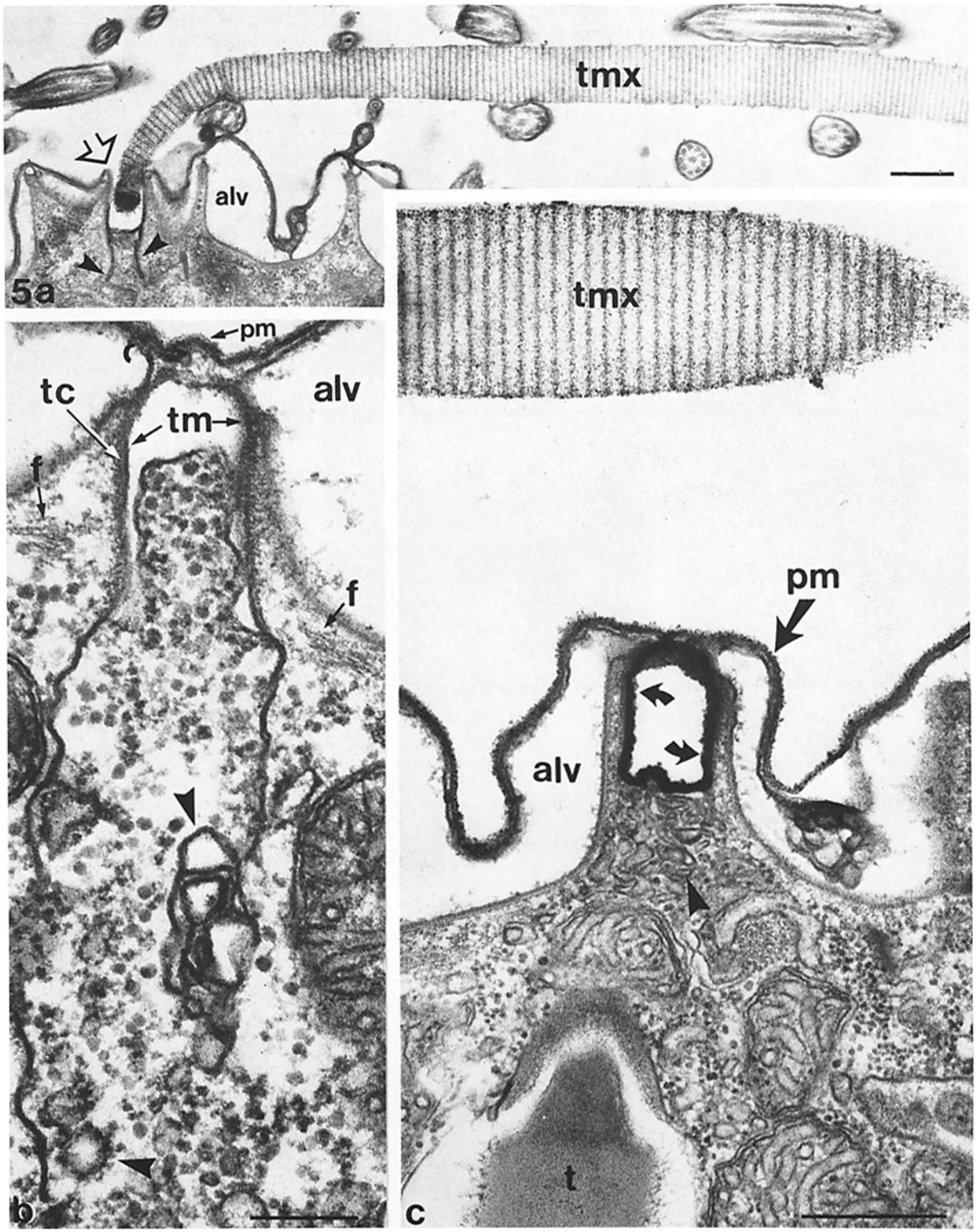

FIGURE 5 A fully expanded (Stage III) trichocyst matrix (*tmx*) is captured by quick fixation just as it is leaving the secretory site in a wt cell stimulated with 15 μM A23187 in the presence of Ca^{2+} (*a*); alveolar sacs, *alv*. The "ghost" of trichocyst membrane (*tm*) and trichocyst coat (*tc*) marks a site of recent exocytosis in a wt cell stimulated by PA (*b*); the trichocyst membrane is now separated from the plasma membrane (*pm*) and there are many filaments (*f*) and vesicles (arrowheads) associated with this site of membrane turnover. Wild type cells stimulated with A23187-Ca^{2+} may exhibit sites of trichocyst membrane coated with a DMSO-OsO_4 precipitate (arrows, *c*) representing a region that was once continuous with the plasma membrane (*pm*). These sites were used to determine whether normal exocytosis occurred in mutant cells after exposure to A23187-Ca^{2+}. (*a*) Bar, 0.5 μm; × 20,750. (*b*) Bar, 0.25 μm; × 70,950. (*c*) Bar, 0.5 μm; × 48,100.

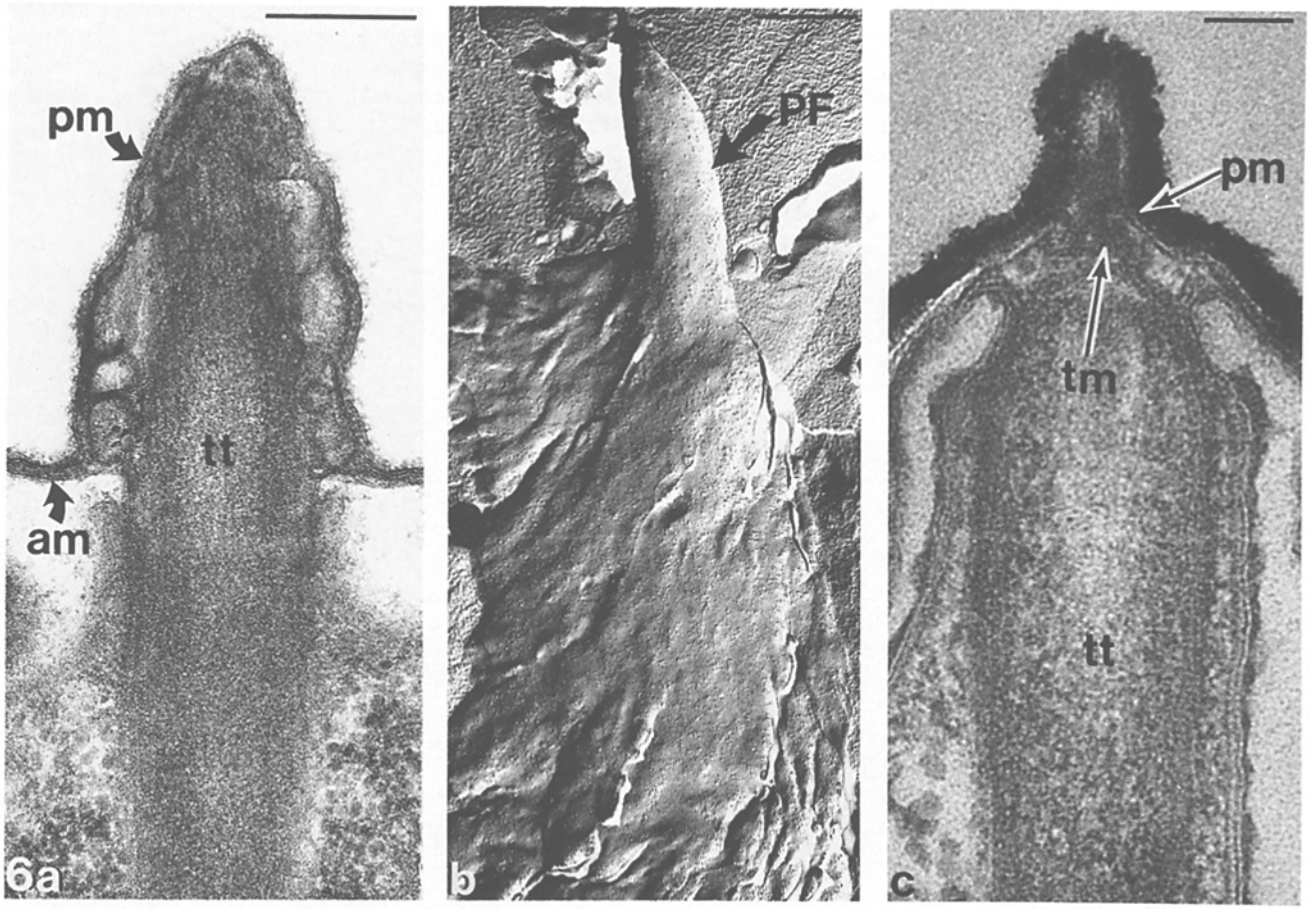

FIGURE 6 Mutant cells grown at the nonpermissive temperature (lacking rosettes) stimulated with 15 μM A23187 and 5 mM Ca^{2+} mainly exhibit trichocyst matrix expansion without membrane fusion as in (*a*) thin section and (*b*) freeze fracture. The matrix of the trichocyst in (*a*) has expanded to stage II (not shown) and the trichocyst tip (*tt*) is forcing its way out of the cell pushing up the plasma membrane (*pm*) without distorting the alveolar membrane (*am*). In FEM, a replica of the protoplasmic face (*PF*) of the plasma membrane shows an expanding trichocyst matrix pulling plasma membrane with it away from the cell (*b*). Fusion of plasma and trichocyst membranes (*tm*) is occasionally seen in nd 9 27°C cells stimulated with 15 μM A23187 and Ca^{2+}, and an example of this is shown in (*c*). (*a*) Bar, 0.25 μm; × 78,000. (*b*) Bar, 0.5 μm; × 28,000. (*c*) Bar, 0.1 μm; × 120,000.

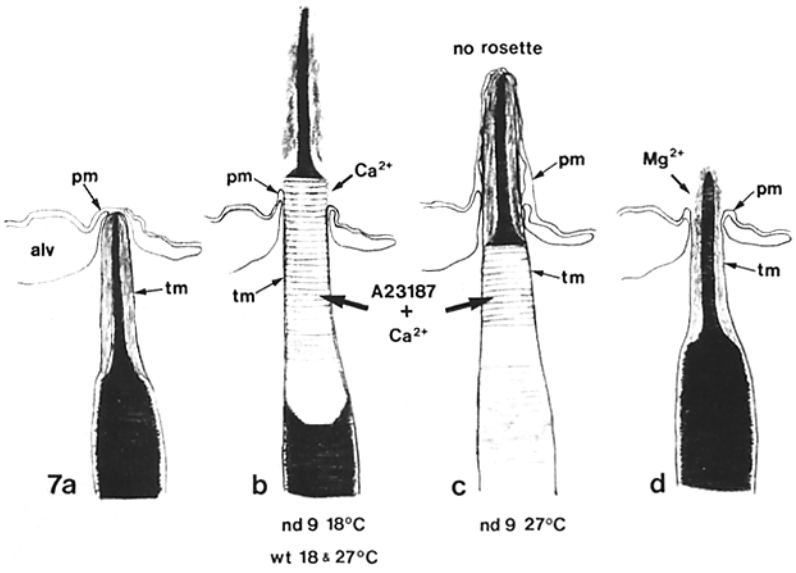

FIGURE 7 Ca^{2+}-dependent events of trichocyst secretion are summarized in this diagram, which shows a dissection of the roles for Ca^{2+}. A trichocyst docked between the alveolar sacs (*alv*) in an unstimulated cell does not exhibit fusion of plasma membrane (*pm*) and trichocyst membrane (*tm*) and the trichocyst matrix is in Stage I (*a*). PA-Ca^{2+} or A23187-Ca^{2+} induces both membrane fusion and tmx expansion in wt and nd 9 18°C cells which both possess assembled rosettes (*b*). In contrast, A23187-Ca^{2+} induces tmx expansion without membrane fusion in nd 9 cells grown at 27°C (lacking rosettes) (17). Wild type cells stimulated with PA in the presence of Mg^{2+} generally do not show membrane fusion, but when fusion does occur (via residual Ca^{2+}) Mg^{2+} fails to induce tmx expansion (*d*).

In Fig. 7*b*, membrane fusion and tmx expansion are depicted simultaneously, as found in the case of wt or nd 9 18°C cells stimulated with A23187-Ca^{2+}. The trichocyst body is shown in a partially expanded state consisting of Stages I-II-III, from the base towards the tip. In vivo the tmx may expand in this sequential manner and such images can be captured with fixation (9).

Under the conditions in Fig. 7*b* it is not possible to separate the effects of Ca^{2+} on membrane fusion vs. tmx expansion. However, we have succeeded in separating tmx expansion from membrane fusion as shown in Fig. 7, *c* and *d* using a secretory mutant and Mg^{2+}. When nd 9 27°C cells (rosette missing) are stimulated with A23187 and Ca^{2+} (Fig. 7*c*), tmx expansion occurs because Ca^{2+} reaches the trichocyst matrix, but membrane fusion does not take place. This event in the mutant cell has been called "pseudoexocytosis" (17). However, this phenomenon is not unique to the mutant, but can be seen occasionally in wt cells. In wt cells it is likely that pseudoexocytosis occurs because Ca^{2+} reaches the tmx before assembly of a complete rosette.

Satir and Oberg (28) used A23187 to raise the intracellular Ca^{2+} concentration in nd 9 27°C cells to determine if an increase of cytoplasmic Ca^{2+} per se would cause exocytosis in mutant cells lacking assembled rosettes. Their light microscopic observations suggested that nd 9 27°C cells secrete in response to A23187 in a Ca^{2+}-dependent manner, with no appreciable secretion in the presence of Mg^{2+}. They hypothesized that the rosettes normally act as Ca^{2+} gates or channels regulating site-specific Ca^{2+} concentration. In the mutant lacking rosettes the addition of A23187 and Ca^{2+} would then phenocopy the function of the rosette.

In wt or nd 9 18°C cells, where docked trichocysts have complete rosettes, certain membrane events may be bypassed by A23187-Ca^{2+} and true exocytosis will still result (Figs. 5 and 7*b*). It is sufficient, therefore, to raise the intracellular Ca^{2+} concentration in order to achieve both membrane fusion and tmx expansion when all components are normal. However, in the case of nd 9 27°C cells, where docked trichocysts have incomplete rosettes (4), raising the intracellular (and intratrichocyst) Ca^{2+} via A23187 is only sufficient to achieve tmx expansion, but not sufficient to achieve membrane fusion. A23187 floods the cell with Ca^{2+} leading to premature expansion of trichocyst matrices without concomitant fusion of the plasma and trichocyst membranes. This suggests that the molecules represented by rosette particles are necessary for membrane fusion. The possibility that rosettes also function as Ca^{2+} gates cannot be ruled out by these results.

Mg^{2+} Inhibition of Secretion

By incubating wt and nd 9 18°C cells in Mg^{2+}, we are able to inhibit PA-induced secretion (Fig. 1, *a* and *c*) and elicit a response identical to the PA-Ca^{2+} response of nd 9 27°C cells (Fig. 1, *e*). Whether Mg^{2+} is inhibiting membrane fusion, tmx expansion, or both cannot be determined from the light microscopic observation alone.

Our TEM and FEM results suggest that Mg^{2+} inhibits PA-induced secretion at the level of membrane fusion. Wild type cells fixed in the presence of Mg^{2+} and PA generally do not show fusion of plasma and trichocyst membranes in TEM. Wild type and nd 9 18°C cells fixed with glutaraldehyde in the presence of 10 mM Mg^{2+} in FEM contain statistically the same frequency of "docked" trichocysts (estimated by rings, Table III) as nd 9 27°C cells, which we know are blocked in membrane fusion. When membrane fusion does occur with significant frequency (as in wt cells exposed to A23187-Ca^{2+}, Table III) the frequency of rings is decreased significantly. This suggests that glutaraldehyde-induced trichocyst release is blocked by Mg^{2+} at the level of membrane fusion. Extracellular Mg^{2+} apparently inhibits secretion such that wt and nd 9 18°C cells have the same frequency of rings in FEM as nd 9 27°C cells.

Ionophore A23187 transports Ca^{2+} or Mg^{2+} across lipid bilayers resulting in a net movement of ions down their concentration gradient (19). Since membrane fusion requires Ca^{2+} and normally precedes tmx expansion, the ability of Mg^{2+} to promote tmx expansion in vivo can be assayed by using A23187 to bypass the normal sequence of events. Mg^{2+} has been shown to inhibit A23187-induced secretion in *Tetrahymena thermophila* (32). Quantitatively, A23187-Mg^{2+} induces very little tmx expansion in contrast with A23187-Ca^{2+} in wt and nd 9 cells (Table II). In the presence of 10–15 mM Mg^{2+}, A23187 may be releasing Ca^{2+} from internal sites, allowing low levels of secretion to occur (1–10 tmx per cell) but clearly, flooding the cell (and trichocysts) with Mg^{2+} does not induce tmx expansion as does A23187-Ca^{2+}.

Thus, Mg^{2+} has provided another separation of tmx expansion and membrane fusion in *Paramecium*; that is, membrane fusion without trichocyst matrix expansion. This separation, diagrammed in Fig. 7*d*, was achieved by incubating wt cells in Mg^{2+} instead of Ca^{2+} and stimulating exocytosis with PA. Although extracellular Mg^{2+} generally inhibits both membrane fusion and tmx expansion, in a few instances membrane fusion is observed, but without concomitant tmx expansion. From this morphological dissection of secretion in *Paramecium* we conclude that Mg^{2+} in vivo (*a*) inhibits membrane fusion and (*b*) inhibits trichocyst matrix expansion and therefore Ca^{2+} has a role in both membrane fusion and tmx expansion in vivo.

Quantitation of Secretion by LM

Electron microscopic observations of the secretory responses of wt and mutant cells suggest possible interpretations of the observed quantitative light microscopic differences. Pseudoexocytosis may explain the lower number of tmx counted when nd 9 27°C cells are stimulated with A23187-Ca^{2+} as compared with nd 9 18°C cells (Table II). Reduced levels are also observed in wt and nd 9 18°C cells stimulated with A23187 vs. PA. This may be due to an inefficiency in the "bypass" mechanism of A23187, which floods the cell with Ca^{2+}, vs. the normal mechanism of stimulating exocytosis, which is local and site specific.

The numbers of tmx released by the three cell types at three different stages of culture growth in response to PA-Ca^{2+} are remarkably similar (Table I) and consistent with the observation that phospholipids of bacterized *P. tetraurelia* do not change with culture age (1). Wohlfarth-Bottermann (33) found that *Paramecium caudatum* released ~300 tmx and only at much later stages of cultural growth (~15 d) after repeated stimulation with electric shock did he find up to 3,000 tmx released per cell. The maximum response observed in our PA assay was 393 tmx, which correlates well with electric shock-induced release for the same age culture of *P. caudatum*.

An important finding is that nd 9 cells grown at 18°C (permissive temperature) release the same number of tmx as wt cells when stimulated with either PA or A23187 (Table II).

If nd 9 cells do have an altered gene product at 18°C, as suggested by Beisson et al. (3), then at this temperature it is working as efficiently as the normal gene product in wt cells. The observation that two cell types release significantly fewer tmx when stimulated by A23187-Ca^{2+} than when stimulated by PA-Ca^{2+} demonstrates that quantitative differences in secretory response can be detected by our technique.

Biochemical Role of Ca^{2+} in Secretion

The molecular mechanisms whereby Ca^{2+} promotes membrane fusion and tmx expansion have not yet been elucidated. We are currently investigating the biochemical role of Ca^{2+} in stimulus-secretion coupling, and the ultrastructural data presented here are the basis for interpretation of our biochemical studies. A molecule that is likely to be involved is calmodulin. Calmodulin, a 17,000-M_r, Ca^{2+}-dependent regulatory protein, has been detected in *P. tetraurelia* (15). Calmodulin antagonists trifluoperazine (30) and W7 (9) inhibit PA-induced secretion in wt *P. tetraurelia*. One of the effects of trifluoperazine when studied in TEM is to prevent expansion of the trichocyst matrix (7) and this effect can be partially reversed by the addition of A23187-Ca^{2+} (8, 9). Calmodulin has been isolated from expanded tmx (23) and therefore may be involved in Ca^{2+}-dependent tmx expansion.

Some of the conditions that stimulate or inhibit secretion, as described morphologically and quantitatively in this paper, have been examined with regard to protein phosphorylation and dephosphorylation (11). We have recently shown that PA-Ca^{2+} induces wt cells to dephosphorylate a 65,000-M_r phosphoprotein. This dephosphorylation can be blocked by incubating cells in Mg^{2+} instead of Ca^{2+} which, as shown here, inhibits both membrane fusion and tmx expansion. In addition, the ts secretion of nd 9 cells is correlated with ts dephosphorylation of the 65,000-M_r phosphoprotein in response to PA-Ca^{2+}. Further ultrastructural and biochemical studies should enable clarification of the roles of Ca^{2+}, rosettes, and the 65,000-M_r phosphoprotein in secretion in *Paramecium*.

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