Quenched Flow Analysis of Exocytosis in *Paramecium* Cells: Time Course, Changes in Membrane Structure, and Calcium Requirements Revealed after Rapid Mixing and Rapid Freezing of Intact Cells

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Abstract. Synchronous exocytosis in *Paramecium* cells was analyzed on a subsecond time scale. For this purpose we developed a quenched flow device for rapid mixing and rapid freezing of cells without impairment (time resolution in the millisecond range, dead time ∼30 ms). Cells frozen at defined times after stimulation with the noncytotoxic secretagogue aminoethyldextran were processed by freeze substitution for electron microscopic analysis. With ultrathin sections the time required for complete extrusion of secretory contents was determined to be <80 ms. Using freeze-fracture replicas the time required for resealing of the fused membranes was found to be <350 ms. During membrane fusion (visible 30 ms after stimulation) specific intramembranous particles in the cell membrane at the attachment sites of secretory organelles ("fusion rosette") disappear, possibly by dissociation of formerly oligomeric proteins. This hitherto unknown type of rapid change in membrane architecture may reflect molecular changes in protein–protein or protein–lipid interactions, presumably crucial for membrane fusion. By a modification of the quenched flow procedure extracellular [Ca^{++}] during stimulation was adjusted to $\leqslant 3 \times 10^{-8}$ M, i.e., below intracellular [Ca^{++}]. Only extrusion of the secretory contents, but not membrane fusion, was inhibited. Thus it was possible to separate both secretory events (membrane fusion from contents extrusion) and to discriminate their Ca^{++} requirements. We conclude that no Ca^{++} influx is necessary for induction of membrane fusion.

As yet, the mechanisms of biological membrane fusion remain obscure (Düzgünes and Bronner, 1988; Ohki et al., 1988; Plattner, 1989; Almers, 1990; Hoekstra and Wilschut, 1990). Whereas at least in some viral systems fusogenic proteins have been identified (Stegmann et al., 1989; White, 1990), no comparable molecular effectors are known for membrane fusion during exocytosis. The regulatory control of exocytosis is also rather unclear (Plattner, 1989). While Ca^{++} was quite generally established as a second messenger, evidence has been obtained now for secretion occurring without any increase of $[Ca^{++}]_i$ (for example see Neher, 1988; Gomperts, 1990).

Serious problems in the analysis of exocytotic membrane fusion involve the short life time and the low frequency of membrane fusion events (see Knoll et al., 1987; Plattner, 1989 for discussion). Appropriate techniques and experimental systems are much needed to overcome these problems. The recent use of patch clamp techniques has allowed the study of some aspects in real time (Neher and Marty, 1982; Penner and Neher, 1989). Thus single fusion events have been correlated to a variety of phenomena including $[Ca^{++}]_i$ changes. Patch clamp observations have also led to the proposal of a junction-like "fusion pore" that connects the membranes just before the actual joining of the bilayers by intercalation of lipids (Almers, 1990). This model is compatible with a "focal (point) fusion mechanism" (Plattner, 1981), based on electron microscopic findings with cells rapidly frozen during exocytosis (Heuser et al., 1979; Chandler and Heuser, 1980; Ornberg and Reese, 1980; Schmidt et al., 1983, Olbricht et al., 1984).

Rapid freezing allows the fixation of dynamic events with a millisecond time resolution. This approach has provided solid experimental evidence for the correlation of transmitter release and exocytosis from synaptic vesicles (Heuser et al., 1979, Torri-Tarelli et al., 1985). However, even in such a well-synchronized system, the frequency of vesicle exocytosis must be artificially increased in order to obtain significant data.

This situation is much more favorable with the ciliated protozoan *Paramecium tetraurelia.* More than 1,000 secretory vesicles (trichocysts) are docked at the plasma membrane ready for exocytosis within 1 s after stimulation with the noncytotoxic secretagogue aminoethyldextran (AED)[1] (Plattner et al., 1984, 1985; Plattner, 1987). Since the time range of interest lies within 1 s, we have now used rapid mixing of

1. *Abbreviations used in this paper*: AED, aminothyldextran; EF-face, exoplasmic fracture face; IMP, intramembranous particle; PF-face, protoplasmic fracture face.

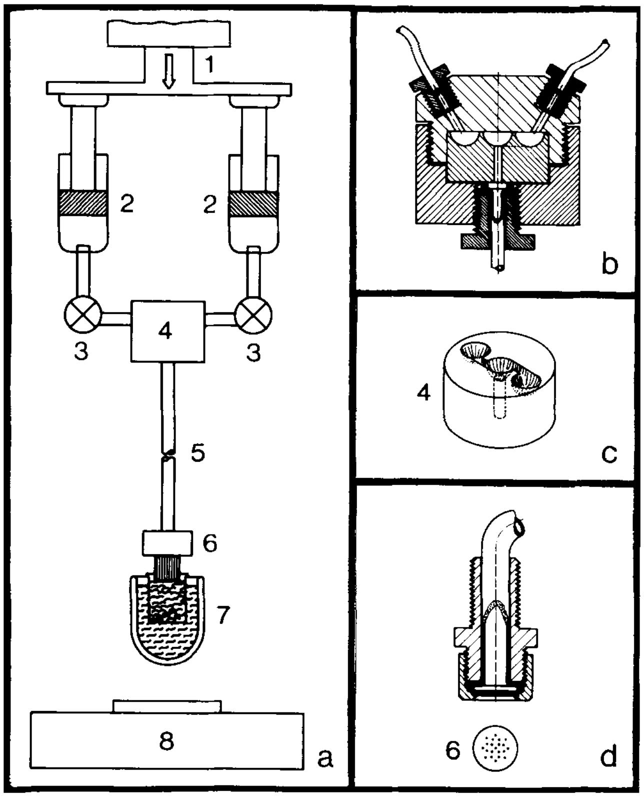

Figure 1. Set-up for rapid mixing and rapid freezing of cells. (*a*) Two cylinders (*2*) are filled via 3-way valves (*3*). By operation of a ram (*1*) the pistons in the cylinders push the reactants (cell and secretagogue) through a mixing chamber (*4*). After passage through defined tubing (*5*) the mixture is sprayed through a sieve plate (*6*) into heavily stirred liquid propane (*7*), cooled by LN_2 on a magnetic stirrer (*8*). (*b*) Housing of the mixing chamber. (*c*) Mixing chamber. (*d*) Sieve plate holder. For the shortest trigger time (30 ms) any tubing is omitted and the sieve plate fixed immediately to the outlet of the mixing chamber. For 80 ms the sieve plate holder (including tubing of 1.5 mm inner diameter) is screwed directly into the housing of the mixing chamber. For all longer time periods the length of the tubings (1.5 mm inner diameter) is specified, thus resulting in different but defined time periods until freezing.

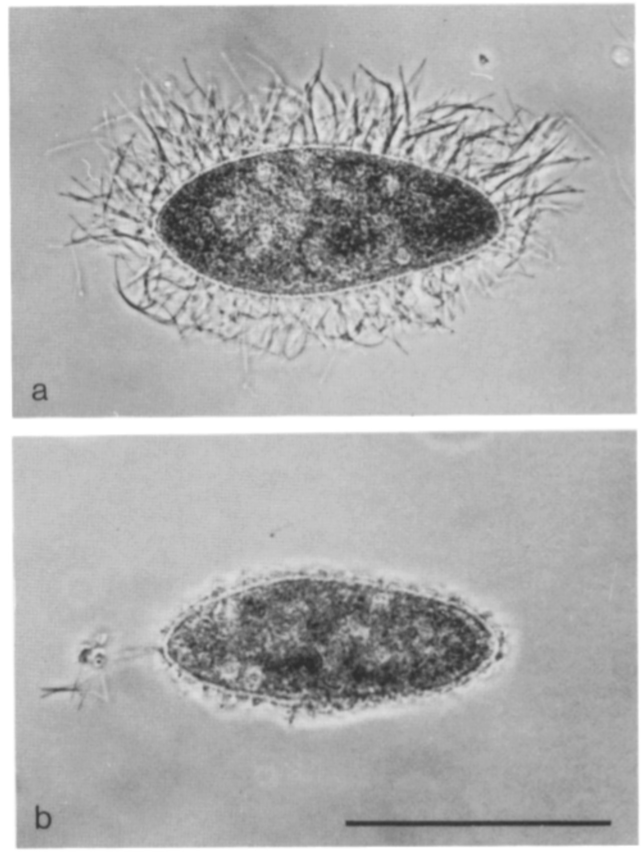

Figure 2. Light microscopic assay (*a*) of cellular integrity after rapid mixing and spraying (*b*) of the efficiency of AED as a secretagogue. After passage of the mixing chamber, cells were sprayed through the nozzle into a beaker (rather than into cold propane for freezing as usual). Picric acid was then applied as a simultaneous trigger and fixative. This allows for an estimation of the residual trichocysts, because with picric acid trichocysts are not completely extruded and remain anchored in the cell. (*a*) To check whether mechanical impairment had caused artifactual trichocyst release, cells were mixed with water (instead of the secretagogue AED). They completely retained their trichocysts during the mixing and spraying procedure. Trichocysts can then be expelled upon adding picric acid. (*b*) Cells challenged by the secretagogue AED (inducing vital exocytosis with complete extrusion of the trichocyst contents) reveal only a small number of trichocysts retained (left side, extruded upon addition of picric acid). Bar, 100 μm.

the cells, followed by rapid freezing (principally spray freezing; Bachmann and Schmitt, 1971, Plattner et al., 1972). This general procedure is known as quenched flow (Bray, 1961; Ballou, 1983; Rand et al., 1985), but has not yet been used for the preparation of intact cells. Once the cells are frozen at defined times after stimulation, different analytical follow-up procedures may be carried out.

Initially we determined the time course of events during exocytosis. For this purpose we made use of morphological criteria, as trichocysts are regularly arranged between the cilia and are easily recognized. Freeze-fracture replicas indicate whether membranes are fused, and thin sections allow determination of extrusion of secretory contents. Using the results of the time-course study, it was possible to establish a close correlation of specific changes in membrane structure (indicating for the first time dissociation of intramembranous particles [IMPs]) with membrane fusion.

Although Ca^{++} is well known to be necessary for exocytosis of trichocysts in *Paramecium* (Matt et al., 1978; Plattner, 1987; Satir et al., 1988; Satir, 1989; Kerboeuf and Cohen, 1990), its distinct (regulatory) role in membrane fusion and extrusion of secretory contents has yet to be established (Adoutte, 1988). Therefore we chelated Ca^{++}_e during stimulation to a concentration below the intracellular level. The quenched flow device was necessary to overcome the problem of rapid lethal effects of Ca^{++} deprivation (Plattner et al., 1985). Analysis by electron microscopy enabled a safe differentiation between membrane fusion and trichocyst decondensation. We demonstrate that membrane fusion occurs at $[Ca^{++}]_e$ even below the resting $[Ca^{++}]_i$, but trichocyst extrusion is inhibited.

Materials and Methods

Cells and Stimulus

Paramecium tetraurelia 7S (wild type) cells were grown axenically until early stationary phase, washed in buffer (5 mM Pipes, 1 mM $CaCl_2$, 1 mM KCl, pH 7) and starved in this buffer overnight (for details and for AED triggering, see Plattner et al., 1985).

Stimulation by Rapid Mixing with AED followed by Rapid Freezing

A scheme of the quenched-flow device used is presented in Fig. 1. Two glass cylinders (of variable diameter) with tightly fitting Teflon pistons are filled via threeway valves (HVX, ports with 3 mm diam for cells; HV, ports 1.5 mm diam for the second component, such as AED; Hamilton, Darmstadt, Germany) by reservoir syringes. For mixing and subsequent freezing the pistons are pushed by a pneumatic ram (Festo ZY-35-80-B; Festo Pneumatic, Esslingen, Germany) with an adjustable integrated hydraulic speed limiting system. Linearity of movement during the sampling period was assessed by recording the signal of an attached potentiometer wheel. The speed in each actual experiment was determined by a home-made light bar system.

During continuous flow, cells (30,000/ml) are challenged by the same volume of trigger agent (0.01% final concentration AED) in a home-made two-jet vortex mixer (7 μl vol, see Fig. 1 *c*). Efficiency of mixing just at the outlet of the mixing chamber was confirmed by the color change of acidic bromophenol blue after contact with a high pH buffer.

After passage through Teflon tubing (1.5 mm inner diameter) of varying length (allowing the cells to be exposed to the trigger agent for different time periods; see Fig. 1 for details) cells pass through an aluminum plate containing a defined number of drilled holes with 100-μm-diam each (Fig. 1 *d*). The actual number of holes is chosen depending on the desired overall flow rate (volume/time) in order to adjust the flow speed through each hole. In the current experiments *Paramecium* cells were sprayed with 3 meter/second into forcefully stirred liquid propane (<−180°C).

The device and the performance were optimized for both high time resolution and preservation of cell integrity.

Efficiency of Mixing and Freezing

A semiquantitative test, using the well-characterized dehydration of carbonic acid according to Bray (1961), was performed to check the efficiency of mixing. Similarly to Bray (1961), who used a comparable set-up, we estimate a gross dead time (from mixing till completion of freezing) of ~30 ms with the moderate conditions found to be essential for preservation of cell integrity.

Integrity of Cells

Cells were sprayed into a beaker (rather than into propane) and by light microscopy we verified vitality and trichocyst content before each actual experiment. The latter parameter was checked by triggering the cells (after passing the apparatus) with the fixative picric acid, resulting in simultaneous fixation and partial extrusion of elongated trichocysts that remain stuck within the cells (Jennings, 1906). Experimental conditions were accepted only when >90% of the cells were apparently unaffected by the handling procedure (see Fig. 2).

Withdrawal of Calcium before Stimulation

Cells were mixed with EGTA-buffer before stimulation with AED: Three syringes (two with the same volume and one with a double volume) and two mixing chambers were connected for mixing cells (60,000/ml in 5 mM Pipes, 1 mM KCl, 1 mM $CaCl_2$, pH 7) with EGTA (9 mM, in 5 mM Pipes, pH 7) for 500 ms, followed by a second mixing of cells with AED (0.02% in 5 mM Pipes, 4.5 mM EGTA, pH 7) for 80 ms before freezing. The $[Ca^{++}]_e$ is expected to be adjusted to ~30 nM (calculated according to Bulos and Sacktor, 1979) already ~10 ms after mixing (Smith et al., 1984), and to decrease below this value during the actual stimulation with AED/EGTA.

Preparation for Microscopy

As a first step after freezing, propane is evaporated at −100°C under vacuum in a freeze dryer (GT1; Leybold Heraeus, Köln, Germany). The remaining powder of frozen hydrated material may be stored under liquid nitrogen.

For freeze fracturing and thin sectioning the material was freeze substituted for 2 d in methanol at −80°C, followed by slow rise of temperature (~5°C/h). Both media contained 3% (vol/vol) glutaraldehyde and 0.5% (wt/vol) uranyl acetate; in addition to this, for thin sectioning 1% (wt/vol) osmium tetroxide was included. Before embedding the temperature was allowed to rise to 5°C, the cells were washed in methanol and embedded in Spurr's resin at room temperature. After polymerization ultrathin sections were stained with uranyl acetate and lead citrate. For freeze fracturing, cells were allowed to warm up only to −30°C, then they were centrifuged at that temperature and rehydrated in ice cold water. After gradual glycerination (10, 20, 30% [vol/vol] for 1 h each) at room temperature, cells were pelleted and frozen either on gold holders or in Cu-sandwiches by dipping into liquid propane. Freeze fracturing was performed in a Balzers unit type 360 M at −100°C and a vacuum of 4–6 × 10^{-5} Pa with occasional 1 min etching before shadowing with Pt/C and C.

Evaluation

To determine the content of trichocysts in cells, grazing sections were evaluated by counting all docking sites, whether hosting a trichocyst or not (Pape and Plattner, 1985). The percentage of occupied sites was determined for each cell, and the median of all individual percentages was calculated.

For evaluation of freeze fractured exocytosis sites a set of structural-functional categories was elaborated (see Results). The number of docking sites belonging to the different categories was counted for each cell, the percentage computed and the median for all cells was calculated.

IMPs were counted within the double ring (~300 nm diam) of IMPs delineating fusion spots in the plasma membrane, both in a central area of 130 nm diam (where at rest almost exclusively rosette IMPs are found) and in the peripheral area between the central area and the double ring of IMPs mentioned above.

Results

Time Course of Exo-Endocytosis

Trichocyst Extrusion. First we analyzed the lag time for the extrusion of trichocysts. For this purpose cells frozen at defined time points after stimulation were freeze substituted and embedded for sectioning. In sections grazing the cell surface, the regular arrangement of predetermined trichocyst docking sites enables the counting of the percentage of sites actually occupied (Fig. 3, *a* and *b*). After membrane fusion and extrusion of the secretory contents an empty membrane compartment is visible only in the plane of trichocyst tips (for terminology, see Plattner, 1987), and the membrane formerly enclosing the trichocyst body is collapsed (see also Hausmann and Allen, 1976; Allen and Fok, 1984). The results of the evaluation are shown in Fig. 3 *c*: after 80 ms, all trichocysts have been extruded. The nearly complete occupation of docking sites in untriggered controls after passing the quenched flow apparatus indicates the absence of cell impairment by the procedure (see also Fig. 2).

Membrane Fusion and Resealing. To analyze the time course of membrane coalescence we subjected the frozen cells to freeze fracturing. The predetermined docking sites of the plasma membrane exhibit a well characterized freeze fracture appearance (Plattner et al., 1973, Plattner, 1987): When a trichocyst is docked ready for exocytosis, this is indicated by a "fusion rosette"; its name indicates the correlation with competence for membrane fusion (Beisson et al., 1976, 1980; Pouphile et al., 1986). This rosette consists of about seven to eight IMPs encircled by a 300-nm-large double ring of smaller IMPs (Fig. 4, *a–c*). A collapsed "parenthesis" without rosette IMPs occurs when the site is not occupied

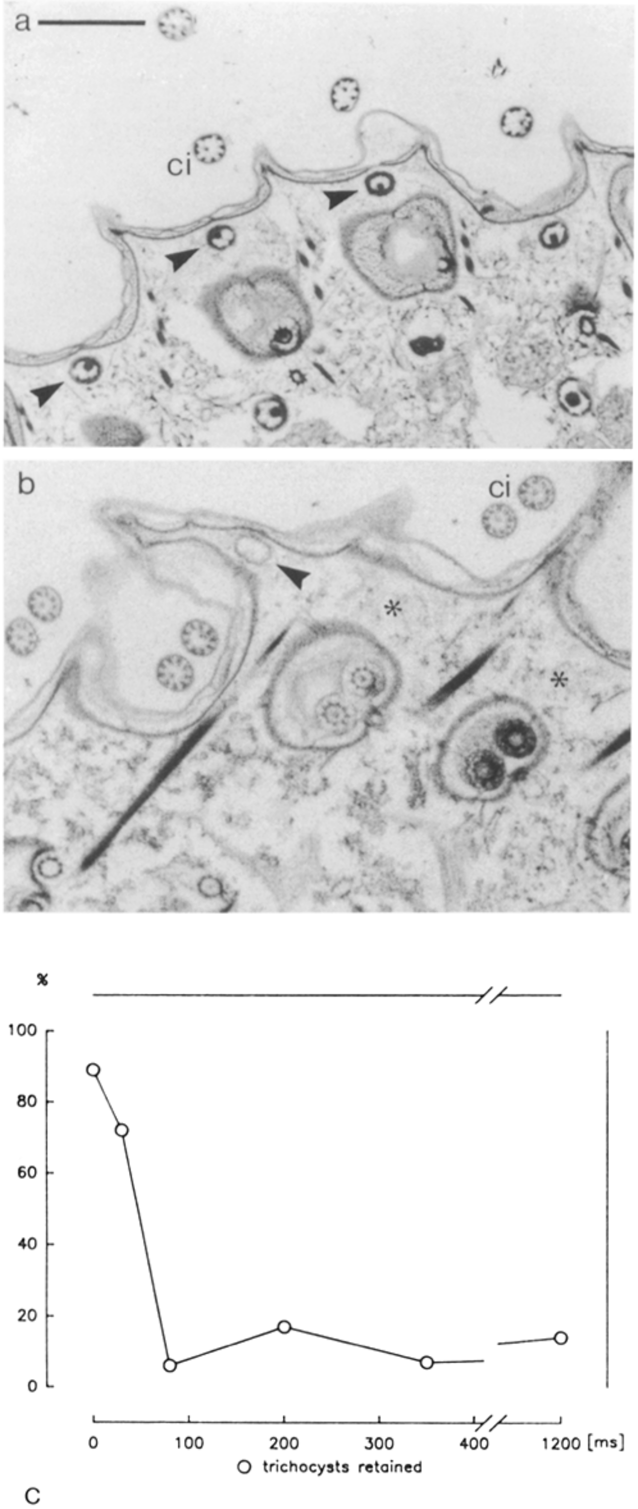

Figure 3. Trichocyst content of cells analyzed on ultrathin sections. Cells were rapidly mixed with AED (or with water for controls), frozen and further processed by freeze substitution and plastic embedding. In tangentional sections the regular arrangement of trichocysts allowed their quantitation at the docking sites (located between the cilia or their basal bodies, respectively, depending on the section plane). *ci*, cilium. (*a*) A control cell, frozen after mixing with an equal volume of water, shows virtually complete retainment of trichocysts (*arrowheads*). (*b*) A cell frozen 80 ms after mixing with AED shows docking sites devoid of a trichocyst (*asterisks*) and frequently exhibits trichocyst membrane ghosts with a varying degree of collapse (*arrowhead*). (*c*) Evaluation of ultrathin sections as shown in *a* and *b*, as described in Materials and Methods. The percentage of trichocysts retained (in relation to the total number of docking sites analyzed) after AED triggering is presented. The time periods indicated include the dead time of the experimental set up (see text). In at least three independent experiments ~20 cells were analyzed for each time point. As early as 80 ms after contact with the secretagogue, AED, trichocyst extrusion was completed. Bar, 1 μm.

(Fig. 4, *j–l*). The rosette IMPs are located at the contact site of the plasma membrane with the trichocyst membrane, and the ring IMPs at contacts of the plasma membrane with the "alveolar sacs" (Plattner et al., 1973).

In control cells most docking sites in the plasma membrane reveal the usual fusion rosette (Fig. 4, *a–c*). 30 ms after triggering by AED exocytotic openings prevail (Fig. 4, *d–f*) and after 350 ms the membranes are resealed (Fig. 4, *g–i*). However, the freshly resealed plasma membrane shows no parenthesis or "oval ring" as described earlier (Olbricht et al., 1984). Instead, the double ring of IMPs is still round and it is filled with numerous small IMPs instead of rosette IMPs. The density of the small IMPs declines with time, and only after almost 1 min do we observe a substantial increase of parenthesis stages (data not shown). The time dependence of the consecutive stages of exocytosis as defined in Fig. 4 is quantified in Fig. 5.

Changes in Membrane Structure

The most significant aspect during membrane fusion and resealing is the disappearance of the rosette IMPs, and the appearance of a new population of smaller IMPs on the protoplasmic fracture face (PF-face) (Fig. 6, Fig. 4 *g*). To test the assumption that this is due to dissociation of the rosette IMPs, we counted the IMPs in the inner central area (130 nm diam) of the double ring, where at rest almost exclusively rosette IMPs (seven to eight) are found. The result is presented in Fig. 7. After stimulation by AED the number of small IMPs suddenly (30 ms) increases to ~35, whereas only a few, on the average below two, prominent rosette particles are still recognized. The number of small IMPs always present in the more peripheral area of the double ring ("b-type" particles as defined by Plattner et al., 1973) remains unchanged. Thus the disappearance of the rosette IMPs is correlated with a local increase of small IMPs by a factor of ~6. The situation does not change significantly during the first second after membrane resealing (Fig. 7). Only after 40 s (first point on a longer time scale analyzed as yet) we observed a reduction of IMP densities close to the number found in the final parentheses (Fig. 4, *j–l*) (data not shown).

Examples for presumable early stages of membrane coalescence are shown in Fig. 6. The plasma membrane shows a protrusion towards the trichocyst tip in exoplasmic fracture (EF-) faces and a complementary dip in the PF-faces. While the EF-face is nearly completely smooth, the PF-face shows a very high density of the small particles just at the fusion spot. A high density of IMPs occurs particularly around exocytotic openings in the PF-face (see Fig. 4 *d*).

Requirements of Extracellular Calcium

Membrane fusion and decondensation of secretory products

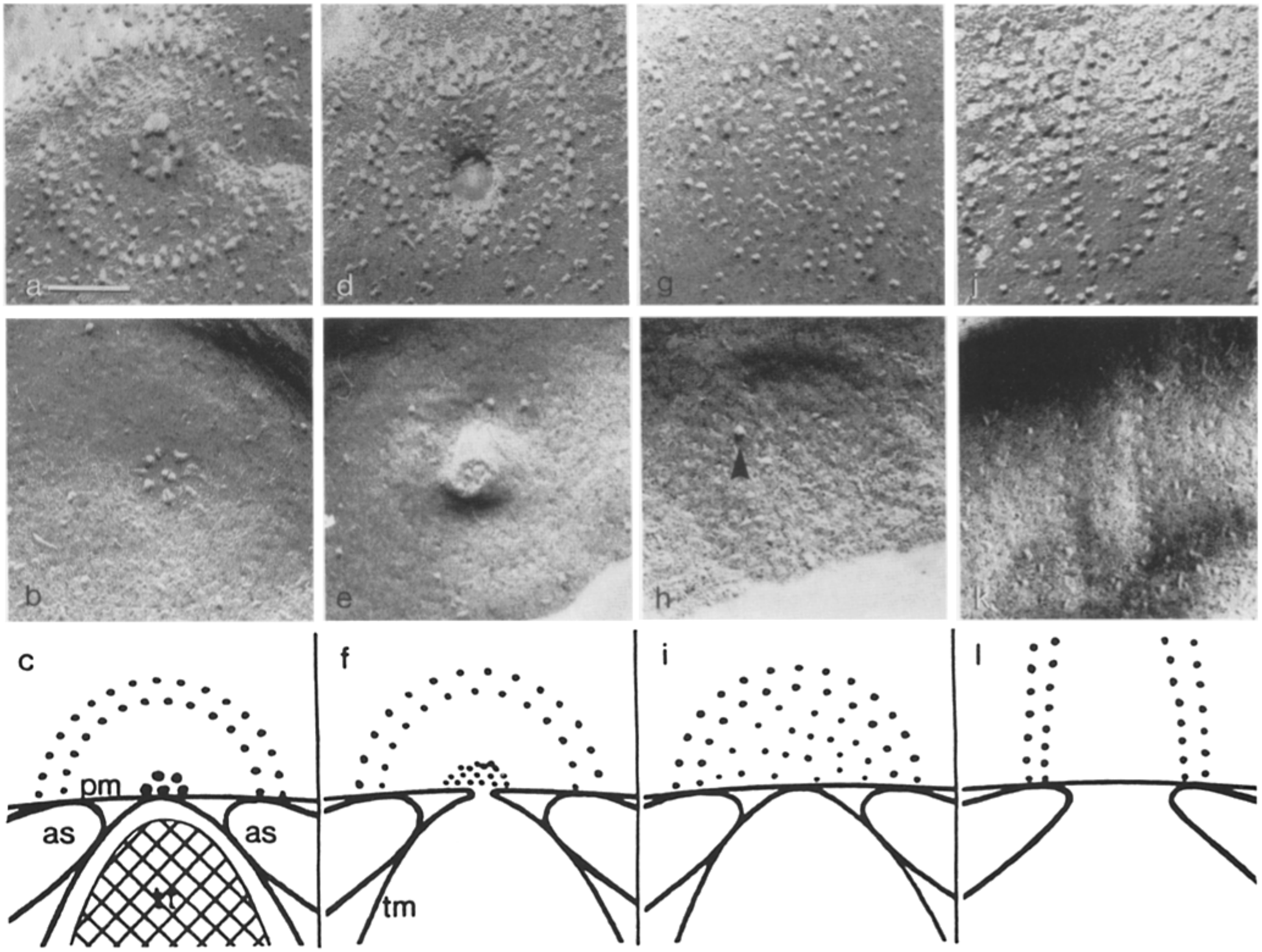

Figure 4. Ultrastructural changes in the plasma membrane during exo-endocytosis. Freeze-fracture aspects of both membrane leaflets, PF- (*top*) and EF-face (*middle*) are depicted as representative of the categories of exo-endocytosis stages considered in the quantitative evaluation of Fig. 5. Drawings illustrate our interpretation by a combination of freeze fracture and ultrathin section aspects of docking sites (see text). *as*, alveolar sacs; *pm*, plasma membrane; *tm*, trichocyst membrane; *tt*, trichocyst tip. (*a–c*) Preformed exocytosis sites containing a central aggregate of rosette particles, encircled by a double ring of IMPs (on PF-faces) or pits (on EF-faces), are indicative of "resting stages" with an exocytosis-competent trichocyst docked underneath. Note also the more peripheral small b-type IMPs (Plattner et al., 1973, see also Fig. 7) within the double ring on the PF-face. Cells were mixed with water instead of AED. (*d–f*) Exocytotic openings are seen during the period when plasma membrane and trichocyst membrane are actually fused. Some prominent rosette particles are diffusely distributed around the exocytotic opening just formed, and several small IMPs are visible in the PF-face near the exocytotic opening. Cells were frozen 80 ms after stimulation with AED. (*g–i*) The resealed plasma membrane displays a round double ring filled with numerous small and "ill-defined" IMPs on the PF-face, whereas the EF-face is totally smooth. No or only a few rosette IMPs (*arrowhead*) are seen on either fracture face at the exocytosis sites. Cells were frozen 1,200 ms after stimulation with AED. (*j–l*) "Parentheses" are indicative for nonoccupied exocytosis sites (after detachment of ghosts). Cells were frozen 40 s after stimulation with AED. Bar, 100 nm.

during exocytosis are distinct steps in *Paramecium* (Bilinski et al., 1981; Matt and Plattner, 1983). Because Ca^{++} is assumed to be involved in these processes, we tried to answer the question whether Ca^{++}_e is necessary for exocytotic membrane fusion. Since these cells are very sensitive to prolonged withdrawal of Ca^{++}_e, we made use of the quenched flow device to chelate Ca^{++}_e for a defined short time (500 ms) before stimulation. On the basis of the experiments described above, we know that in the presence of $[Ca^{++}]_e = 10^{-3}$ M, membranes are fused and the trichocyst contents are extruded 80 ms after AED stimulation. We chose this time point to freeze the cells stimulated under conditions of reduced $[Ca^{++}]_e$ (i.e., 500 ms EGTA and 80 ms AED + EGTA, $[Ca^{++}]_e \leq 30$ nM). Evaluation of grazing thin sections revealed retention of trichocyst contents (Fig. 8; Table I). Freeze-fracture replicas, however, showed the membranes to be fused, as can also be seen in median thin sections (Fig. 9; Table I). Thus, only decondensation of the trichocyst matrix, but not membrane fusion depends on Ca^{++}_e.

Discussion

In the present study we report on three major findings. (*a*) The time course of exo-endocytosis in *Paramecium* cells is described . (*b*) Specific membrane integrated ("rosette") particles dissociate into smaller IMPs during membrane fusion.

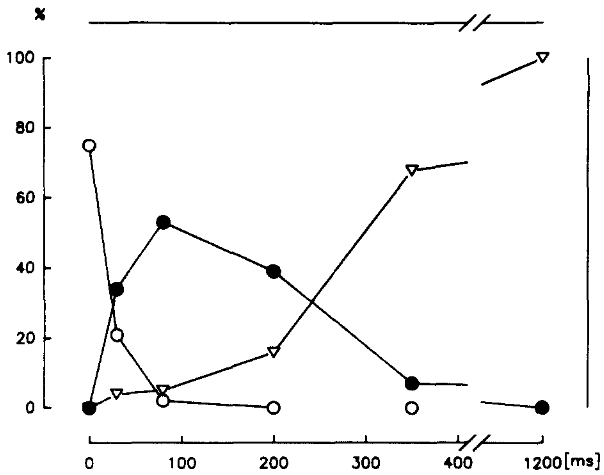

Figure 5. Time dependence of ultrastructural changes in the plasma membrane. Fracture faces of cells frozen at defined time points after exocytosis stimulation were analyzed as indicated in Materials and Methods, to determine the frequency of stages (defined and described in Fig. 4). In at least three independent experiments approximately 20 cells were analyzed for each data point. (*Open circles*) Resting stages with fusion rosettes, (*filled circles*) exocytotic openings of variable size, (*open triangles*) double ring filled with numerous small particles (resealing stage). Parentheses depicted in Fig. 4 were not considered here, since they were observed only at a very low and not significantly changing frequency during the time period analyzed; preliminary data indicated an increase only at times where retrieval of the trichocyst membrane has been observed (Pape and Plattner, 1985).

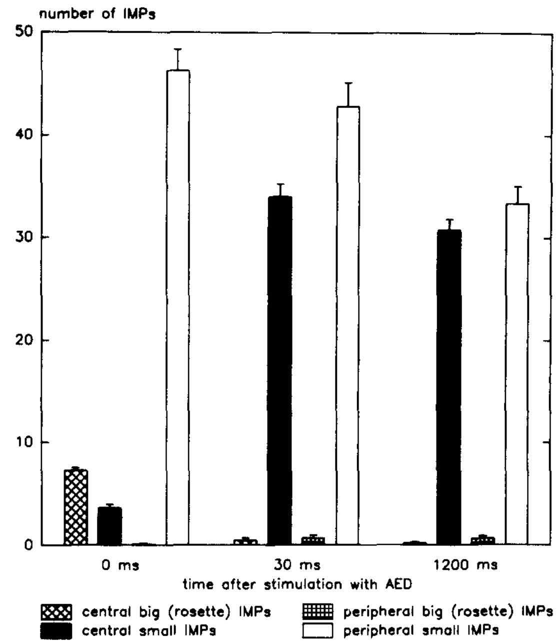

Figure 7. Change in frequency of rosette IMPs and small central IMPs (c.f. Fig. 6) during membrane fusion. The number of rosette IMPs and small IMPs at fusion spots (central area of 130 nm diam) during rest and after AED stimulation (30 and 1,200 ms) was counted. (As an internal control we also determined the more peripheral b-type IMPs in the area adjacent to the double ring, see also Fig. 4). In the fusogenic (central) area the number of rosette IMPs is reduced from ∼7–8 to below 2, whereas the number of central small IMPs increases from ∼3 (some very central b-type IMPs) to ∼35 upon AED stimulation. (The number of small peripheral IMPs did not change). More than 20 docking sites obtained in different experiments were evaluated for controls and stimulated cells.

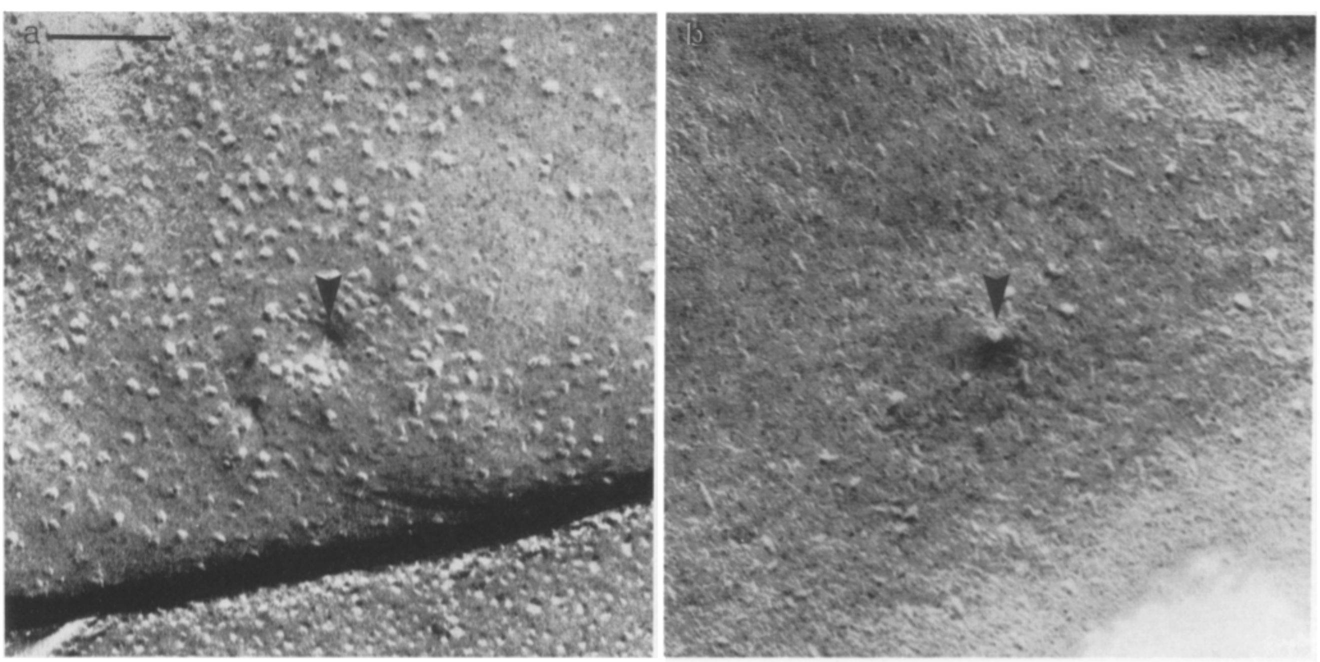

Figure 6. Fusion intermediates. Restructuring of the fusogenic zone in the plasma membrane during exocytotic membrane fusion (30–50 ms AED), i.e., at a stage between those depicted in Fig. 4, *a–c* and *d–f.* (*a*) PF-face. (*b*) EF-face. Note the funnel-like depression of the cell membrane, the occurrence of a central focal (point) fusion intermediate at arrowheads (represented by a pit [*a*] or an IMP [*b*], respectively, as well as the numerous surrounding small IMPs in the PF-face and few rosette IMPs in both fracture faces. Bar, 100 nm.

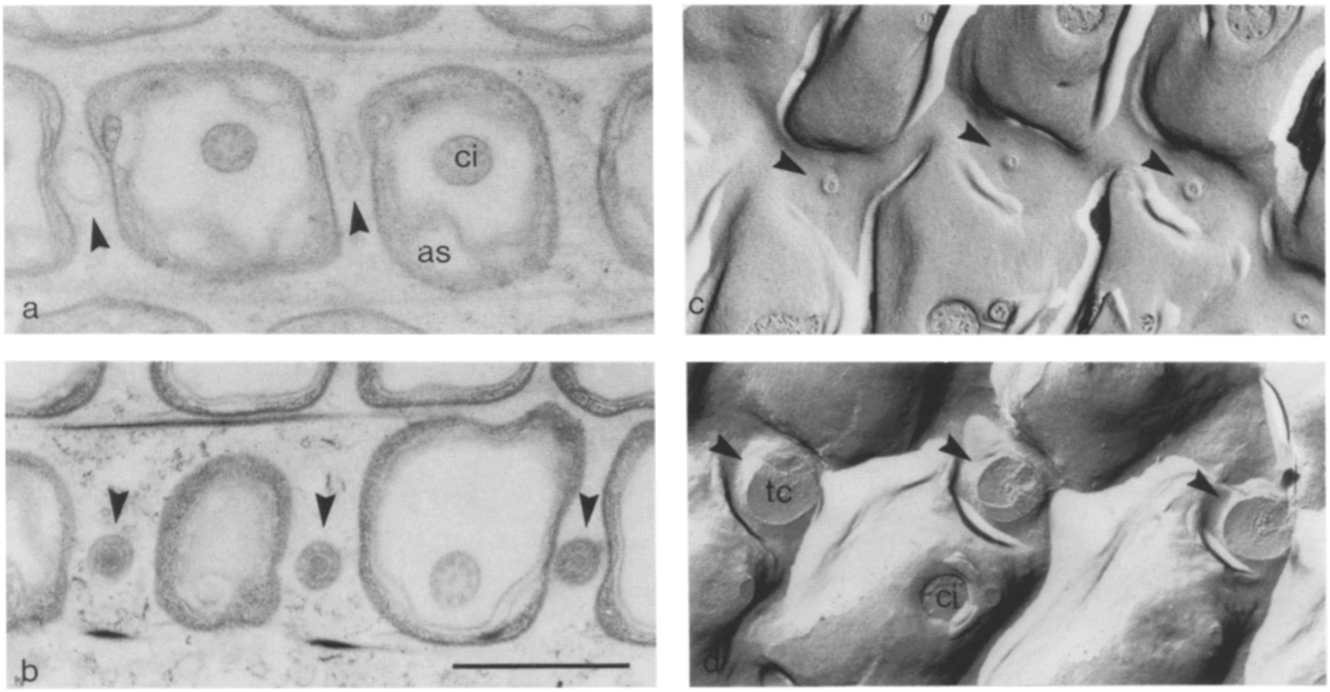

Figure 8. Trichocyst content and membrane fusion in cells stimulated in the absence or presence of EGTA. *as*, alveolar sacs; *ci*, cilium; *tc*, trichocyst contents. (*a*) Thin section of control cell stimulated in the absence of EGTA ($[Ca^{++}]_e = 1$ mM) for 80 ms as indicated in Materials and Methods. All trichocysts are extruded here; empty trichocyst membrane ghosts (only visible in the plane of the tips) are marked by arrowheads. (*b*) Thin section of a cell stimulated in the presence of 4.5 mM EGTA ($[Ca^{++}]_e < 30$ nM) for 80 ms. Trichocyst contents are completely retained and marked by arrowheads. (*c*) Freeze-fractured plasma membrane (EF-face) of a control cell stimulated in the absence of EGTA ($[Ca^{++}]_e = 1$ mM) for 80 ms. Small exocytotic openings characteristic of membrane fusion spots after discharge of trichocysts are visible (*arrowheads*). (*d*) Freeze-fractured plasma membrane (EF-face) of a cell stimulated in the presence of 4.5 mM EGTA ($[Ca^{++}]_e < 30$ nM) after 80 ms. Exocytotic openings indicative of fused membranes are visible. The diameter of the openings is larger than in control cells and trichocyst contents, though still in a condensed state, are also recognized. Bar, 1 μm.

(*c*) No Ca^{++} influx is required for induction of membrane fusion. All results are based on experiments using rapid mixing followed by rapid freezing, i.e., quenched flow.

Quenched Flow as a Tool for the Analysis of Subcellular Dynamics

Flow techniques (with a time resolution in the millisecond range) as a means for the analysis of rapid kinetics have been used since 1923 (Hartridge and Roughton), as continuous, stopped or quenched flow (Chance et al., 1964). But only occasionally have flow techniques (in whatever form) been used for the analysis of whole cells (Utsunomiya et al., 1986; Sage and Rink, 1987; Merritt and Rink, 1987; Carty et al., 1986; Jones et al., 1989). Cell damage induced by the procedure (a serious risk, as we had learned in pilot experiments with commercial equipment) might have hampered its more general use. This problem has now been overcome for cells even as big and as fragile as *Paramecium tetraurelia*.

After mixing we stopped cellular responses and collected the cells by rapid freezing. For analytical techniques, where real-time observation of living cells is not possible, this represents a generally applicable alternative. In this study we used electron microscopy for a first description and definition of events during exocytosis; but the same cell batches may be used for correlated alternative biochemical analyses.

Table I. Influence of $[Ca^{++}]_e$ on Membrane Fusion and Trichocyst Decondensation 80 ms after AED Stimulation

$[Ca^{2+}_e]$	Trichocysts retained	Membranes fused
	%	%
1 mM	29	52
<30 nM	79	50

The values (medians from 7 to 25 cells from two independent experiments) have been determined by counting retained trichocysts (on ultrathin sections) and fused membranes (analyzed on freeze-fracture replicas). While the difference in trichocyst content is highly significant ($p < 0.0001$), the amount of membranes fused is not different (even at $p > 0.1$; determined by U-test).

Time Course of Membrane Fusion and Resealing

By the use of quenched flow and electron microscopy, single events during synchronous exocytosis in *Paramecium tetraurelia* were discerned on a subsecond time scale: membrane fusion (∼30 ms), extrusion of secretory contents (⩽80 ms) and resealing of the fused membranes (⩽350 ms). (The time periods indicated apply to all events in the whole population of cells analyzed.) To our knowledge this is the best defined exocytotic system as yet, and therefore represents a solid basis to address various questions specific to each of the single events, particularly since all secretory organelles are released synchronously.

Changes in Membrane Structure

During membrane fusion and resealing the morphology of

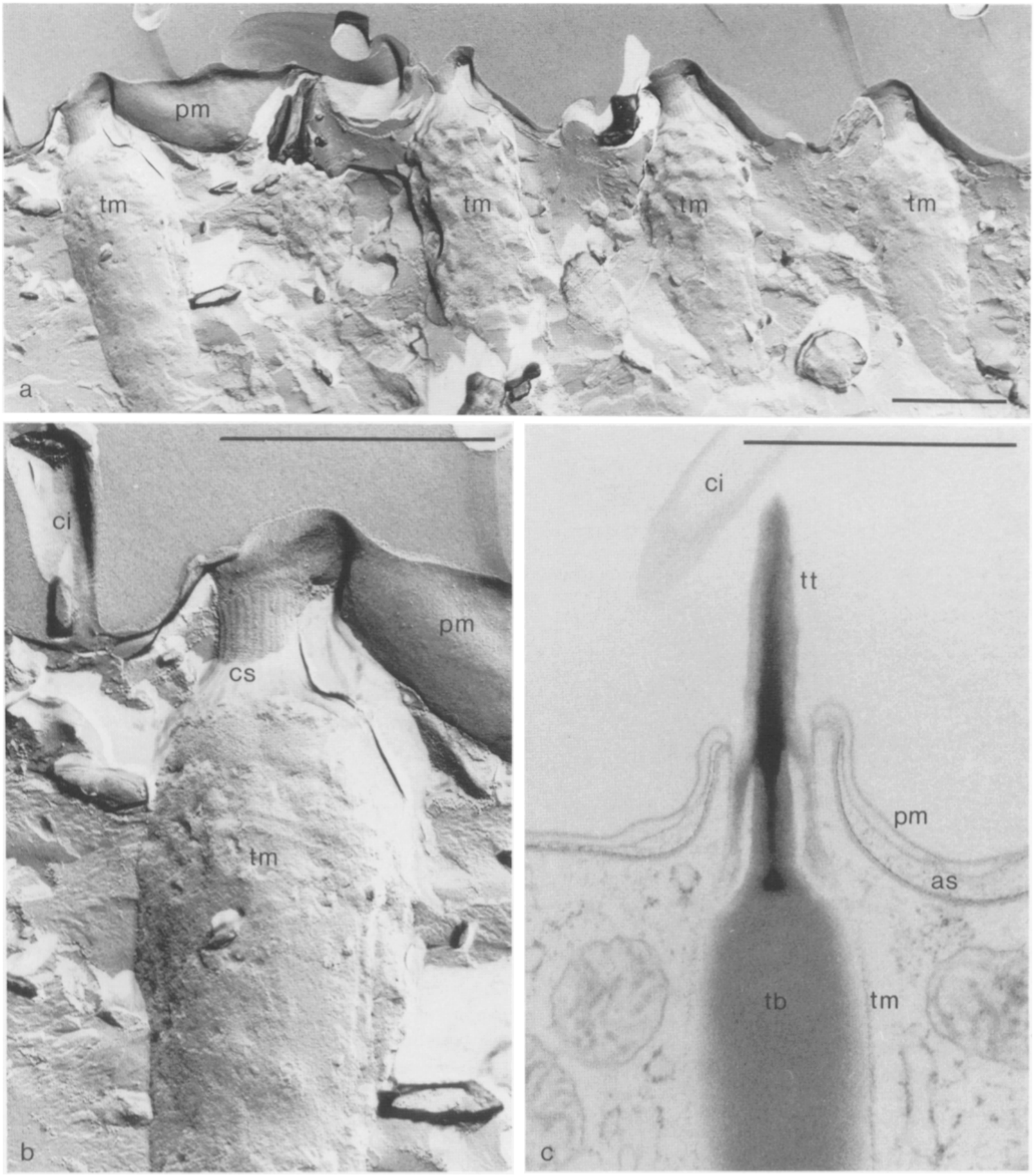

Figure 9. Membrane fusion aspects of cells stimulated in the presence of EGTA. *as*, alveolar sacs; *ci*, cilium; *cs*, collar striations; *pm*, plasma membrane; *tm*, trichocyst membrane; *tb*, trichocyst body; *tt*, trichocyst tip. (*a*) In a cross-fractured cell, continuity of the plasma membrane (*pm*) with the trichocyst membranes (*tm*) is apparent. (*b*) Detail from *a*; *cs*, collar striations of trichocyst membrane in continuity with the plasma membrane. (*c*) Median section of a trichocyst revealing continuity of the plasma membrane with the trichocyst membrane. The trichocyst body shows no sign of decondensation, but the trichocyst tip sticks through a 0.3-μm-wide exocytotic opening. Bars, 1 μm.

the freeze-fractured plasma membrane displays a significant change: a rosette of about seven to eight prominent IMPs at the docking site is replaced by numerous small IMPs. This transition is already obvious 30 ms after stimulation, a time during which membrane fusion, but not resealing, is considered to take place. Most trichocysts are not yet extruded. Two possibilities can be envisaged. (*a*) The rosette IMPs diffuse very rapidly out of the double ring encircling the fusion spot (Olbricht et al., 1984) and the numerous small IMPs would be rapidly inserted. (*b*) The rosette IMPs dissociate giving rise to smaller subunits. The following arguments are in favor of the second possibility. (*a*) Rosette IMPs were identified as proteins due to their sensitivity to proteolytic enzymes (Vilmart and Plattner, 1983), and integral membrane proteins exhibit diffusion coefficients in the order of 10^{-10} cm^2/s (McCloskey and Poo, 1984). This is too slow to allow most rosette IMPs to diffuse out of the double ring, even if any restraints holding them in place before would have been lost very rapidly. (*b*) It is suggested that rosette IMPs are assembled from smaller subunits by the underlying "connecting material" (Beisson et al., 1980), and indeed during docking of the trichocysts a "filled ring" exhibiting small IMPs has been observed preceding the assembly of rosette IMPs (Pape and Plattner, 1985). (*c*) A ratio of about six smaller IMPs to one rosette IMP has been observed. This would be a reasonable stoichiometry for an oligomeric protein.

Therefore we conclude that dissociation of the fusion rosette IMPs is correlated with membrane fusion, and that this is likely a causal relationship. The dissociation of oligomeric proteins as a means to expose previously hidden hydrophobic moieties (and thus turn proteins fusogenic) is discussed by Stegmann et al. (1989). The authors are attracted by the fact that such a change in protein conformation could easily be induced under physiological conditions. Oligomeric protein dissociation as a possible mechanism for membrane fusion has also been proposed by Lew et al. (1988) based on observations of inside-out vesiculation of erythrocytes. The disruption of the spectrin network liberates integral membrane proteins for monomerization and free diffusion. These monomers can disturb the continuous bilayer by the formation of openings, of free edges and of various fusion and fission events.

Such a mechanism would be compatible with our observations. The rosette IMPs are stabilized and kept in place by a connecting material between the plasma membrane and the trichocyst membrane (Beisson et al., 1980; Plattner et al., 1980; Westphal and Plattner, 1981; Pouphile et al., 1986). The trigger for membrane fusion would then affect this link, thereby setting the monomers of the rosette IMPs free to turn fusogenic.

The Role and Possible Source of Calcium

Chelation of Ca^{++} to 30 nM before, and considerably below this value during AED stimulation, resulted in inhibition of trichocyst decondensation, but membrane fusion was not affected. This result provides strong evidence against a gradient-driven Ca^{++} influx as a step necessary for induction of membrane fusion during trichocyst exocytosis (Satir et al., 1988; Satir, 1989; Kerboeuf and Cohen, 1990). Instead, the signal transduction pathway between AED binding and membrane fusion might require the liberation of Ca^{++} from intracellular stores, presumably the alveolar sacs underlying the plasma membrane (Stelly et al., 1991). On the other hand, extrusion of secretory contents is obviously tightly coupled to the forming of exocytotic openings: membrane fusion allows a Ca^{++} influx for decondensation of the trichocyst matrix, as suggested previously (Bilinski et al., 1981).

This apparent contradiction may be explained by the fact, that in several reports a light microscopic assay was applied to study Ca^{++} requirements for trichocyst exocytosis. Therefore membrane fusion and trichocyst decondensation could not be discriminated. However, these are distinct events, as can be seen clearly in electron micrographs (Figs. 8 and 9) in this study and former reports (Gilligan and Satir, 1983; Matt and Plattner, 1983). Apparently trichocyst extrusion is not suited as the sole parameter to assay membrane fusion.

In their study Kerboeuf and Cohen (1990) observed an AED-induced Ca^{++} influx a few seconds after AED stimulation. The correlation between this influx and membrane fusion is not very strict, since we show completion of the membrane fusion step already after 80 ms. At present it seems more likely that a Ca^{++} influx occurs only after membrane fusion, but more highly resolved time measurements are clearly necessary. A secondary Ca^{++} influx could serve for replenishment of Ca^{++}_i stores exhausted during stimulation, as has been proposed as one general mechanism for IP_3-induced Ca^{++} influx (Putney, 1990).

Since we have made evident that no Ca^{++} influx is necessary for membrane fusion, we suggest two other possible alternatives. (*a*) A rise in $[Ca^{++}]_i$ might not be necessary for membrane fusion at all. This would be in line with recent findings showing that GTP, rather than a rise in $[Ca^{++}]_i$, can stimulate exocytosis in some cells (Gomperts, 1990; Plattner, 1989). In vitro experiments involving the addition of GTP to cortices from *Paramecium* cells have also shown a shift of the Ca^{++} sensitivity of the exocytotic response close to resting levels (Lumpert et al., 1990). Possibly AED, a basic secretagogue as compound 48/80, could directly activate a putative GTP-binding protein closely coupled to membrane fusion (Mousli et al., 1990; Aridor et al., 1990). (*b*) There might be a rapid liberation of Ca^{++} from intracellular stores. This would be possible, since Ca-storing alveolar sacs are in close vicinity of the fusion sites (Schmitz et al., 1985; Stelly et al., 1991).

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