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"Frustrated Exocytosis" — A Novel Phenomenon: Membrane Fusion without Contents Release, Followed by Detachment and Reattachment of Dense Core Vesicles in *Paramecium* Cells

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Abstract. The lipophilic fluorescent dye, FM1-43, as now frequently used to stain cell membranes and to monitor exo-endocytosis and membrane recycling, induces a cortical $[Ca^{2+}]_i$ transient and exocytosis of dense core vesicles ("trichocysts") in *Paramecium* cells, when applied at usual concentrations ($\leqslant$10 μM) in presence of extracellular Ca^{2+} ($[Ca^{2+}]_o$ = 50 μM). When $[Ca^{2+}]_o$ is kept at 30 nM ($<[Ca^{2+}]_i^{rest}$), in about one third of the population of extrudable trichocysts docked at the cell membrane, FM1-43 induces membrane fusion, visible by FM1-43 fluorescence of the vesicle membrane. However, in this system extrusion of secretory contents cannot occur in absence of any sufficient Ca_o^{2+}. Upon readdition of Ca_o^{2+} or some other appropriate Me_o^{2+} at 90 μM, secretory contents can be released (complete exocytosis). Resulting ghosts formed in presence of Ca^{2+}, Sr^{2+} or Mn^{2+} are vesicular, but when formed in presence of Mg^{2+}, for reasons to be elucidated, they are tubular, though both types are endocytosed and lose their FM1-43 stain. In contrast, in presence of $[Mg^{2+}]_o$ = 3 mM (which inhibits contents release), the exocytotic openings reseal and intact trichocysts with labeled membranes and with still condensed contents are detached from the cell surface ("frustrated exocytosis") within ~15 min. They undergo cytoplasmic streaming and saltatory redocking, with a half-time of ~35 min. During this time, the population of redocked trichocysts amenable to exocytosis upon a second stimulus increases with a half-time of ~35 min. Therefore, acquirement of competence for exocytotic membrane fusion may occur with only a small delay after docking, and this maturation process may last only a short time. A similar number of trichocysts can be detached by merely increasing $[Mg^{2+}]_o$ to 3 mM, or by application of the anti-calmodulin drug, R21547 (calmidazolium). Essentially we show (i) requirement of calmodulin and appropriate $[Me^{2+}]$ to maintain docking sites in a functional state, (ii) requirement of Ca_o^{2+} or of some other Me_o^{2+} to drive membrane resealing during exo-endocytosis, (iii) requirement of an "empty" signal to go to the regular endocytotic pathway (with fading fluorescence), and (iv) occurrence of a "filled" signal for trichocysts to undergo detachment and redocking (with fluorescence) after "frustrated exocytosis".

Key words: Ca^{2+} — Calcium — Endocytosis — Exocytosis — Membrane fusion — *Paramecium*

Introduction

Recently a fluorescent dye, FM1-43, has been introduced as a valuable tool in exo-endocytosis research (Ryan et al., 1993; Henkel, Lübke & Betz, 1996; Cochilla, Angelson & Betz, 1999). This lipophilic but water-soluble cationic styrene compound, used at ~10 μM concentration, is spontaneously inserted into the outer leaflet of the cell membrane from where it can diffuse into membranes of exocytotic vesicles after fusion. Internalization of "ghosts" derived from labeled vesicles and recycling of synaptic vesicles can thus be analyzed. Fusion after reloading and redocking of vesicles can be demonstrated by destaining through the exocytotic opening in absence of extracellular FM1-43 during a second round of fusion (Henkel et al., 1996; Klingauf et al., 1998; Kavalali, Klingauf & Tsien, 1999), as reviewed by Cochilla, Angleson & Betz (1999) and by Cousin and Robinson (1999).

We now apply FM1-43 to *Paramecium* cells to label membranes of dense core vesicles ("trichocysts") during

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exo-endocytosis and we find some surprising novel aspects, based on the following presuppositions. A cell contains ~1000 trichocysts docked at the cell membrane, which all are ready for immediate release upon stimulation (Plattner, Stürzl & Matt, 1985*b*). Trichocyst membranes normally fuse with the cell membrane very rapidly and synchronously upon stimulation, e.g., by the polycationic secretagogue, aminoethyldextran (AED, Plattner et al., 1985*a*, *b*; 1993; Knoll et al., 1991). Probably due to its positive charge, FM1-43, applied at a usual concentration of 5–10 μM (Henkel et al., 1996; Cochilla et al., 1999; Murthy & Stevens, 1999), per se induces fusion of trichocyst membranes with the cell membrane, as we find. Normally trichocyst exocytosis includes "decondensation" of trichocyst contents, i.e., explosive elongation of paracrystalline secretory materials, from carrot-shape to long needles which are extruded into the extracellular space. This process requires extracellular Ca^{2+}, $[Ca^{2+}]_o > 10^{-5}$ M, but is inhibited by $[Mg^{2+}]_o \geqslant 1$ mM (Bilinski, Plattner & Matt, 1981). When $[Ca^{2+}]_o$ is reduced, Ca^{2+} mobilization from subplasmalemmal pools (Klauke & Plattner, 1997; 1998) can still cause some exocytotic membrane fusion (Knoll et al., 1993; Erxleben et al., 1997; Plattner, Braun & Hentschel, 1997*b*), yet no contents discharge can occur. We now demonstrate that, with FM1-43, this situation can be exploited to induce a novel phenomenon, "frustrated exocytosis". This involves resealing of FM1-43 tagged trichocyst membranes, followed by detachment ("free" trichocysts) and reattachment of intact, labeled trichocysts at the cell surface. (Thus, the phenomenon we describe is widely different from usual recycling which involves refilling of empty vesicles.) Upon a second stimulus these redocked trichocysts can undergo exocytosis in a regular way. Since this involves some synchronization, it is possible to roughly estimate the time scale required for achieving exocytosis competence after docking. Furthermore, we show requirement of appropriate concentrations of Ca^{2+}, or of some other Me^{2+}, in the medium, to support exocytosis-coupled endocytosis, whose morphology widely differs depending on the type of Me^{2+} used. Most important is the novel implication that internalization may go through different routes, which evidently depend on a signal indicating the "filled" or "empty" state of an exo-endocytotic vesicle.

In search of some rational clues to the novel phenomenon, "frustrated exocytosis", we take into consideration the presence of calmodulin at trichocyst docking sites (Momayezi et al., 1986) and the requirement of calmodulin for maintaining the assembly of a functional docking site in *Paramecium* (Kerboeuf et al., 1993), the interference of Mg^{2+} in the Ca^{2+} binding capacity of the calmodulin molecule (Malmendal et al., 1999) and the easy exchange of bivalent cations in *Paramecium* (Erxleben et al., 1997, Plattner & Klauke, 2000), possibly via an unspecific cation channel (Saitow, Nakaoka & Oosawa, 1997). Therefore, we expose cells to high $[Mg^{2+}]_o$ and/or to the calmodulin antagonist, R24547. These treatments can cause trichocyst de-docking without membrane fusion (no release of secretory contents at decondensation-permissive $[Ca^{2+}]_o = 50$ μM) and, thus, may give us some insight into the mechanism of "frustrated exocytosis" induced by FM1-43.

Materials and Methods

Paramecium tetraurelia cells were cultivated and used for Ca^{2+} imaging and FM1-43 staining as indicated previously (Klauke & Plattner, 1997; 1998). We used wildtype (7S) cells in axenic culture to avoid FM1-43 labeling of interiorized bacteria.

Usually $[Ca^{2+}]_o$ was kept at 50 μM, but eventually Ca_o^{2+} was chelated to a calculated value of 30 nM by adding EGTA or the fast Ca^{2+} buffer, BAPTA, each at 1 mM. FM1-43 (Molecular Probes, Eugene, OR) was dissolved in 5 mM Pipes-NaOH buffer pH 7.0 to a final concentration of 5 to 10 μM. For staining the complete trichocyst membranes, membrane fusion was induced at low $[Ca^{2+}]_o$ to prevent both, release of secretory contents (depending on Ca_o^{2+} in this system [Bilinski et al., 1981]), and rapid closure of exocytotic openings (normally occurring within 350 msec [Knoll, Braun & Plattner, 1991]). Under normal conditions, dynamics of exo-endocytosis coupling would be much too rapid (Plattner et al., 1993) to allow the dye to diffuse into the secretory vesicle membranes (*not shown*). Protracted resealing of the fusion pore was ascertained by adding, under light microscope control, $[Ca^{2+}]_o = 90$ μM to aliquots where it can cause trichocyst release and, thus, indicate open pores. It indicates a closed, resealed state, when no trichocyst contents are released under these assay conditions. Only one third of the docked trichocysts could perform membrane fusion upon exposure to 10 μM FM1-43 at low $[Ca^{2+}]_o = 30$ nM. This amount could not be increased by adding any other established stimulant, like caffeine (Klauke & Plattner, 1998) or 4-chloro-m-cresol (Klauke, Blanchard & Plattner, 2000), as we found in pilot experiments (*not shown*).

We also exposed cells to $[Mg^{2+}]_o = 3$ mM and/or to the calmodulin antagonist, R24571 (calmidazolium, Boehringer Mannheim, Mannheim, Germany), 50 μM in DMSO, at $[Ca^{2+}]_o = 50$ μM, without FM1-43 application. After 30 min we took random Nomarski interference contrast pictures for counting the relative number of "free" (nondocked) trichocysts in the cytoplasm, in a square field placed over a terminal cell segment, for evaluation at a final magnification of 1,500 times. (DMSO controls were without any effect).

For $[Ca^{2+}]_i$ measurements, 100 μM Fluo-3 (Molecular Probes) dissolved in 10 mM Tris-HCl buffer pH 7.2 was injected into single cells, as outlined previously (Klauke & Plattner, 1997; 1998). Wavelength used for evaluation of Fluo-3 was $\lambda_{excitation} = 488$ nm and $\lambda_{emission} = 520$ nm (or 520–560 nm when combined with FM1-43 analysis). FM1-43 labeling in different membranes was documented using $\lambda_{excitation} = 488$ nm and $\lambda_{emission} \geqslant 520$ nm (or $\geqslant 560$ nm in combination with Fluo-3) on frames taken in videorate by a fast confocal laser scanning microscope system (CLSM, Odyssey XL, Noran, Bruchsal, Germany) mounted on an inverted microscope (Axiovert from Zeiss, Oberkochen and Jena). Confocal z-series in 0.5 μM steps were taken to follow internalization of resealed trichocysts under conditions of "frustrated exocytosis" or of empty vesicles (ghosts) after complete exocytosis when fluorescently labeled trichocysts had been dedocked and redocked and cells stimulated at different redocking times for a second time by AED. Median optical sections were selected to count the number of FM1-43 labeled trichocysts in their different

states, i.e., docked, detached (free), redocked or emptied (ghosts). Counts were referred to 1,000 μM^2 area evaluated, containing peripheral and central regions of a cell.

Results

FM1-43 molecules are not only capable to spontaneously integrate into the cell membrane but, at the usual $[Ca^{2+}]_o$ = 50 μM, also to induce exocytosis of some trichocysts (Fig. 1*a*-*a*″). Their "decondensing" contents and "ghost" membranes become fluorescently labeled. This FM1-43-induced exocytosis is accompanied by a sevenfold transient cortical $[Ca^{2+}]_i$ increase (Fig. 1*b*). The time point of this $[Ca^{2+}]_i$ peak coincides with the onset of trichocyst exocytosis in Fig. 1*a*.

Intense labeling of trichocyst membranes, in the absence of any intense labeling of the condensed contents, can be achieved as follows. In Fig. 1*c*-*c*″, trichocyst membrane fusion is induced by FM1-43 at $[Ca^{2+}]_o$ = 30 nM, i.e., $[Ca^{2+}]_o < [Ca^{2+}]_i^{rest}$ (~60 nM, Klauke & Plattner, 1997). This $[Ca^{2+}]_o$ is not sufficient to cause Ca_o^{2+}-dependent decondensation of trichocyst contents which requires $[Ca^{2+}]_o \sim 10^{-5}$ M by influx through exocytotic openings and which is followed by rapid rearrangement of paracrystalline secretory contents (Bilinski et al., 1981) under participation of several Ca^{2+}-binding proteins in secretory contents (Klauke et al., 1998). FM1-43 stains membranes of docked trichocysts within seconds (Fig. 1*c*″), thus indicating formation of a membrane continuum, i.e., exocytotic membrane fusion, yet without rearrangement or release of secretory contents. This process is again accompanied by a cortical $[Ca^{2+}]$ transient, though this is now less pronounced (1.75-fold, as seen in Fig. 1*d*) than shown in Fig. 1*b* for the same manipulation at high $[Ca^{2+}]_o$. The only source of Ca^{2+} may be cortical stores, in this case, since the $[Ca^{2+}]_i$ increase observed is of a similar size as with other secretagogues at low $[Ca^{2+}]_o$ (Erxleben et al., 1997; Klauke & Plattner, 1998). Membrane resealing seems to be inhibited due to insufficient $[Ca^{2+}]_i$ increase, under these conditions. Staining is more intense when FM1-43 is applied in the absence of Ca_o^{2+} for ≤1 min, as can be recognized by comparing Fig. 1*c*′, *c*″ with Fig. 1*a*′, *a*″. As described below, trichocysts can then undergo detachment in labeled, intact form ("frustrated exocytosis").

Next we checked whether readdition of any Me^{2+} (90 μM) may drive exocytosis and any subsequent retrieval of labeled ghosts. This is indeed what we found with Ca^{2+}, Sr^{2+} or Mn^{2+} which results in formation of globular ghosts (Fig. 2*a*, *a*′), in contrast to Mg^{2+} which causes formation of tubular ghosts (Fig. 2*b*, *b*′). Globular ghosts may fragment into smaller balls, but some additional tubular extensions may also occur with time (Fig. 3*a*). Both, globular and tubular types of ghosts are internalized in a nonsaltatory manner. Yet their label fades out (Fig. 3*b*), so that their final determination is impossible to determine.

In the following we show how intact docked trichocysts, with intensely labeled membranes, can be detached. We call this "frustrated exocytosis" since dense core vesicles undergo fusion but do not empty upon FM1-43 stimulation, in presence of low $[Ca^{2+}]_o$, i.e., 30 nM. For resealing, 3 mM Mg^{2+} was added for at least 3–5 min. While this slowly promotes closure of exocytotic openings, it prevents contents release. This is shown by substituting Mg_o^{2+} for Ca_o^{2+} which then can no more induce Ca_o^{2+}-dependent stretching ("decondensation") of the trichocyst matrix (Fig. 4). Detachement of intact trichocysts labeled by "frustrated exocytosis", with subsequent cyclosis and reattachment, can be induced in two ways (Fig. 5), (i) by replacing $[Mg^{2+}]_o$ = 3 mM, after only 5 min, by $[Ca^{2+}]_o$ = 90 μM (Fig. 5, top), or (ii) by adding $[Mg^{2+}]_o$ = 3 mM for 60 min (Fig. 5, bottom). This implies that high $[Mg^{2+}]_o$ suffices to drive membrane resealing, while either Mg^{2+} or Ca^{2+} can drive detachment and allow for the following steps to occur. One has to recall that only about one third of the total population of trichocysts can thus be labeled and detached from the cell surface. The time required for detachment is ~15 to 20 min. Re-attachment under conditions of maintained high Mg_o^{2+} requires ~40 min (Fig. 5, bottom, while data in Fig. 5, top, are less consistent). The hatched area in Fig. 5 takes into account some uncertainty due to the fact that initial changes develop with variable speed from one cell to another.

In an attempt to obtain more detailed insight into the mechanism of trichocyst detachment we then analyzed any effect of $[Mg^{2+}]_o$ = 3 mM and/or of compound R24547 upon de-docking of trichocysts with nonfused membranes at $[Ca^{2+}]_o$ = 50 μM (Table). Either treatment causes a significant, ~7-fold increase of the relative number of free trichocysts. (Unfortunately, without fluorescence labeling we cannot easily analyze the population of docked trichocysts.) The effects of Mg^{2+} and of R24547 are not additive, which may indicate the involvement of the same target. When free trichocysts normally represent ~5% of the total trichocyst population, i.e., 50 per cell (Plattner, Stürzl & Matt, 1985), their number in the present experiments would increase to ~350. This is about one third of the total population, and would, thus, just correspond to the fraction of trichocysts amenable to "frustrated exocytosis".

An example of a time sequence series obtained with FM1-43 labeled cells (Fig. 6) shows cytoplasmic streaming of labeled trichocysts which then undergo saltatory docking to the cell membrane. Redocked trichocysts can be released by exocytosis, e.g., in response to AED (Fig. 7). Globular ghosts with subsequent fragmentation and eventual tubule formation follows, as described above.

Does redocking of FM1-43 labeled trichocysts in-

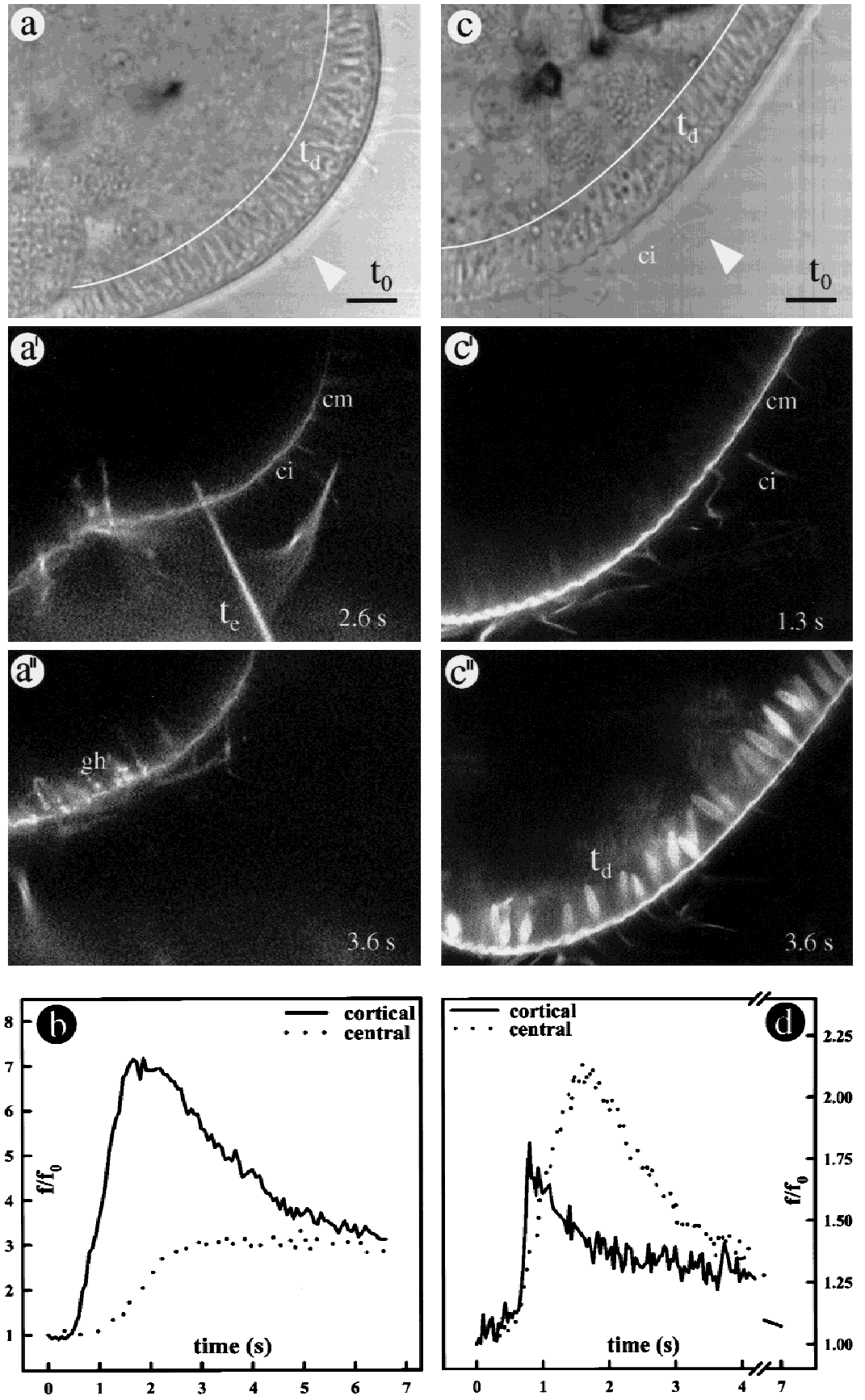
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Fig. 1.

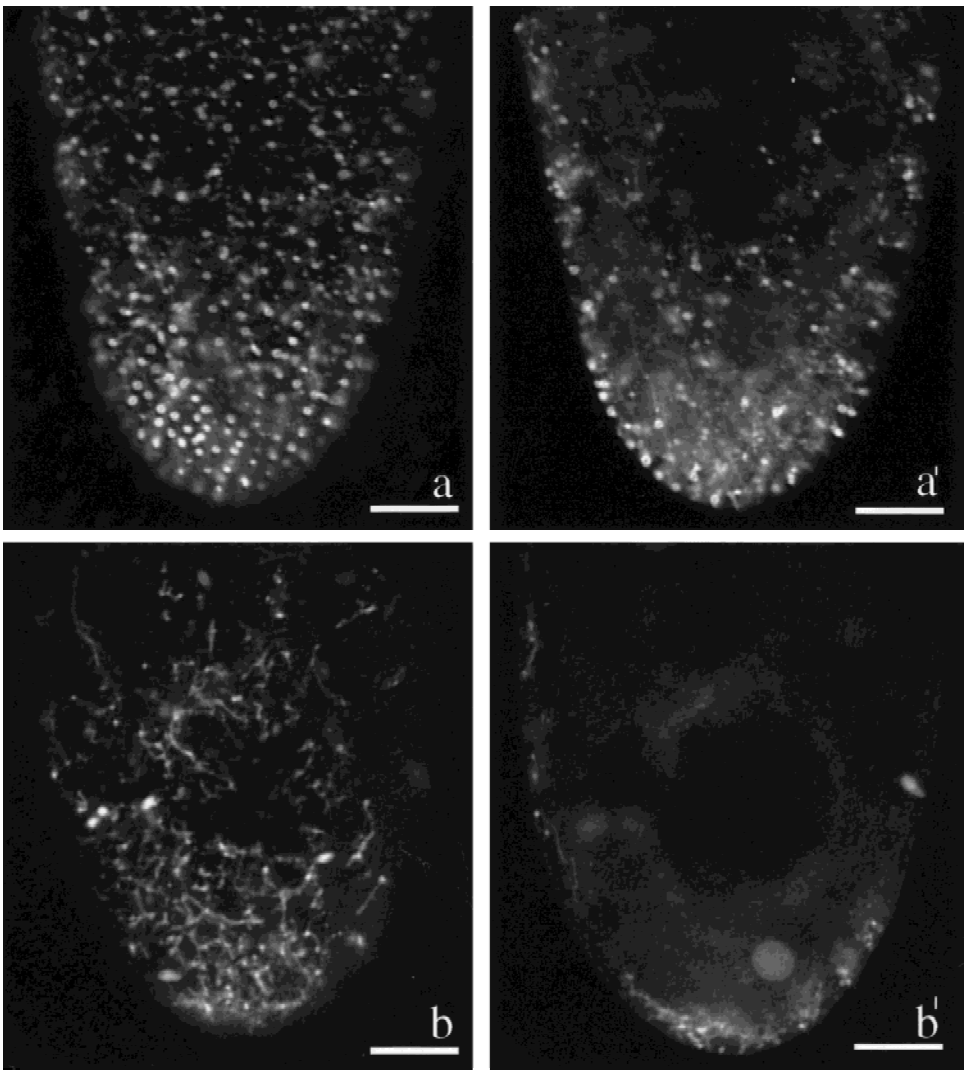

Fig. 2. FM1-43 induces membrane fusion and regular exocytosis with formation of ghosts at high $[Ca^{2+}]_o$. (*a*, *a′*) Superficial (*a*) and median view (*a′*) of a cell exposed to FM1-43 at $[Ca^{2+}]_o$ = 30 nM for 5 sec, and immediate readdition of $[Ca^{2+}]_o$ = 90 μM for 5 min. Only the fused trichocysts release their contents, causing intensely labeled globular ghosts which in part already have been internalized in globular or in tubular form. (*b*, *b′*) Superficial (*b*) and median view (*b′*) of a cell taken 5 min after exposure to FM1-43 at $[Ca^{2+}]_o$ = 30 nM for 5 sec, and immediate addition of $[Mg^{2+}]_o$ = 90 μM. Note formation and internalization of many tubular ghosts. Bars = 10 μM.

volve maturation of docking/fusion sites? We tried to answer this by applying an AED stimulus at different times of the redocking phase. As Fig. 8 shows, this entails a decrease in the number of docked (releasable) trichocysts, while the number of ghosts increases. In Fig. 9 we evaluate the response from the time of the second AED stimulation on (0 sec) until reestablishment of a set of redocked trichocysts with full fusion compe-

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Fig. 1. FM1-43, at usual concentrations of ≤10 μM, causes regular exocytosis and a regular $[Ca^{2+}]_i$ transient when $Ca^{2+}{}_o$ is present (left side, *a*, *a′*, *a″*,*b*), but "frustrated exocytosis" and a much more modest $[Ca^{2+}]_i$ transient when $Ca^{2+}{}_o$ is reduced (right side, *c*, *c′*, *c″*, *d*). (*a*, *c*) are transmitted light, (*a′*, *a″*, *c′*, *c″*) are fluorescence images. Bars = 5 μM. Left: Time sequence of a cell exposed, from t_o on, for the different times indicated to 10 μM FM1-43 at $[Ca^{2+}]_o$ = 50 μM, analyzed in transmitted light (*a*), fluorescence (*a′*, *a″*) and by f/f_o ratio determination of a $[Ca^{2+}]_i$ transient formed (*b*). Note induction of exocytosis as well as occurrence of label in the cell membrane (cm), in contents of trichocysts undergoing exocytosis (t_e) and in trichocyst ghosts (gh); ci = cilia. Right: Similar series as on the left, obtained under the same conditions but at low $[Ca^{2+}]_o$. = 30 nM. Cell exposed to FM1-43 after preincubation at low $[Ca^{2+}]_o$, 30 nM for 1 min, evoking membrane fusion in about one third of docked trichocysts (t_o). Note heavy labeling of cell membrane and subsequent staining of fused trichocyst membranes within ≤4 sec, while secretory contents are retained in condensed form ("frustrated exocytosis").

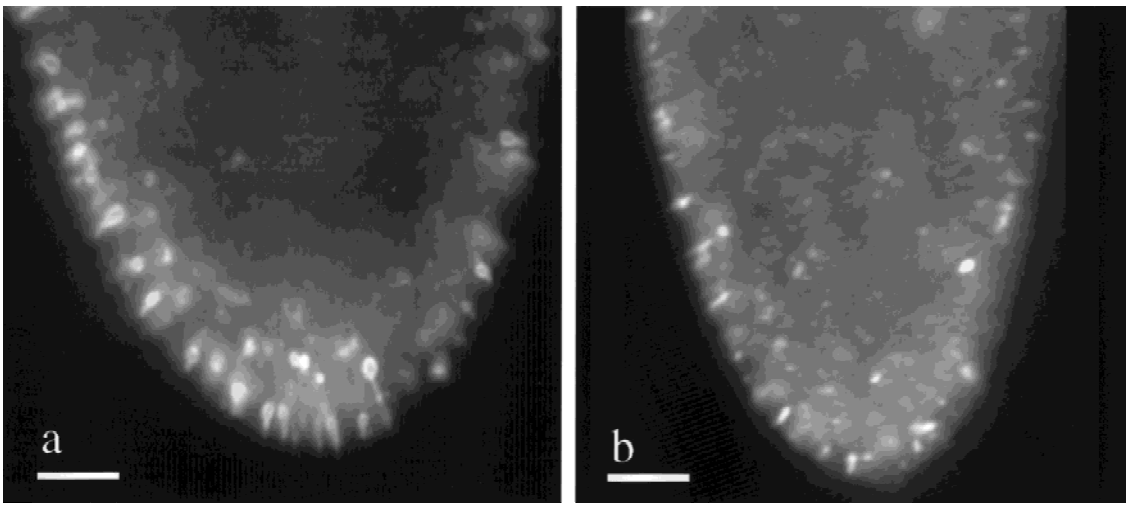

Fig. 3. Median view of a cell taken 5 (*a*) or 30 min (*b*) after ghost formation under conditions specified in Fig. 2*a*, *a*′, i.e., at high $[Ca^{2+}]_o$. Individual ghosts may fragment into 2 to 3 balloonlike structures and eventually form tubular extensions for internalization. Bar = 5 μM.

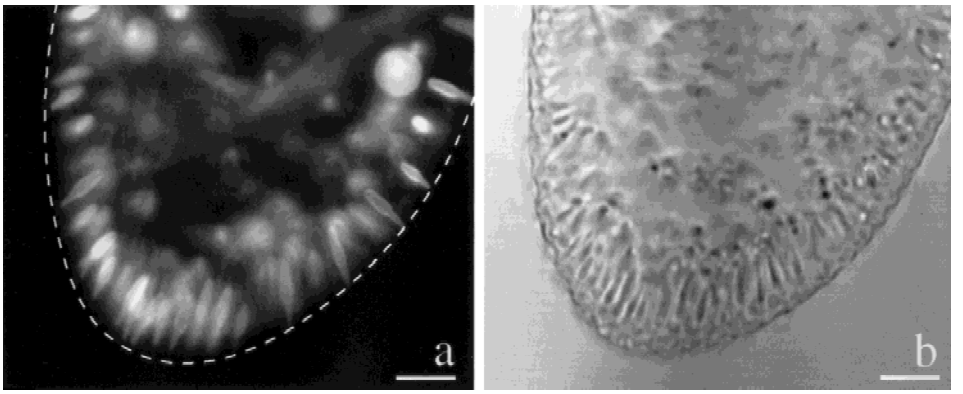

Fig. 4. Median view of a cell exposed for 5 sec to FM1-43 at $[Ca^{2+}]_o$ = 30 nM before incubation in $[Mg^{2+}]_o$ = 3 mM for 5 min. (*a*) and (*b*) are fluorescence and transmitted light images, respectively. This results in "frustrated exocytosis", with trichocysts whose membrane is intensely labeled, but now resealed, and which later on will be detached from the cell membrane. Note destaining of the cell membrane (dashed outlines) after washout of FM1-43. Bar = 5 μM.

tence. This is compared with the number of free trichocysts disappearing due to increasing docking. From the slopes of curves we estimate the half-time required for redocking (disappearance of free trichocysts) as ~35 min, and for acquiring fusion capacity also as 35 min. (Absolute numbers for the two data sets to be compared are different because each state of trichocysts, docked or free, are distributed over widely different reference area sizes, as outlined in Materials and Methods). We are aware of the statistical error inherent to such morphological evaluation, but clearly there is no difference recognizable between the half-times of the two processes, as derived from the slopes in Fig. 9, i.e., for free and extrudable trichocysts, respectively. This indicates that the time required for assembly of a functional docking/fusion site is small, i.e., at most in the range of minutes.

Discussion

To our knowledge there is no precedent for "frustrated exocytosis" and its systematic induction in the literature. Although part of our discussion, therefore, is descriptive, we try to find some clues to the underlying mechanisms. We argue on the basis of the established presence of calmodulin at trichocyst docking sites, its involvement in maintaining docking sites in a functional state, and the binding of Ca^{2+} and Mg^{2+}, respectively, to the calmodulin molecule, probably with some overlapping effects on its functional state (*see* "Introduction").

General Aspects Pertinent To Our Study

Docking of a secretory (or synaptic) vesicle involves co-assembly of a multitude of molecules, including a

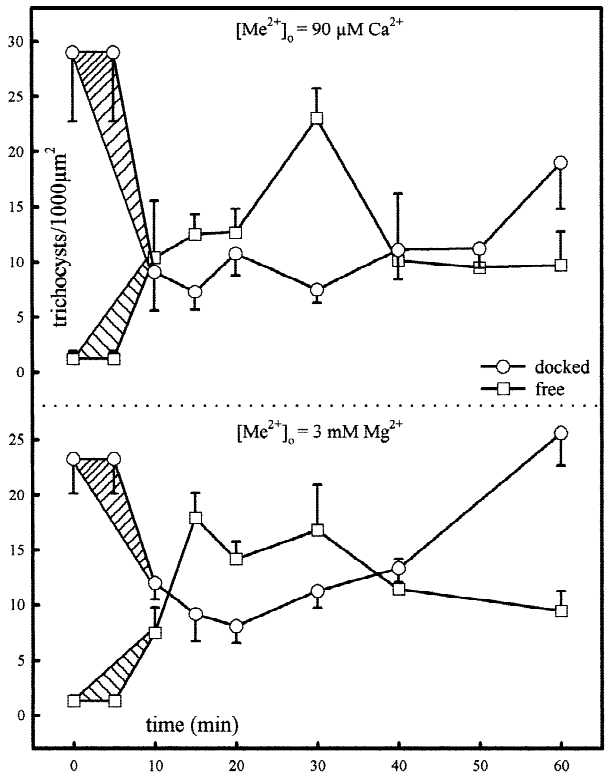

Fig. 5. Quantitative evaluation of frustrated exocytosis, trichocyst detachment and reattachment in cells exposed for 5 sec to FM1-43 at $[Ca^{2+}]_o$ = 30 nM before incubation in $[Mg^{2+}]_o$ = 3 mM for 5 min to allow for membrane resealing and subsequent addition of $[Ca^{2+}]_o$ = 90 µM, with a residual $[Mg^{2+}]_o$ = 100 µM (top), or for 60 min in $[Mg^{2+}]_o$ = 3 mM (bottom). Note that initial decrease of docked trichocysts and antiparallel increase of free trichocysts in the cytoplasm show similar tendency in the top and bottom figure, with some occasional fluctuation. Hatched area signifies some variable reaction at the beginning. Means from 3 experiments (± SD).

Ca^{2+} sensor (Südhof & Rizo, 1996) which mediates competence for exocytosis in response to an extracellular stimulus (Lin & Scheller, 1997; Burgoyne & Morgan, 1998; Edwards, 1998; Robinson & Martin, 1998; Xu et al., 1998). Comparison of the population of vesicles physically docked at the cell membrane with the size of the readily releasable pool, determined by patch-clamp analysis, reveals that only a fraction of vesicles may be competent for membrane fusion (Morgan & Burgoyne, 1997; Plattner, Artalejo & Neher, 1997*a*; Xu et al., 1998). Some individual vesicles may again be detached from the cell membrane, e.g., in chromaffin cells (Oheim et al., 1999; Steyrer & Almers, 1999), before acquiring fusion competence. Exocytosis stimulation can cause release only of "mature" vesicles, i.e., with a fully assembled molecular docking/fusion complex. Though reversibility of docking of "clear" (Murthy & Stevens, 1999) and of "dense" vesicles (Xu et al., 1999) has been observed, the underlying mechanism could not be settled

Table. Effect of the calmodulin inhibitor, R24571 (calmidazolium), and/or of increased $[Mg^{2+}]_o$, added to the culture medium, on the relative number of free trichocysts in the cytoplasm, determined as described in Materials and Methods

Medium	Free trichocysts ±SEM	*n*	*N*	Increase factor
No addition	0.86 ± 0.45	28	5	1.0
R24571, 50 µM	6.64 ± 1.23	34	5	7.7
Mg^{2+}, 3 mM	6.40 ± 1.61	10	3	7.4
R24571, 50 µM + Mg^{2+}, 3 mM	5.31 ± 1.76	13	5	6.2

Exposure time 30 min, values ± SEM, *n* = number of cells analyzed, *N* = number of independent experiments.

in any detail and in no case has it been observed up to now that exocytosis-competent vesicles would again be deprived of their competence, as we show for *Paramecium* (whose docked trichocysts normally are practically all exocytosis-competent [Plattner et al., 1985*b*; Knoll et al., 1991]). Only after releasing their contents are fusion competent vesicles easily detached in the different systems analyzed so far ("exocytosis-coupled endocytosis"). Since exocytotic membrane fusion involves irreversible rearrangement of molecular components there was no reason to look for reversibility. By implication the assumption was that fusion competence can only be acquired by reassembly of these components after docking of a newly formed "virgin" secretory vesicle.

Fusion occurs in response to a local increase of $[Ca^{2+}]_i$ generated by influx and/or mobilization from cortical stores (Berridge, 1998; Barritt, 1999; Mackrill, 1999). Internalization of empty ghosts may be strictly coupled to exocytosis since in a variety of systems this also requires increased $[Ca^{2+}]_i$ (Heinemann et al., 1994; Henkel & Almers, 1996; Vogel et al., 1999) or a similar Me^{2+}, like Sr^{2+} (Guatimosim et al., 1998). A novel aspect in our analysis is decoupling of membrane retrieval from contents release.

Specific Aspects of the *Paramecium* System

In *Paramecium*, trichocyst docking and acquirement of fusion competence requires co-assembly of docking proteins with calmodulin (Kerboeuf et al., 1993). Once docked at the cell membrane, all trichocysts can undergo exocytosis (Plattner et al., 1985*b*; 1993; Knoll et al., 1991), provided a sufficiently intense $[Ca^{2+}]$ transient is generated (Plattner et al., 1997*b*). This implies activation of cortical stores ("alveolar sacs"), a vast system of established Ca-stores (Stelly et al., 1991; Länge et al., 1995; Plattner et al., 1997*c*), superimposed by Ca^{2+}-influx from the outside medium (Kerboeuf & Cohen, 1990; Knoll et al., 1992; Erxleben & Plattner, 1994; Erx-

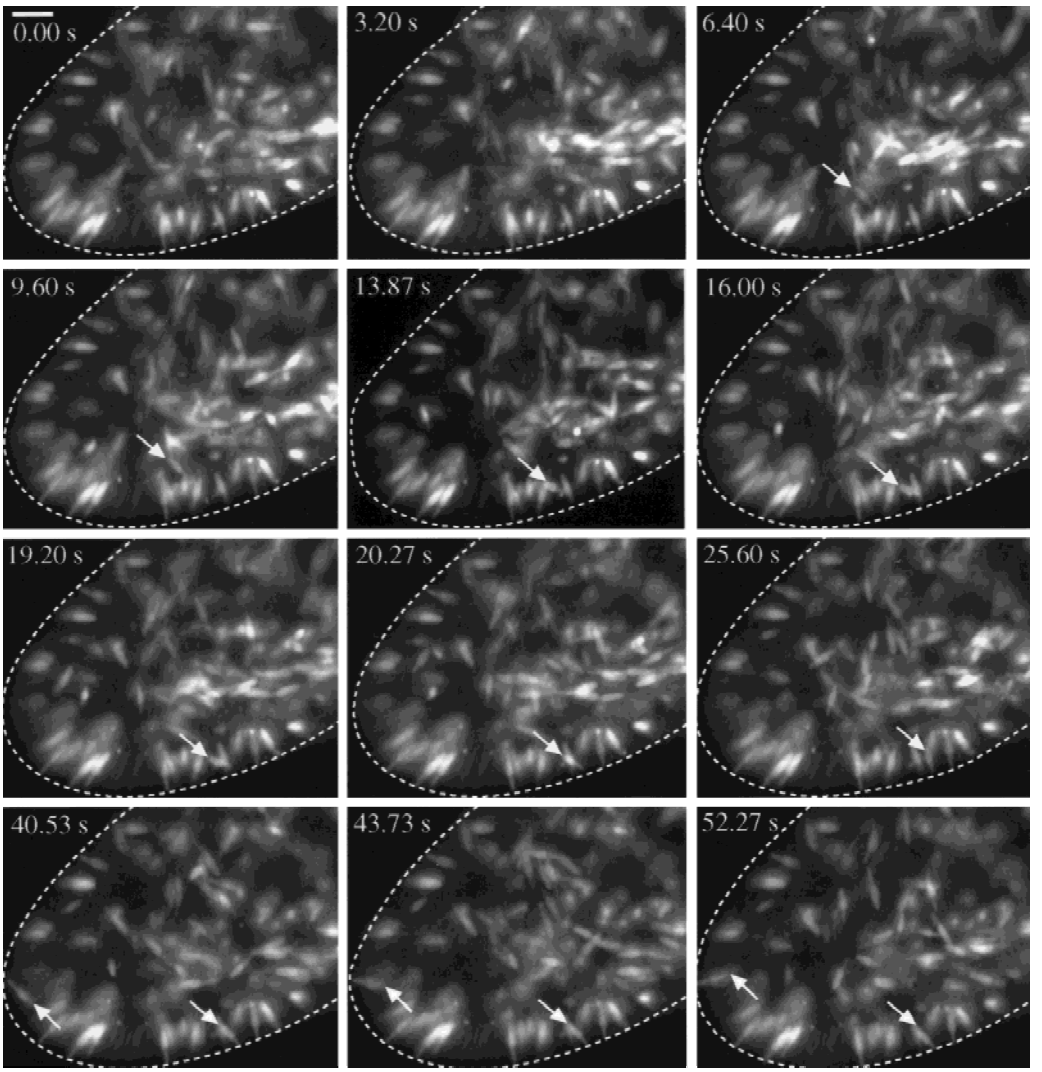

Fig. 6. Time-sequence of intracellular movements of labeled trichocysts following frustrated exocytosis under conditions specified in Fig. 4. Note docking of two trichocysts, labeled by arrows, to the cell membrane (dotted line) which has been destained by removal of FM1-43. Bar = 5 μM.

leben et al., 1997; Klauke & Plattner, 1997). Normally exocytosis takes only 80 msec for all trichocysts of a cell suspension (Knoll et al., 1991). In ~40% of the entire docked, i.e., releasable trichocyst population, exocytotic membrane fusion can be induced without contents release, when $[Ca^{2+}]_o$ is kept at levels of, or below, $[Ca^{2+}]_i^{rest}$ (Erxleben et al., 1997; Plattner et al., 1997*b*). This is due to the requirement of extracellular Ca^{2+} to drive extrusion ("decondensation") of trichocyst contents in our system, by binding to Ca^{2+}-binding proteins contained in the secretory material, in *Paramecium* (Klauke et al., 1998) as in *Tetrahymena* (Chilcoat et al., 1996).

Induction of Frustrated Exocytosis and of Organelle Dedocking

This was the starting point of our experiments. As we show for the first time, we can induce frustrated exocytosis, i.e., membrane fusion without contents release, followed by membrane resealing and detachment of secretory vesicles. In our cells, formation of exocytotic openings is stimulated by FM1-43, possibly due to its lipophilicity and its double positive charge in appropriate spacing (Kavalali et al., 1999). Compounds of this di- to polyamine type are known to induce exocytotic membrane fusion in many cells, like in *Paramecium* (Plattner et al., 1985*a*, *b*). FM1-43 also produces a large $[Ca^{2+}]_i$ signal at $[Ca^{2+}]_o = 50$ μM, and, though a much smaller one, even at $[Ca^{2+}]_o < [Ca^{2+}]_i^{rest}$, as seen from comparison of Figs. 1*b* and 1*d*. This is similar to the response to AED (Erxleben et al., 1997) or to caffeine (Klauke & Plattner, 1998) where the $[Ca^{2+}]_i$ signal generated at low $[Ca^{2+}]_o$ suffices to induce membrane fusion in ~40% of trichocyst docking sites, yet without contents release (Erxleben et al., 1997; Plattner et al., 1997*b*). Exogenous polyamines activate Ca^{2+} release from cortical stores (al-

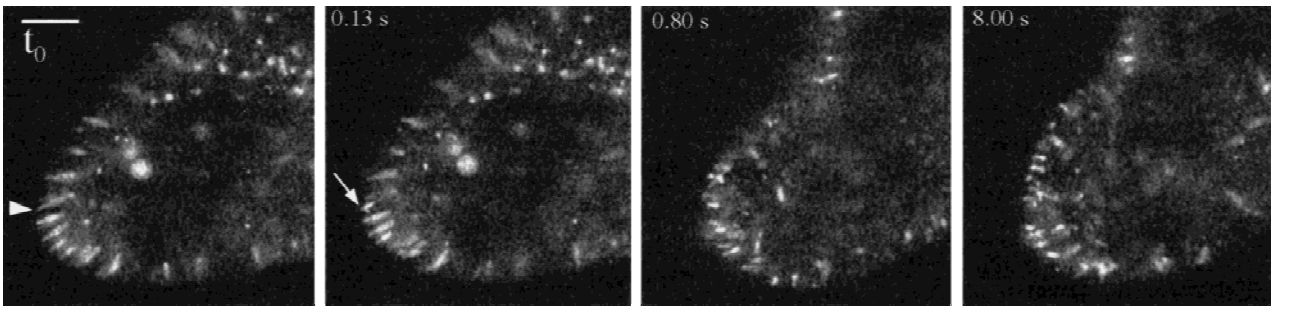

Fig. 7. Example showing that reattached labeled trichocysts are capable of undergoing exocytosis upon AED stimulation. Labeling was under conditions of "frustrated exocytosis" and internalization as specified in Fig. 4. Note that, at t_o, docked trichocysts are of regular shape, while, after adding AED at arrowhead, many collapsed ghosts are formed within ≤1 sec. Bar = 10 μM.

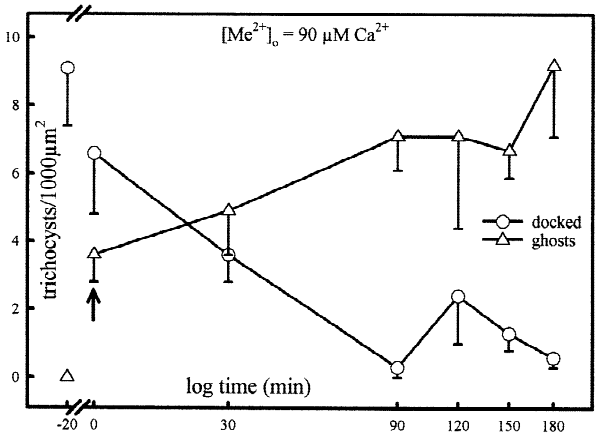

Fig. 8. Quantitative analysis of time-dependent acquirement of exocytosis capacity of redocked labeled trichocysts. Means of 3 experimental series, with ± SD indicated. An AED stimulus was applied at different times after redocking of trichocysts, FM1-43 labeled according to Fig. 4. The experimental period shown in the figure is preceded by a period of "frustrated exocytosis" in presence of FM1-43 (5 sec) at $[Ca^{2+}]_o$ = 30 nM, followed by $[Mg^{2+}]_o$ = 3 mM for 5 min, and subsequent detachment and reattachment at $[Ca^{2+}]_o$ = 90 μM (as specified in Fig. 4) for 60 min. After redocking, from t_o on (arrow), aliquots were exposed each to one AED stimulus at times indicated in the abscissa. Note increase of ghosts on account of decreasing docked trichocysts.

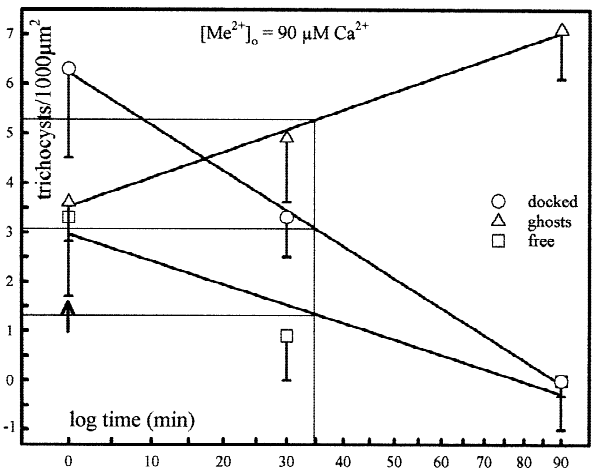

Fig. 9. Evaluation of the first 90 min shown in Fig. 8, obtained by analyzing redocked, labeled trichocysts, as well as free trichocysts. Note antiparallel changes of docked trichocysts (releasable in response to a AED stimulus after redocking) and of ghosts (formed by such an AED stimulus), while free trichocysts decrease due to docking. The first AED stimulus of the second round of stimulation, i.e., after redocking, was applied at the arrow and from then on at the different time points of the abscissa, as specified in Fig. 8. Values are from 3 experiments, ± SD. Note that organelle numbers indicated per 1,000 μM² evaluated area are referring to areas where trichocysts are densely packed in a narrow zone adjacent to the cell membrane (docked trichocysts), and distributed over a much larger intracellular area (free trichocysts), respectively.

veolar sacs) via a "Ca^{2+}/(polyvalent cation)-sensing receptor" in the cell surface and this in turn activates a "store-operated Ca^{2+}-influx" via unspecific cation channels (Klauke et al., 2000). As with AED, mobilization of Ca^{2+} by FM1-43 from cortical stores may just suffice to induce membrane fusion in about one third of the docked trichocyst population, when $[Ca^{2+}]_o$ is kept low. Evidently a $[Ca^{2+}]_i$ signal has to achieve a certain threshold level to allow (i) fusion to occur at all trichocyst docking sites and (ii) the fusion pores formed to expand. According to our observations, Ca^{2+} and Mg^{2+} may interact in regulating these processes. For instance, it appears from Fig. 5 that, over longer time periods, Mg^{2+} at sufficiently high concentrations in the medium can achieve similar effects as short time Ca^{2+} application.

Instead of our assumption that FM1-43 triggers membrane fusion, could one also assume (as suggested by one of our reviewers) that in our cells trichocyst membranes would be permanently liable to spontaneous membrane fusion, possibly with silently ongoing "frustrated exocytosis"? We deny this possibility mainly for two reasons: (i) The extent of "frustrated exocytosis" we see is incompatible with the persistently low change of occupied docking sites, unless membrane fusion is triggered (Plattner et al. 1997*b*). (ii) FM1-43 causes massive exocytosis unless conditions are manipulated in a way to inhibit contents release (this study).

In the present study we observe the diffusion of FM1-43 from the cell membrane into trichocyst membranes within ⩽3 sec. Prerequisite for this is the continuum between the two different membranes during formation of the fusion pore which may not expand completely under our experimental conditions, i.e., at $[Ca^{2+}]_o$ = 30 nM. After the time in low $[Ca^{2+}]_o$ the medium is supplemented with $[Mg^{2+}]_o$ = 3 mM, instead of Ca_o^{2+}, not only to avoid cell damage which would have to be expected in absence of Ca_o^{2+}, but also to avoid rapid closure of the fusion pore. In fact, it is well documented in some other systems that expansion of the fusion pore is a distinct, Ca^{2+}-dependent step (Rosenboom & Lindau, 1994; Fernández-Chacón & Alvárez De Toledo, 1995; Hartmann & Lindau, 1995; Lindau & Almers, 1995; Monck & Fernández, 1996). Holding the fusion pore open for a sufficiently long time may require an additional regulating mechanism (Fernández-Chacón et al., 1999). However, the molecular mechanisms of these observations are not understood. In our cells, at $[Mg^{2+}]_o$ = 3 mM, fusion pores have all resealed within 5 min in $[Mg^{2+}]_o$ = 3 mM, tested by readdition of Ca_o^{2+} (which would cause contents discharge visible in the light microscope) and the entire population of FM1-43-tagged trichocysts is internalized into the cyclosis stream, before they finally all undergo redocking. How may Ca^{2+} and Mg^{2+} influence these mechanisms?

It may be more than circumstantial that trichocysts can be detached from the cell membrane by exposure to high $[Mg^{2+}]_o$ or by calmidazolium (Table), i.e., without previous membrane fusion, even at $[Ca^{2+}]_o$ = 50 μM. We consider this possibility since it correlates with several facts established for *Paramecium*, i.e., (i) presence of calmodulin at docking sites (Momayezi et al., 1986), (ii) its requirement for mediating exocytosis compentence to docked trichocysts (Kerboeuf et al., 1993), (iii) the nonadditive effect of high $[Mg^{2+}]_o$ and of calmidazolium (Table) which both can affect calmodulin function, and (iv) detachment of trichocysts under conditions of "frustrated exocytosis" which may be based on the sensitivity of calmodulin not only to Ca^{2+}, but also to sufficiently high $[Mg^{2+}]$ (Malmendal et al., 1999). Though still speculative, our current experiments may allow in the future more detailed analysis on a molecular scale.

We underscore that (i) we could for the first time detatch exocytosis-competent secretory vesicles and (ii) that these are all handled like newly formed vesicles, as they are amenable to redocking and fusion in response to a second secretory stimulus. Thus, their fate is totally different from those vesicles that have fused and released their contents before internalization as ghosts. Therefore, the respective internalization signals must be different ones. It also must be different from the mechanism underlying occasional dedocking of structurally, but not yet functionally docked vesicles seen in some other cells by evanescent fluorescent wave microscopy (Oheim et al., 1999; Steyrer & Almers, 1999). The current situation is also different from previous experiments with exocytosis-incompetent mutant *Paramecium* strain nd9–28°C whose trichocysts are linked only to alveolar sacs, but not to the cell membrane, and can be detached by Ca^{2+} ionophore treatment (Pape & Plattner, 1990), a procedure which would cause exocytotic membrane fusion and endocytotic membrane retrieval in the wildtype cells used here.

As a consequence we have to postulate that a vesicle membrane must contain a signal indicating the filling state. Possible candidates to be considered are proteins linking secretory contents to the vesicle membrane, as occurring in pancreatic zymogen granules (Kleene, Dartsch & Kern, 1999) and chromaffin granules (Glombik et al., 1999), where they participate as a centrifugal sorting mechanism, as well as in *Paramecium* trichocysts (Momayezi et al., 1993). Clearly this is speculative at this time.

Redocking of Trichocysts and Maturation of Docking/Fusion Sites

FM1-43 labeled detached trichocysts are redocked in a saltatory manner, just like "virgin" trichocysts (Aufderheide, 1977), via microtubules emanating from ciliary basal bodies (Plattner, Westphal & Tiggemann, 1982; Glas-Albrecht et al., 1991). In the Results we tried to estimate the time scale of maturation of newly assembled trichocyst docking/fusion sites. Within the error range inherent to the methods available for our system, we find identical half-times for redocking (mirrored by decrease of free trichocysts) and reestablishment of exocytosis capacity. Therefore, the time required for maturation must be small, i.e., maximally in the range of minutes. This is fully compatible with a previous estimation of ~5 min (Pape & Plattner, 1985), achieved by comparing time-variable numbers of docked trichocysts with numbers of preformed docking/fusion sites containing a "fusion rosette" of integral membrane proteins — an infallible indicator of fusion capacity (Beisson et al., 1976; Pape & Plattner, 1985; Pouphile et al., 1986). No directly comparable estimations from other systems are known.

Variable Endocytotic Vesicle Morphology

Resealing of trichocyst ghosts after exocytosis occurs without a clathrin coat (Plattner et al., 1985*a*), normally within <1 sec (Knoll et al., 1991) and, thus, follows a fast mechanism defined as "kiss-and-run" (Artalejo et al., 1998; Palfrey & Artalejo, 1998). In our case, exocytosis-coupled endocytosis occurs with either Ca^{2+} or Mg^{2+} in

the medium, though vesicle morphology is different, i.e., primarily ball-shaped and tubular, respectively. Possibly a kiss-and-run mechanism can more easily be supported by Mg^{2+} than clathrin-coated vesicle formation since the latter requires dynamin dephosphorylation by the Ca^{2+}/calmodulin activated protein phosphatase 2B, calcineurin (Marks & McMahon, 1998). This difference in morphology of retrieved vesicles may depend on the free charge available to bind to functionally important components of so far unknown identity. Interestingly balloons can also emanate tubules. All these structures are internalized. Unfortunately their label fades out and, therefore, any selective delivery to internal structures could not be analyzed.

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