# Calmodulin Antagonists Inhibit Secretion in *Paramecium*

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ABSTRACT Secretion in *Paramecium* is $Ca^{2+}$-dependent and involves exocytic release of the content of the secretory organelle, known as the trichocyst. The content, called the trichocyst matrix, undergoes a $Ca^{2+}$-induced reordering of its paracrystalline structure during release, and we have defined three stages in this expansion process. The stage I, or fully condensed trichocyst, is the 4 μm-long membrane-bounded form existing prior to stimulation. Stage II, the partially expanded trichocyst, we define as an intermediate stage in the transition, preceding stage III, the fully expanded extruded form which is a 20–40 μm-long needlelike structure. These stages have been used to assay the effects of trifluoperazine (TFP) and W-7, calmodulin (CaM) antagonists, on trichocyst matrix expansion in vivo. TFP and W-7 are shown to reversibly block matrix release induced by picric acid. Ultra-structural examination reveals that one effect of this inhibition is reflected in the organelles themselves, which are prevented from undergoing the stage I–stage II transition by preincubation in 14 μM TFP or 35 μM W-7 before fixation. This inhibition of expansion by TFP can be moderated but not abolished by high extracellular $Ca^{2+}$ (5 mM). The moderation by high $Ca^{2+}$ can be eliminated by raising TFP concentration to 20 μM. A possible explanation for the ability to titrate the inhibition in this manner is that TFP is acting to block expansion by binding to the $Ca^{2+}$-CaM complex. Brief exposure of cells to the $Ca^{2+}$ ionophore A23187 and 5 mM $Ca^{2+}$ following TFP treatment promotes matrix expansion, although in 14 μM TFP a residual level of inhibition remains. These results suggest that, following stimulation, CaM regulates secretion in *Paramecium*, possibly by controlling the $Ca^{2+}$-dependent matrix expansion which accompanies exocytosis in these cells.

The ciliated protozoan *Paramecium* offers an exciting model system for studying the control of secretory exocytosis. Thousands of membrane-bounded secretory organelles known as trichocysts are docked beneath the plasma membrane at specific secretory sites in the highly organized cell cortex (18). The fine structure of these organelles has been well characterized (2, 15, 16) and freeze-fracture studies have revealed specific intramembrane particle arrays in both the plasma and organelle membranes which are required for secretion (3, 21, 41, 42). The trichocysts remain docked in position at the secretory sites until a stimulus triggers release of the vesicle content, which will be called the trichocyst matrix, via exocytosis. The proteinaceous matrix has recently been found to contain significant (1–10%) amounts of the $Ca^{2+}$-dependent regulatory protein calmodulin (CaM) (38). The role of this matrix CaM, which appears to be a structural part of the trichocyst, is not yet understood. An essential part of the secretory product release in these cells is an easily visualized transformation of the trichocyst matrix, from a highly condensed resting form to a greatly elongated secreted form, with an accompanying change in its paracrystalline structure. This makes it possible to assay release at the light microscope level, and concomitant changes in the state of the organelle can be monitored with the electron microscope.

As with many other secretory systems (39), external $Ca^{2+}$ is required for trichocyst release (29, 44). The role of $Ca^{2+}$ in secretion is not yet understood, but a likely candidate for mediating its action is the Cal-dependent regulatory protein, CaM. CaM has been shown to be present in *Paramecium* in both cytoplasmic fractions (41, 45) and localized to specific cellular structures (28), in addition to being present as a component of the trichocyst matrix itself (13, 38). This ubiquitous protein is involved in the regulation of an increasingly larger number of $Ca^{2+}$-regulated events (6, 30, 56) and its involvement in the process of secretion has been under investigation in this laboratory (10–13, 45) as well as others (1, 8, 9, 23, 33, 47, 50, 51).

Initial experiments indicated that preincubation of *Paramecium* in 14 μM trifluoperazine (TFP), a drug known to inhibit CaM-regulated enzymes (26), inhibits picric acid (PA, the routine stimulus)-induced trichocyst release (45). This result suggests a possible involvement of CaM at some stage of the



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release process in these cells. However, since a large number of enzymes are known to be regulated by CaM (6, 30, 56), numerous pathways are potential targets for TFP action. In addition, TFP inhibition alone, while suggestive, is not a firm enough criterion to implicate CaM in a cellular process. In this report we have used two approaches to address the problem of a possible CaM-sensitive step in stimulus-secretion coupling in *Paramecium.*

First, cells were examined in the transmission electron microscope (TEM) to determine whether any manifestations of TFP inhibition would be evident in any of the structurally well-defined components of the secretory apparatus, such as the secretory organelles themselves. We define three stages in trichocyst morphology during secretion and demonstrate a clear ultrastructural correlate to the drug-induced inhibition observed in the earlier light microscope study (45). Second, another anti-CaM drug, the naphthalenesulfonamide derivative W-7, and its less active dechlorinated analog, W-5 (18–20, 54), were used to examine whether the inhibition of secretion by TFP is due to specific interactions with the $Ca^{2+}$-CaM complex, or to nonspecific effects. The results show that effects of W-7 are similar to those of TFP in both light and electron microscope assays, whereas W-5 has no effect at concentrations similar to those of W-7. These results further support a role for CaM in the regulation of trichocyst secretion in *Paramecium*, and in addition provide information as to possible sites of action for this molecule during exocytosis.

## MATERIALS AND METHODS

*Culture Conditions:* Cells used in all experiments are *Paramecium tetraurelia*, wild type, grown at 27°C in bacterized (*Enterobacter aerogenes*) Cerophyll medium (48).

*Picric Acid-induced Release:* 200 ml of late log phase cultures were harvested, washed once in a buffer containing 1 mM HEPES, 20 μM Na-Citrate, 20 μM $CaCl_2$, and 0.5–1.5 mM Tris to adjust pH to 7.0 (HEPES-Citrate buffer) (5), and suspended to 10–15 ml in the same buffer. Aliquots of this cell suspension were then exposed to TFP, W-7 or W-5 at the indicated concentrations in a volume totaling 250 μl. Samples of this suspension were withdrawn at various time intervals and rapidly added to a drop of saturated picric acid on a microscope slide. PA is a secretagogue routinely used to induce trichocyst release in *Paramecium* (36). Cells were then scored for release (more than or less than 50 trichocysts per cell) with a Wild light microscope equipped with phase-contrast optics. TFP, W-7, or W-5 was dissolved in distilled, deionized water (stock solutions: TFP, 1 mM; W-7 and W-5, 5 mM) and stored in small aliquots at −20°C. In experiments where drug exposure was carried out in the presence of high (5 mM) $Ca^{2+}$, a sufficient amount of a stock solution (0.5 M $CaCl_2$ in HEPES-Citrate buffer) was added directly to the cell suspension immediately before drug addition. Raising the $Ca^{2+}$ concentration to 5 mM during W-7 exposure was achieved by adding a small volume of this stock $Ca^{2+}$ solution so that drug dilution was minimal. All incubations were performed at room temperature.

Reversal of TFP-induced inhibition was achieved by diluting the sample (200 μl) to 5 ml with HEPES-citrate buffer containing 10% bacterized cerophyll medium. After 5 min the cells were pelleted in a clinical centrifuge, the buffer removed except for ~250–300 μl in which cells were resuspended by gentle agitation. Samples were taken from this suspension and scored for trichocyst release as described.

For electron microscope observations, 400 ml of late log phase cultures were harvested, washed as for the light microscope studies, and suspended to 20 ml in HEPES-citrate buffer. Aliquots of this cell suspension were then exposed to TFP, W-7, or W-5 at the indicated concentrations for varying times before fixation. Controls (no drug addition) received equivalent amounts of water. $Ca^{2+}$ concentration was raised as in the light microscope experiments, by addition of a small volume of 0.5 M $CaCl_2$ in HEPES-citrate buffer immediately before drug exposure. The final volume of cell suspension, $CaCl_2$ (if added), and drug was always 1 ml. All incubations were performed at room temperature.

For treatment of cells with $Ca^{2+}$ ionophore, a 1 mM stock solution of A23187 in dimethylsulfoxide (DMSO) was prepared, which was diluted to the working concentration (100 μM) in HEPES-citrate buffer. Cells were prepared as described above, and incubated in either 8 or 14 μM TFP for 10 min in a volume of 850 μl. After 10-min exposure, 150 μl of 100 μM A23187 was added rapidly, to a final volume of 1 ml (final A23187 concentration, 15 μM) and final DMSO concentration of <2%. Control cells exposed to this concentration of DMSO were indistinguishable from untreated controls. A23187 exposure was carried out for 60 s at room temperature.

*Electron Microscopy:* Cells were fixed at the indicated times by rapid addition of 1 ml of osmium tetroxide (final concentration 0.5%) for 20 s, followed by 2 ml of 4% glutaraldehyde (final concentration 2%) for 1 h at room temperature.

Fixed cells were washed twice in HEPES-citrate buffer and processed for TEM. Cells were stained en bloc for 1 h in 1% aqueous uranyl acetate followed by alcohol dehydration and embedding in Epon-Araldite or Epon. Thin sections were cut on an LKB 8801/2 (LKB Instruments, Inc., Gaithersburg, MD) or a Reichart ultramicrotome and stained with 4% uranyl acetate in 40% ethanol for 30 min, followed by lead citrate (55) for 10 min at room temperature. Electron micrographs were taken with either a Seimens 1A or a JEOL 100CX electron microscope.

*Quantitation of Electron Micrographs:* Micrographs for quantitation were taken at magnifications ranging from × 1,500 to 2,500, depending on size and orientation of the cell section, to obtain the entire cell section in one or two micrographs. Negatives were then further enlarged × 2.5. The images were scored by counting trichocyst profiles, determining the number of condensed (I) and expanded (II and III) stages, and calculating the percentage of trichocysts in each stage per cell. Stage II (partially expanded) and stage III (fully expanded) trichocysts were considered a single category, because stage III trichocysts inside the cell were only seen infrequently.

## RESULTS

### *Effects of Trifluoperazine and W-7 on Picric Acid-induced Release*

Secretion of trichocysts induced by PA is inhibited when *Paramecium* cells are incubated in buffer containing TFP (47; Fig. 1 *a*). Exposure of cells to 16 μM TFP (Fig. 1 *a*) results in a gradual decrease in the number of cells exhibiting a normal level of trichocyst release. After 15 min of drug exposure, ~35% of cells are releasing more than 50 trichocysts per cell (Fig. 1 *a*, arrow). Dilution of the drug at this point (final concentration <1 μM TFP) results in recovery of secretory capacity, following a lag time of 10 min during which the inhibition progresses further. The reason for the continued inhibitory effect of TFP after dilution of the drug is not clear, but recovery is evident within 15 min of dilution, and near control levels of secretion are achieved 30–45 min after dilution of TFP.

Inhibition of secretion is also seen following exposure to another anti-CaM drug, the naphthalenesulfonamide derivative, W-7 (18, 19), (Fig. 1 *b*). Cells exposed to 35 μM W-7 in HEPES-citrate buffer (20 μM $Ca^{2+}$) show rapid inhibition (within 1 min) of PA-induced trichocyst release. 5 min of exposure to W-7 (35 μM) in low $Ca^{2+}$ (20 μM) results in 80–90% inhibition of trichocyst release. However, most of this inhibitory effect of W-7 can be prevented if the drug exposure is carried out in the presence of high $Ca^{2+}$ (5 mM) (Fig. 1 *b*). In addition, inhibition of secretion induced by W-7 (35 μM) is rapidly reversible following addition of $Ca^{2+}$ to a final concentration of 5 mM (Fig. 1 *b*, arrow). 2 min after $Ca^{2+}$ is raised to 5 mM, 50% of the cells tested are secreting more than 50 trichocysts per cell. This recovery appears to plateau at ~75% after 7 min of exposure to the increased $Ca^{2+}$. Interestingly, this level of cells secreting normally following $Ca^{2+}$-induced recovery is similar to that of cells exposed to W-7 in high $Ca^{2+}$ from the start of the experiment.

W-5, structurally very similar to W-7, but with much lower affinity for CaM (20, 54), has no effect on trichocyst release at a concentration of 50 μM (Fig. 1 *b*).

These results show that two different CaM antagonists are

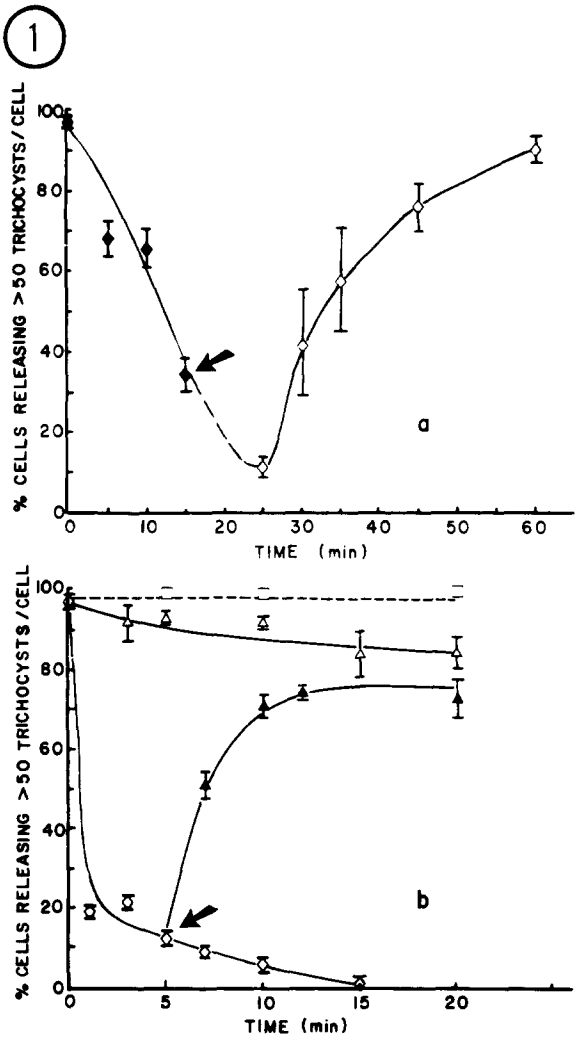


FIGURE 1 Effect of CaM antagonists on PA-induced secretion. Trichocyst release was assayed at the light microscope. (*a*) Cells exposed to 16 μM TFP in HEPES-citrate buffer (20 μM $Ca^{2+}$). At the arrow, the sample was diluted to 5 ml (TFP, <1 μM). The dashed portion of the curve indicates the time during which cells were centrifuged and resuspended. Closed diamonds: 16 μM TFP; open diamonds; <1 μM TFP after washout. (*b*) Cells exposed to 35 μM W-7 in HEPES-citrate buffer (20 μM $Ca^{2+}$) (open diamonds), or in HEPES-citrate buffer + 5 mM $Ca^{2+}$ (open triangles). At arrow, $Ca^{2+}$ concentration was raised from 20 μM to 5 mM (without significant drug dilution) and cells recover secretory capacity (closed triangles). 50 μM W-5 (open squares) in HEPES-citrate buffer (20 μM $Ca^{2+}$) has no effect.

capable of inhibiting trichocyst release in *Paramecium*. These data, in addition to the differential effect of the W-7/W-5 analogs, suggest that CaM is involved at some stage of exocytosis in these cells. It therefore became important to examine whether there were any observable effects of these drugs at the ultrastructural level on the secretory organelles themselves or related structures.

## Three Stages of Matrix Expansion

Fig. 2 shows examples of the three hypothesized stages defining the sequence through which the trichocyst matrix passes from stimulation to final release into the medium. Fig. 2*a* shows the in vivo trichocysts prior to release docked in position under the cell membrane in between two adjacent alveolar sacs. Earlier descriptions of this form of the trichocyst (2, 16) identified it as the mature form of the organelle. At this stage the trichocysts are characterized by their uniform shape and size, ~4 μm in length and 0.9 μm in width. The membrane

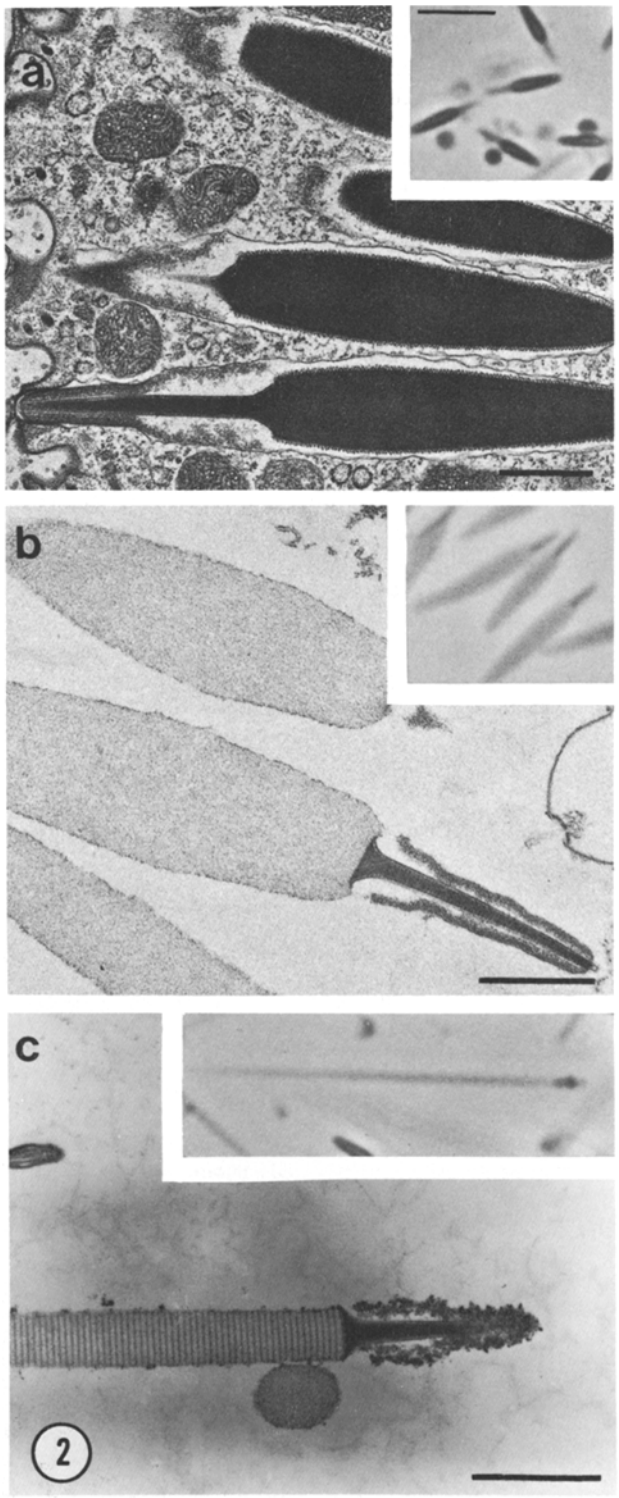


FIGURE 2 The three hypothesized stages defining the sequence through which the trichocyst matrix passes from stimulation to final discharge. (*a*) Stage I, fully condensed; (*b*) stage II, partial expansion; (*c*) stage III, full expansion. *Insets* show trichocyst stages as they appear in phase contrast microscopy. (*a–c*) Bar, 5 μm. (*Inset* to *a*) Bar, 1 μm. (*a*) × 2,000; (*inset*) × 12,480. (*b*) × 2,000; (*inset*) × 16,340. (*c*) × 2,000; (*inset*) × 16,950.

surrounding the trichocyst matrix is clearly visible. The ultrastructural characteristics of the trichocyst matrix itself can be subdivided into the lower two-thirds consisting of the broader body region and the upper one-third consisting of the conical tip. The tip region mediates the insertion of the organelle into its secretory site, and the tip itself remains unchanged during the entire release event. The body of the resting trichocyst is electron-dense and appears crystalline, exhibiting regular transverse dense lines spaced at ~7-nm intervals as reported earlier (16). These resting trichocysts will be referred to as "condensed" or stage I trichocysts.

The *inset* of Fig. 2*a* shows the light microscope image of such isolated, stage I trichocyst matrices. Homogenization of *Paramecium* in the presence of $Ca^{2+}$ chelators yields preparations enriched in stage I trichocysts, allowing direct observation of this form of the organelle. They appear phase-dense, and their characteristic tip and body features make them easily recognizable even at this low magnification.

Upon stimulation for release, the matrix of the trichocyst undergoes a reorganization to form a 20–40-μm-long needle-like, paracrystalline structure, with a distinct and increased periodicity of ~55 nm (nearly eight times that of the resting stage) (2, 15, 50). An example is shown in Fig. 2*c*. The tip maintains its characteristic morphology but now surmounts a greatly elongated body. This structure is the secreted form of the matrix, normally found outside the cell, and will be referred to as the "fully expanded" or stage III trichocyst. The *inset* of Fig. 2*c* shows the corresponding image in phase-contrast microscopy, which again is characteristic and easily distinguished.

Fig. 2*b* shows the predominant form of the trichocyst matrix visualized following routine fixation of cells for electron microscopy. The majority of trichocysts observed within *Paramecium* cells fixed for TEM can be seen to have undergone (*a*) an increase in size from 4 μm to 8–12 μm in length, and from 0.9 μm to 1.2 μm in width, and (*b*) a loss in electron density and paracrystalline order. The *inset* of Fig. 2*b* again shows the corresponding phase microscope appearance of this form as illustrated by isolated trichocysts matrices.

A number of observations suggest that this form, which we call the "partially expanded" or stage II trichocyst, represents an intermediate stage in the transition from the condensed, resting form (stage I) to the fully expanded form (stage III). Images of trichocysts exhibiting all three stages of expansion in succession down their length can be found (Fig. 3) (also reference 17). The portion of the body closest to the tip has undergone a transition to stage III, a small segment deeper in the cell is in stage II, and the most distal portion of the body remains in stage I. Images such as these suggest that the transition of the matrix from stage I to stage III passes down the body of the trichocyst like a wave: stage I progresses to stage II and then stage III as matrix reorganization proceeds down the length of the organelle in response to a spatial and temporal gradient of stimulation.

In *Paramecium*, as well as in other cells, low concentrations of fixatives can serve as secretagogues (2, 35, 43) eliciting complete release of secretory products. It is probable that at higher concentrations, such as those used here, the release process, which in *Paramecium* includes matrix expansion, is initiated but proceeds only to an intermediate stage (II) before fixation occurs. Inhibition of trichocyst discharge can be induced with potassium ferrocyanide which has been shown to generate a number of inhibited states, one of which corresponds to stage II. Hausmann et al. (16) called this form a "type II" trichocyst and suggested that it might represent a stage of discharge.

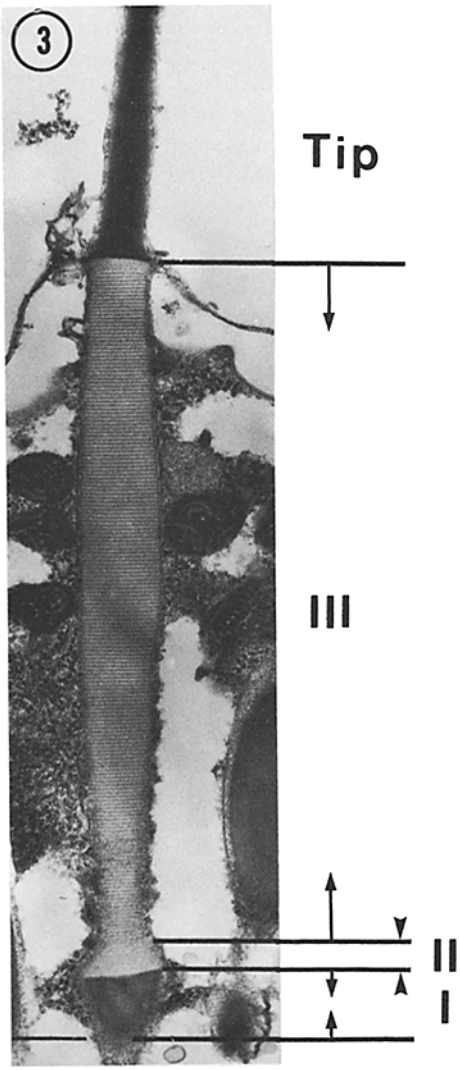


FIGURE 3 TEM image of a single trichocyst exhibiting all three stages of the trichocyst matrix. The tip remains unchanged, and the body consists of matrix in stages III, II, and I ordered consecutively from tip to base of the organelle. Bar, 0.5 μm. × 19,800.

TABLE I

*Requirements for PA-induced Release and Osm/glut-induced Expansion*

| | PA release | Osm/glut-induced expansion |
|---|---|---|
| Extracellular $Ca^{2+}$ | Required | Required |
| High extracellular $Mg^{2+}$ | Inhibits | Inhibits |
| Slow cooling | Inhibits | Inhibits |
| TFP | Inhibits | Inhibits |
| W-7 | Inhibits | Inhibits |

A comparison of requirements for PA-induced trichocyst release and fixative-induced partial expansion to stage II suggests that both stimuli are eliciting the same cellular response. Table I shows that both the I–II transition and complete release (I–III) require extracellular $Ca^{2+}$, are inhibited by high extracellular $Mg^{2+}$, slow cooling, and the anti-CaM drugs TFP and W-7. The similarity of response to a variety of different treat-

ments suggests that stimulation both by osm/glut fixation and by PA (also a fixative) invokes the same cellular processes, but the more rapidly acting fixatives will arrest matrix reorganization at the intermediate stage we call stage II. Thus, the formation of stage II trichocysts is indicative of the initial events leading to secretion, and monitoring the effects of agents, such as TFP, on stage II formation should allow dissection of early cellular events following stimulation.

### *Effect of TFP on Matrix Expansion*

This hypothesis forms the basis for the ultrastructural approach to the question of CaM involvement in secretion described in the following experiments. To test whether the observed TFP inhibition of PA-induced release might be reflected in the structural components of the secretory apparatus, we exposed late log phase cells to the CaM inhibitor TFP (14 μM) for 0–10 min. Cells were fixed at different timepoints and examined in the TEM. Fig. 4*a* shows a control cell exposed to buffer only. In this case, virtually all of the trichocysts are in the partially expanded state (stage II). Trichocysts are shown sectioned both longitudinally (arrows) and in cross section (arrowheads). Although the trichocyst tips are for the most part out of the plane of section in this cell, several tips are visible in a number of longitudinally sectioned organelles (*). It is clear that trichocysts are docked in their correct places beneath the cell membrane.

Fig. 4*b* shows an example of a *Paramecium* cell after a 3-min exposure to TFP which leads to a slight increase in the number of stage I trichocysts; a few images of condensed trichocyst matrices are now evident. After 5 min of exposure (Fig. 4*c*), ~50% of the trichocysts remain condensed (stage I). This effect is progressive and Fig. 4*d* shows a sample cell after 10-min of exposure to TFP which causes nearly complete inhibition of matrix expansion, that is, the majority of trichocysts are now found in stage I.

The intimate relationship of the trichocyst tip to the plasma membrane appears unaltered after TFP treatment. Condensed stage I trichocysts seen after TFP exposure remain docked in position between the alveolar sacs, with the trichocyst tip membrane close to (but not fused with) the plasma membrane, identical to stage II trichocysts seen in control cells. Therefore the increase in condensed trichocysts cannot be accounted for by any gross alteration in organelle-plasma membrane relationship, nor by an increase in undocked trichocysts.

The rise in the percentage of stage I trichocysts is not due to an overall depletion of trichocysts resulting from increased release induced by TFP. If this were the case, a fixed number of stage I trichocysts would comprise a higher percentage of the total trichocysts remaining in each cell, thus giving an artificial impression of an increase in the condensed form. Table II indicates that the average number of trichocysts in the cell sections counted does not decrease following 10 min of TFP exposure. Thus, the increase in stage I trichocysts per cell occurs in cells exhibiting a fairly constant level of trichocysts

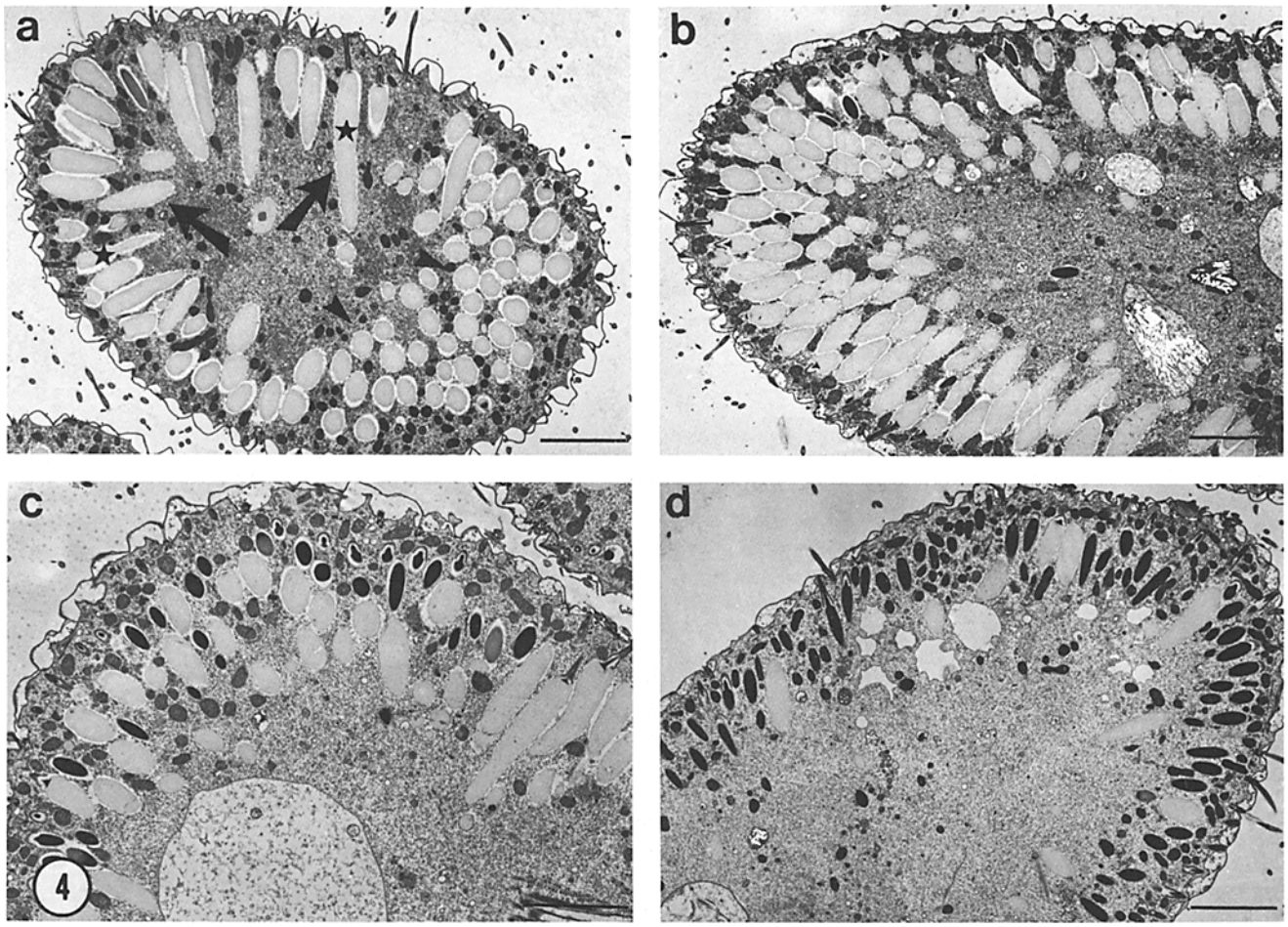


FIGURE 4 TEM images of *Paramecium* cells exposed to 14 μM TFP, low extracellular $Ca^{2+}$ (~20 μM). (*a*) Control cell, no TFP exposure. (*b*) 3 min. (*c*) 5 min. (*d*) 10 min. Bar, 5 μm. *a*, × 2,400. *b*, × 2,250. *c*, × 2,960. *d*, × 2,400.

and reflects a real rise in the condensed form following TFP treatment.

It thus became of interest to us to test if the inhibitory effects of TFP would be affected or overcome by increasing the external $Ca^{2+}$ necessary to drive the expansion of the trichocyst matrix. *Paramecium* cells were therefore exposed to 14 μM TFP in the presence of a 5-mM concentration of external $Ca^{2+}$. Examination of these cells in TEM reveals that TFP still inhibits matrix expansion, although the inhibition is not so complete as in low $Ca^{2+}$. Fig. 5 *a* shows a control cell exposed to 5 mM $Ca^{2+}$ but not to TFP. In this case, nearly all trichocysts are partially expanded (stage II). The remaining Fig. 5 *b–d* demonstrate the extent of TFP-induced inhibition of expansion in the presence of high $Ca^{2+}$. Again, with increasing time of incubation, the drug leads to an increase in the number of stage I trichocysts visible in each cell. Cells after 10 min of exposure to TFP, as shown in Fig. 5 *d*, have ~50% of their trichocysts remaining in stage I. Expansion is therefore clearly inhibited but not to the same extent as the case where the external $Ca^{2+}$ concentration was low (~20 μM), seen in Fig. 4 *d*, where >90% of the trichocysts remain condensed.

TABLE II

*Numbers of Trichocyst Profiles per Cell Section Scored in Untreated Cells, and Cells Exposed to TFP under Various Conditions for 5 and 10 min*

| Conditions | Control | 5 min | 10 min |
|---|---|---|---|
| 14 μM TFP, low $Ca^{2+}$ | 111.6 (±5.5) | 73.8 (±5.9) | 97.4 (±5.7) |
| 14 μM TFP, 5 mM $Ca^{2+}$ | 86.3 (±19.3) | 91.3 (±14.7) | 101.8 (±14.9) |
| 20 μM TFP, 5 mM $Ca^{2+}$ | 76 (±8.1) | 86.4 (±15.7) | 94.3 (±12.5) |

The moderating effect of high extracellular $Ca^{2+}$ is shown quantitatively in Fig. 6 *a*. It is evident from this graph that exposure to 14 μM TFP in both the low (~20 μM) and high (5 mM) $Ca^{2+}$ conditions leads to a decrease in the percentage of expanded trichocysts per cell. The presence of 5 mM external $Ca^{2+}$, however, reduces this inhibitory effect of TFP. The sharp drop in the number of expanded trichocysts per cell seen between 3 and 5 min in low extracellular $Ca^{2+}$ and TFP is tempered by addition of high external $Ca^{2+}$, and the level of trichocyst expansion seen after 10 min of TFP exposure in high $Ca^{2+}$ is approximately ten times that seen in low $Ca^{2+}$ and TFP. The percentage of expanded stage II trichocysts per cell after 5 and 10 min of TFP treatment in both low and high external $Ca^{2+}$ is significantly different from that in control samples.

If the effects observed on trichocyst matrix behavior are real biological manifestations it should be possible to titrate the moderating effect seen in the presence of 5 mM external $Ca^{2+}$ by increasing the concentration of TFP, thereby converting the high $Ca^{2+}$ situation into the low $Ca^{2+}$ case. An experiment was

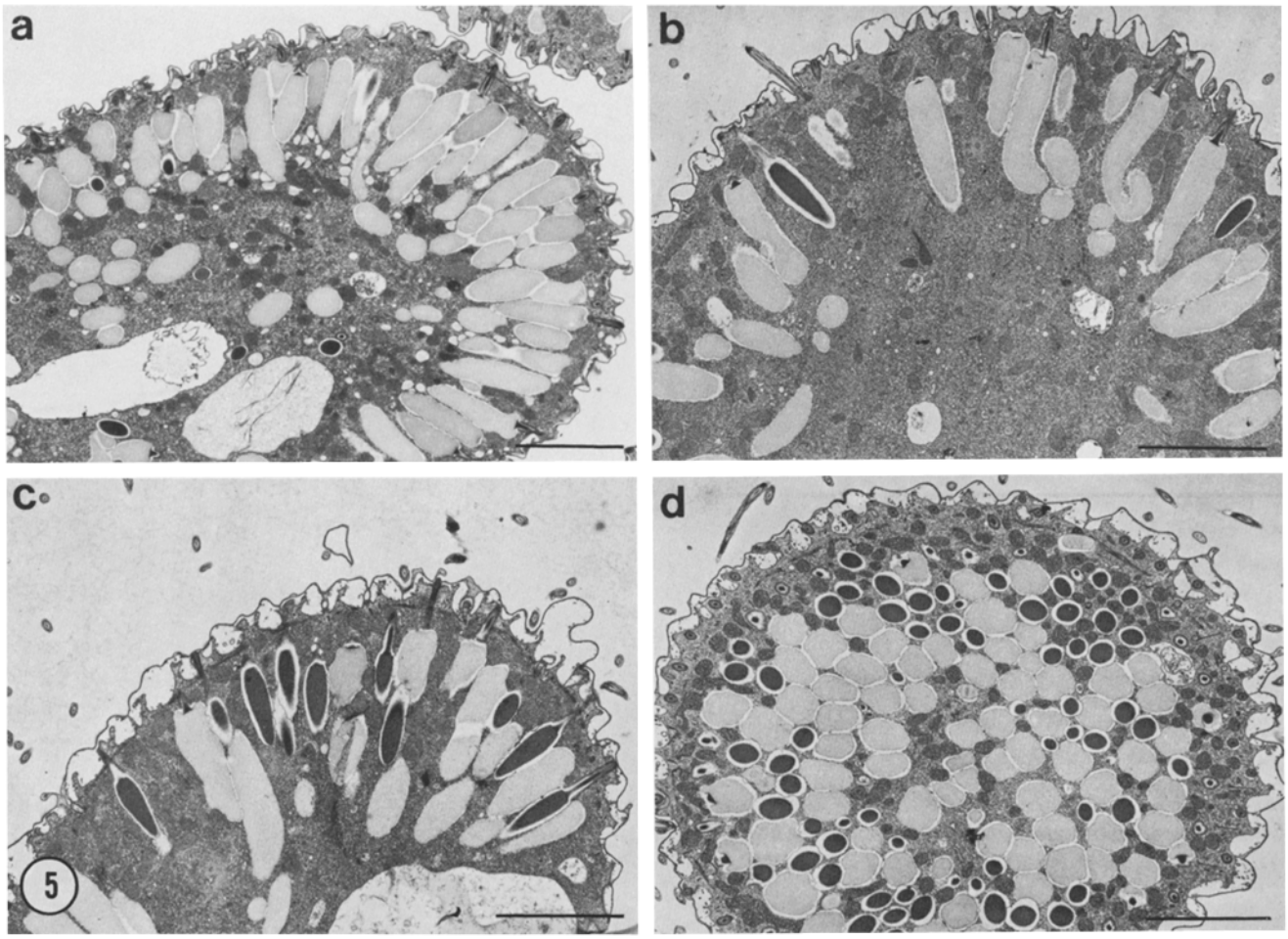


FIGURE 5 TEM images of *Paramecium* cells exposed to 14 μM TFP, high extracellular $Ca^{2+}$ (5 mM). (*a*) Control cell, no TFP exposure. (*b*) 3 min. (*c*) 5 min. (*d*) 10 min. Bar, 5 μm. *a*, × 3,000. *b*, × 3,500. *c*, × 3,500. *d*, × 3,400.

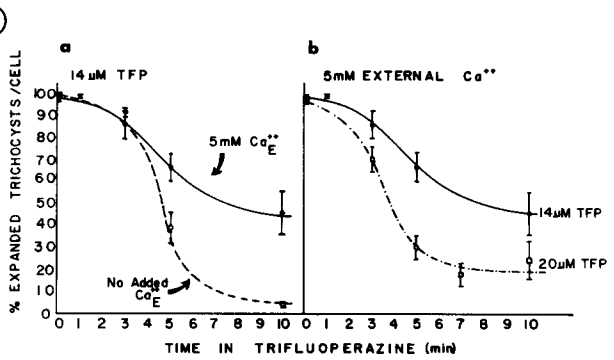


FIGURE 6 (*a*) The effect of extracellular $Ca^{2+}$ concentration on inhibition of matrix expansion by TFP. Increasing extracellular $Ca^{2+}$ moderates the inhibition of expansion by 14 μM TFP. (*b*) The effect of increasing TFP concentration on inhibition of matrix expansion in high extracellular $Ca^{2+}$ (5 mM). Increasing TFP concentration from 14-20 μM overcomes the moderating effect of high extracellular $Ca^{2+}$.

performed in which the TFP concentration was raised to 20 μM and the $Ca^{2+}$ concentration kept as before (5 mM); the results are shown graphically in Fig. 6*b*. It is evident that even in the face of a large inward gradient of $Ca^{2+}$ ion during stimulation, a slight increase in drug concentration can restore the level of condensed stage I trichocyst matrices to nearly that seen in the absence of added $Ca^{2+}$ (Fig. 6*a*). This antagonistic relationship between TFP and $Ca^{2+}$ ion suggests that TFP is acting specifically to block $Ca^{2+}$ action, possibly by affecting a CaM-dependent process which may be necessary for $Ca^{2+}$ to drive matrix expansion.

Another stimulus which has frequently been used to induce secretion in numerous secretory systems (37, 57), and which induces trichocyst release in *Paramecium* (34, 44), is the $Ca^{2+}$ ionophore A23187. The effect of this secretagogue was tested to determine whether an ionophore-induced rise in intracellular $Ca^{2+}$ concentration alone could override the TFP-induced inhibition of expansion. Cells were therefore exposed to TFP for 10 min at concentrations which block trichocyst expansion (8 and 14 μM) followed by brief treatment (60 s) with 15 μM A23187. The results of such experiments are shown in the histogram (Fig. 7). Exposure of cells to 8 μM TFP for 10 min in the presence of 5 mM $Ca^{2+}$ results in inhibition of trichocyst expansion to ~60% of the control value. Another cell sample given identical TFP treatment but followed by 60-s exposure to the $Ca^{2+}$ ionophore A23187 shows trichocyst matrix expansion returning to control levels.

Cells incubated in a higher concentration of TFP (14 μM) show an even greater reduction in the percentage of expanded stage II trichocysts per cell (Fig. 7). Subsequent exposure of these cells to A23187 (15 μM) promotes a substantial expansion (~50%) of the TFP-induced stage I trichocysts to stages II and III (Fig. 7). However, the level of expanded trichocysts per cell under these conditions is only ~70%, which is significantly depressed compared to control levels.

Therefore, it appears that at lower concentrations of TFP (8 μM), brief treatment of cells with ionophore can reverse the inhibition of matrix expansion completely, but when TFP concentration is raised to 14 μM ionophore stimulation can return the level of expansion to ~70% of the control value, leaving a residue which remains in stage I (~25%). Thus, $Ca^{2+}$ entering cells via the ionophore is capable of reversing the TFP-induced inhibition to a large degree, although the effectiveness of ionophore stimulation also appears to be antagonized by increasing concentrations of TFP.

## *Effect of W-7 on Matrix Expansion*

The CaM antagonist W-7 (18, 19) which inhibits picric acid-induced release (Fig. 1) also inhibits the trichocyst matrix expansion to stage II seen following fixation (Fig. 8). Cells were exposed to 35 μM W-7 for 10 min in 1 mM $Ca^{2+}$ prior to osm/glut fixation. The effect of pretreatment with W-7 is similar to that seen with TFP, trichocyst expansion is inhibited, and most matrices remain in stage I (Fig. 8). Control cells without drug treatment are equivalent to those seen in Figs. 4*a* and 5*a*.

Thus, two CaM antagonists, TFP and W-7, exhibit parallel effects on both secretion stimulated by picric acid (Fig. 1) and

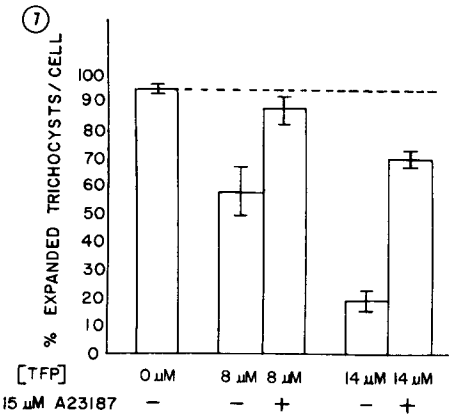


FIGURE 7 TFP treatment followed by $Ca^{2+}$ ionophore A23187. TFP exposure was carried out at the indicated concentrations for 10 min. Samples not treated with A23187 were then fixed immediately, while companion samples given identical TFP exposure were treated for 60 s with 15 μM A23187 prior to fixation. TFP inhibits matrix expansion in a dose-dependent manner. Subsequent exposure to A23187 can promote matrix expansion to control levels when TFP concentration is low (8 μM), and promote substantial expansion (70% of the control) in the presence of 14 μM TFP.

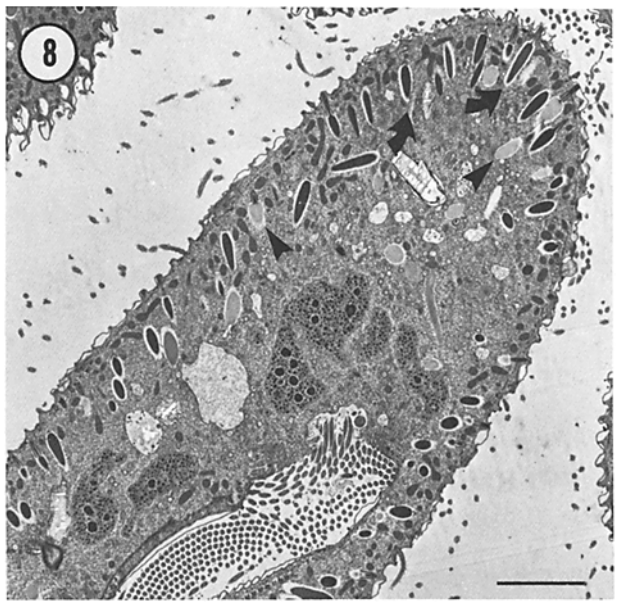

FIGURE 8 TEM image of *Paramecium* cell exposed to 35 μM W-7 (1 mM $Ca^{2+}$) for 10 min. (Arrows) Stage I trichocyst matrices. (Arrowheads) Stage II matrices. Bar, 5 μm. × 2,400.

matrix expansion occurring after osm/glut fixation (Figs. 4, 5, and 8).

## DISCUSSION

### Picric Acid-induced Release

In this report we have shown that two different drugs known to bind to CaM and inhibit its actions (18, 19, 26, 27) will reversibly inhibit picric acid-induced release of the secretory organelle content of *Paramecium* (Fig. 1). The kinetics of inhibition by the two drugs appear to be different judging from the graphs in Fig. 1. However, the decrease in secretory capacity is variable, with some experiments exhibiting faster or slower kinetics than the graphs shown. This variation may be due to slight differences in cell density, culture age, or residual $Ca^{2+}$ concentration, and does not appear to reflect different mechanisms of action for the two drugs. Clearly, the results always indicate inhibition of release with longer exposure to either W-7 or TFP.

There are, however, some differences in the responses of *Paramecium* to the two anti-CaM drugs. The recovery of secretory capacity following TFP exposure occurs more slowly and cannot be achieved by simply raising the $Ca^{2+}$ concentration, as in the case of W-7 (Fig. 1). TFP has a higher affinity for $Ca^{2+}$-CaM complexes than W-7 ($K_d$ = 1 vs. 11 $\mu$M) (19, 27), which may slow its removal from the cell. The rise in $Ca^{2+}$ concentration alone may be insufficient in reversing TFP inhibition because of at least two effects of the drug: (*a*) its specific binding to CaM and (*b*) nonspecific membrane effects resulting from its inherent hydrophobic properties (24). Raising the $Ca^{2+}$ concentration may overcome the specific inhibition of CaM, as suggested by the results from W-7 treatment, but this by itself may not be sufficient to overcome the possible nonspecific effects of exposure to TFP. However, if $Ca^{2+}$ is present from the start of TFP exposure it greatly reduces the extent of inhibition of secretion caused by the drug (Fig. 6). The same protection via $Ca^{2+}$ can be observed in W-7 experiments (Fig. 1).

The differential effects of the W-7/W-5 pair of drugs offer further support for a direct effect on CaM activity. These two drugs differ only slightly in structure but the affinity of W-7 for CaM is five- to sixfold greater than that of W-5 (20, 54). The ability of W-7 to inhibit secretion at concentrations where W-5 has no effect strongly suggests that the drug is acting by interfering with a CaM-mediated process.

### Three Stages of Matrix Expansion

We have defined three distinct stages in the expansion and release of the secretory organelle content of *Paramecium*. Using these stages, we propose a morphological sequence for the trichocyst matrix expansion. The structural reorganization of the trichocyst matrix has been examined (2, 15, 16, 46), and it was determined that the phase and electron-dense, fully condensed trichocysts, here defined as stage I, represent the true mature form of the organelle existing in vivo prior to stimulation. This form is converted by a rearrangement of preformed elements into stage III, the fully expanded trichocyst during the expansion process that normally accompanies extrusion (2, 15). The partially expanded trichocyst, which we call stage II, has also been observed and discussed by a number of authors (2, 16). Forms similar to this were seen in studies using potassium ferrocyanide to inhibit expansion (16). Detailed studies of trichocyst expansion (16, 17, 46) suggest that the matrix reorganization occurs via unfolding of tightly packed filamentous structures to form a more loosely packed matrix of a new periodicity. It is reasonable to suggest that during the unfolding process there may exist a brief transitory period when the periodicity is not clearly visible. This is strongly suggested by images such as Fig. 3 (also reference 17) where the small segment of the matrix which is in stage II is interposed between stage I and stage III, as if it is a region of transition.

Fixatives such as osm/glut and picric acid can stimulate secretion (43); cold osmium tetroxide (2) or low concentrations of glutaraldehyde (<1%) (35) will elicit complete release of trichocyst matrices. At higher concentrations, such as those used in this study, it is probable that stimulation of release by osm/glut is countered by more rapid fixation, halting the process of matrix expansion for the majority of cases at an intermediate stage (stage II). It would appear that the unfolding process of the matrix has begun, but in most cases fixation occurs before full reorganization to stage III matrix can occur. It is possible that in the normal process of release of trichocysts the entire matrix is never actually in stage II all at once but proceeds very rapidly via stage II to stage III as extrusion occurs. In this sense the stage II trichocyst is somewhat artifactual, but we postulate that the molecular events which occur to produce stage II are indicative of the early events in the reorganization of the trichocyst matrix.

The transition to stage II trichocysts can be blocked by treatments which also block secretion, such as high $Mg^{++}$ concentration (15a, 44), slow cooling of cells, and, as shown here, treatment with the anti-CaM drugs TFP and W-7 (10–12, 45). Inhibition of expansion from stage I to stage III (release of trichocysts), and from stage I to stage II by identical treatments, suggests either that they have common requirements, or represent the same pathway. We suggest that stage II trichocyst matrix represent an intermediate form and not an alternate form since images of single trichocysts exhibiting all three stages in succession along their length can be found (Fig. 3; reference 17).

### Effect of CaM Antagonists on Matrix Expansion

*Paramecium* cells preincubated in TFP or W-7 are blocked from promoting trichocyst matrix expansion when fixed (osm/glut = stimulus). The lack of matrix expansion is a visible indication that TFP and W-7 apparently have interrupted the chain of events leading to exocytosis, perhaps by interfering with CaM-activated cellular events. These results provide corroboration for the inhibition of picric acid-induced trichocyst release by TFP and W-7 observed at the light microscope level (47; Fig. 1). The ultrastructural manifestation of this inhibition shown here represents the first clear manipulable demonstration of the effect of CaM antagonists on components of the $Ca^{2+}$-sensitive exocytic machinery.

The mode of action of TFP and W-7 has been shown in vitro to be a $Ca^{2+}$-dependent binding to a hydrophobic site on the CaM molecule (25, 26, 53–54). The resulting $Ca^{2+}$-CaM-drug complex is then incapable of interacting with and activating its target enzymes. Currently, this reaction has not been shown to occur in vivo, although W-7 has been localized throughout the cytoplasm, similar to CaM (20). Phenothiazines in general, and TFP in particular, have been shown to bind to other cellular proteins (7, 31), and could also be interfering with lipid-enzyme interactions due to their hydrophobic nature. However, the ability to block secretion and matrix expansion with a structurally different CaM antagonist provides stronger support for

the view that the primary effect of these agents is the inhibition of CaM function.

The ability to titrate the inhibition by altering the $Ca^{2+}$ concentration also fits predictions based on the model of the "TFP shunt" proposed by Satir et al. (45). This model suggests that increasing the available $Ca^{2+}$ should moderate, but not abolish, the inhibitory effect of CaM antagonists. Higher intracellular $Ca^{2+}$ concentration following stimulation will create a greater "pool" of $Ca^{2+}$-CaM, which, when drug concentration is held constant, will lead to a higher percentage of activated enzyme complexes and a moderation of TFP or W-7 inhibition. An assumption inherent in this model is that within the cell the concentration of CaM exceeds that of TFP, so that excess CaM is available to form $Ca^{2+}$-CaM complexes when intracellular $Ca^{2+}$ is raised.

A moderation of inhibition is indeed what we see in the case of trichocyst expansion (Fig. 6*a*). A similar moderation was reported by Kanamori et al. (22). These authors found that relaxation of rat aortic strips induced by W-7 could be partially reversed by the addition of excess extracellular $Ca^{2+}$.

Another prediction of the "TFP shunt" is that increasing the concentration of CaM antagonists will counter the moderating effects of increased $Ca^{2+}$ concentration. $Ca^{2+}$ and TFP or W-7 should work in opposition, with $Ca^{2+}$ driving the formation of $Ca^{2+}$-CaM complexes, and the drugs acting to lower the availability of these complexes for interaction with target enzymes. The results shown in Fig. 4*b* clearly demonstrate that increasing the TFP concentration from 14 to 20 $\mu$M abolishes the moderating effect of high extracellular $Ca^{2+}$ on the inhibition of trichocyst expansion; most organelles now remain in stage I. Therefore, the second prediction of this model is satisfied.

While this evidence is not conclusive proof of a role for CaM in trichocyst release, it is hard to account for these results by any other model. For instance, CaM antagonists might simply be inhibiting $Ca^{2+}$ entry into cells, so that stimulation does not lead to a rise in intracellular $Ca^{2+}$ concentration sufficient to promote expansion. Results from a study of the effects of CaM antagonists on swimming behavior in *Paramecium* suggest that intracellular $Ca^{2+}$ increases following exposure to the drugs (35a). This would suggest that $Ca^{2+}$ is present in the cytoplasm but unable to activate events leading to matrix expansion and secretion. This is also suggested by the results of experiments in which the $Ca^{2+}$ ionophore A23187 was used to raise cytoplasmic $Ca^{2+}$ directly and thereby stimulate secretion. The effects of exposure to 8 $\mu$M TFP are completely overcome by the ionophore, but when TFP concentration is raised to 14 $\mu$M a small but significant level (~25%) of inhibition remains even after ionophore stimulation (Fig. 7).

## Possible Sites for Calmodulin Action

Where does CaM act in regulating trichocyst expansion and secretion? A possible sequence of events involved in trichocyst release appears to be: (*a*) an influx of extracellular $Ca^{2+}$, raising cytoplasmic $Ca^{2+}$ $>10^{-6}$ M, (*b*) formation of $Ca^{2+}$-CaM complexes, and (*c*) expansion of trichocyst content and fusion of trichocyst and plasma membranes in a coordinated fashion, to result in release of the fully expanded trichocyst matrix.

Observations on the swimming behavior of *Paramecia* exposed to TFP suggest that cytoplasmic $Ca^{2+}$ increases following exposure to CaM antagonists. *Paramecium* exposed to 10–15 $\mu$M TFP or 40 $\mu$M W-7 exhibit a slowing of forward swimming (35a), which is indicative of a rise in cytoplasmic $Ca^{2+}$ concentration from $10^{-7}$ M to the vicinity of $10^{-6}$ M (32). Therefore it would appear that CaM antagonists are capable of inhibiting trichocyst matrix expansion even when cytoplasmic $Ca^{2+}$ concentration is elevated, suggesting that the effect of the CaM antagonists is after $Ca^{2+}$ entry.

Of the events following the rise in intracellular $Ca^{2+}$ concentration, matrix expansion is clearly TFP sensitive. Matrix expansion requires $Ca^{2+}$ and can be induced in isolated organelles in vitro by simple addition of this ion (4, 11). In vivo, spontaneous trichocyst expansion is presumably prevented by the trichocyst membrane, which prevents access of $Ca^{2+}$ to the matrix. Upon stimulation, $Ca^{2+}$ must enter the trichocyst vesicle to induce expansion and can do so via two possible routes: (*a*) through the trichocyst membrane which, following stimulation, is made permeable to $Ca^{2+}$; or (*b*) from the outside via the exocytic opening created by the fusion of trichocyst and plasma membranes. While the second alternative probably contributes to the $Ca^{2+}$ flux involved in expansion, it does not appear necessary, at least in the early stages of trichocyst expansion seen following fixation, the stage I to stage II transition. When the tips of expanded (stage II) trichocysts can be viewed in favorable sections, most of them do not have fused membranes. Indeed, partially expanded trichocysts (stage II) are common, whereas fusion profiles are rarely seen. These observations suggest that the route of $Ca^{2+}$ into the trichocyst vesicle following fixation is across the trichocyst membrane, but do not preclude that in vivo membrane fusion may occur prior to or concurrent with expansion.

The next question is whether TFP inhibits matrix expansion by blocking this $Ca^{2+}$ flux across the trichocyst membrane, or, alternatively, whether the inhibition is due to a direct interaction of TFP with the matrix itself? This latter possibility is especially important to consider in light of a recent report that CaM comprises a significant percentage of the protein of extruded trichocyst matrices (38). In vitro studies on isolated, membrane-free, condensed trichocysts (stage I) in our laboratory suggest that TFP as well as other CaM antagonists appear not to inhibit the $Ca^{2+}$-induced matrix expansion (11, 13). The lack of an inhibitory effect in vitro may suggest that a component(s) conferring drug sensitivity in vivo is lost during the isolation procedure. These may be cytoplasmic components involved in stimulus-secretion coupling, regulatory components associated with the matrix itself, or possibly the trichocyst membrane, absent from the isolated organelle, which may regulate $Ca^{2+}$ access to the trichocyst matrix in a CaM-dependent manner. Another potential site for CaM regulation is suggested by a recent report which correlates a $Ca^{2+}$-dependent dephosphorylation with secretion in these cells (14).

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