

Review

Dense-core secretory vesicle docking and exocytotic membrane fusion in *Paramecium* cells

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Abstract

Work with *Paramecium* has contributed to the actual understanding of certain aspects of exocytosis regulation, including membrane fusion. The system is faster and more synchronous than any other dense-core vesicle system described and its highly regular design facilitates correlation of functional and ultrastructural (freeze–fracture) features. From early times on, several crucial aspects of exocytosis regulation have been found in *Paramecium* cells, e.g. genetically controlled microdomains (with distinct ultrastructure) for organelle docking and membrane fusion, involvement of calmodulin in establishing such microdomains, priming by ATP, occurrence of focal fusion with active participation of integral and peripheral proteins, decay of a population of integral proteins (“rosettes”, mandatory for fusion capacity) into subunits and their lateral dispersal during fusion, etc. The size of rosette particles and their dispersal upon focal fusion would be directly compatible with proteolipid V_0 subunits of a V-ATPase, much better than the size predicted for oligomeric SNARE pins (SCAMPs are unknown from *Paramecium* at this time). However, there are some restrictions for a straightforward interpretation of ultrastructural results. The rather pointed, nipple-like tip of the trichocyst membrane could accommodate only one (or very few) potential V_0 counterpart(s), while the overlaying domain of the cell membrane contains numerous rosette particles. Particle size is compatible with V_0 , but larger than that assumed for the SNARE complexes. When membrane fusion is induced in the presence of antibodies against cell surface components, focal fusion is seen to occur with dispersing rosette particles but without dispersal of their subunits and without pore expansion. Clearly, this is required for completing fusion and pore expansion. After cloning SNARE and V_0 components in *Paramecium* (with increasing details becoming rapidly available), we may soon be able to address the question more directly, whether any of these components or some new ones to be detected, serve exocytotic and/or any other membrane fusions in *Paramecium*.

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1. Conceptual developments

Early on, ultrastructural analysis of *Paramecium* cells, due to some special structural features, was able to provide some new insights into exocytosis regulation. Interestingly, some of these aspects could be verified with higher eukaryotic cells and some are taken into consideration again in recent work. To anticipate, *Paramecium* is the fastest dense-core vesicle system described (Fig. 1).

Paramecium has structurally well defined and regularly arranged exocytosis sites where its dense-core secretory organelles (“trichocysts”) are docked, as reviewed previously by the author [1–6] and by colleagues [7,8]. In freeze-

fracture replicas, such sites are encircled by a 300-nm-wide “ring” of particles, with a “rosette” of about eight or nine relatively large particles in the center, where membrane fusion occurs (Fig. 2). The tip of a trichocyst, where it closely approaches the cell membrane, may be extended to a nipple-like structure, so that membranes to be fused upon stimulation are kept in close contact. With appropriate electron “staining” methods, some amorphous material was seen to connect the two membranes. Almost all docked trichocysts display these features and almost all can be released upon stimulation at once in a rather synchronous fashion [5,9–12]. This means that there is a rather strict correlation between docking sites with the ultrastructural characteristics described and the percentage of docked trichocysts amenable to exocytosis [13,14] and it implies that ~ 95%, of this type of dense-core vesicles are primed and belong to the immediately releasable pool. This is an

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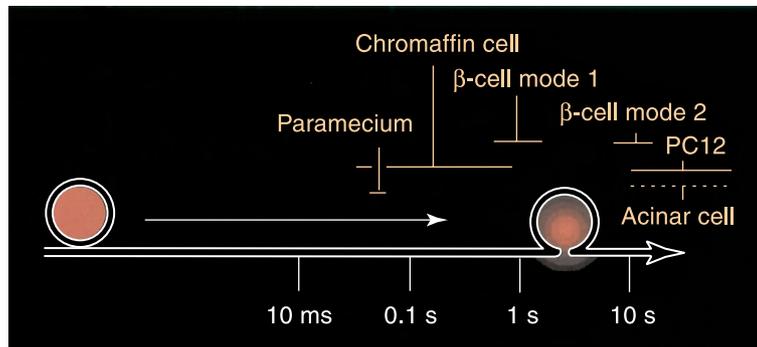


Fig. 1. *Paramecium* is the fastest dense-core vesicle system known. Comparison of time constants of dense-core vesicle systems. Data compiled by Kasai [53] supplemented with data for *Paramecium*, from Plattner et al. [54].

unusually high percentage when compared with other dense-core vesicle systems [15–17].

Docking sites are flanked by “alveolar sacs”, i.e. cortical calcium stores (see below). Such structural elements, more than thousand per cell, are arranged redundantly in regular fields (“kinetids”) and these in rows (“kineties”). Thus, it is possible to pinpoint preformed exocytosis sites and to follow their transformation during exocytosis stimulation.

Most importantly, some groups have collected a series of exocytotic mutants. Examples are the trichocyst-free strain “trichless” (tl) [18] and a series of exocytosis-incompetent strains, termed “non-discharge” (nd), which can dock trichocysts but are unable to release them [7,8,19–21]. The ring delineating potential docking sites in tl cells is collapsed, while in nd cells, it forms a ring without rosette particles. In this case, in the absence of any trichocyst-plasma membrane binding [20,22], trichocysts are kept in place by attachment to alveolar sacs (see below). We therefore differentiated between docking site I and II, i.e. between a trichocyst and alveolar sacs, and between a trichocyst and the cell membrane, respectively [23].

Unfortunately, the molecular identity neither of ring particles, nor of rosette particles is known as yet. However, in the meantime, it was possible to identify a variety of proteins involved in the assembly of a trichocyst docking site. Although genetic complementation has been introduced some time ago [24,25], the dramatic progress in the identification of molecular components in the last few years is mainly due to the systematic use of an indexed genomic library [8,26] and to a recent *Paramecium* genome cloning project [27]. Some of the proteins are novel, some others have also been detected later on in higher eukaryotic systems, and finally some, like calmodulin (CaM), have been re-considered after neglect for some time, as specific components of exocytosis sites in widely different cells.

Early on, we took advantage of the precise structural arrangement of preformed exocytosis sites and of their components. After developing adequate preparation methods, e.g. cryofixation (fast freezing), structural rearrange-

ments could be studied by electron microscope (EM) analysis, particularly once the possibility to induce highly synchronous exocytosis by an appropriate secretagogue [9] had been established.

Up to the early 1980s, Ca^{2+} -triggered membrane fusion was explained by lipid phase transitions, while the relevance of integral- and membrane-associated proteins was hardly envisaged and certainly far from established. Based on fast freezing/freeze-fracturing work in our lab and on work in a very few other labs, from 1981 onwards, we propagated a “focal membrane fusion concept” [1–3,5,11] with the following aspects. (i) Not only does membrane fusion occur as a small pore (~ 10 nm large, the smallest size recognizable on freeze-fracture replicas), as suggested also by Heuser [28,29] for other cells, but also work with *Paramecium* showed in addition (ii) that integral and peripheral proteins are required for a regulatory role in organelle docking and membrane fusion. In fact, similar, though essentially less distinct details have also been seen in other systems, but most stringent evidence for a functional relevance of proteins came from work with *Paramecium*, especially from the analysis of different exocytosis-incompetent *Paramecium* strains [19]. From 1991 onwards, we were also able to follow membrane fusion during synchronous exocytosis by precisely timed quenched-flow/freeze-fracture analyses [12].

We were aware of the restrictions of the methods available to us at any time, before Neher and Marty [30] introduced the patch-clamp method in exocytosis research. This improved temporal and spatial (fusion pore size) resolution by more than one order of magnitude and allowed to detect formation of the fusion pore from a size of ~ 1 nm on and its subsequent expansion [31].

1.1. Conclusions

The *Paramecium* system proved an important system to set a baseline for important conceptual aspects, i.e. fusion pore formation regulated by integral and peripheral proteins. As shown below, its further analysis has also highlighted additional aspects of importance for higher eukaryotic cells.

Later on, the question will be addressed whether SNARE proteins and/or V_0 components of the H^+ -transporting V -ATPase may be potentially involved in the docking and/or fusion process.

2. Molecular components

An ever increasing number of proteins have been identified at dense-core vesicle docking sites in mammalian cells [32,33], so that the question arises how many may be accommodated in how many copies in the small area/volume available for focal fusion. Only some are known in *Paramecium*, while others have been known first or even exclusively from this system and are now also found in mammals. Among the proteins currently discussed for docking and/or membrane fusion, SNAREs [34–36] and V_0 components [37] are of paramount importance, as well as some other candidates to be tested [38].

2.1. NSF, α -SNAP, SNAREs

SNAREs have been implied to mediate specific membrane–membrane interactions, which may be facilitated by (less specific) monomeric GTP-binding proteins [39]. Recent cloning work with *Paramecium* revealed two genes for the SNARE-specific chaperone [40], *N*-ethylmaleimide-sensitive factor (NSF), *PtNSF1* and *PtNSF2* [41]. They both have 87% identity to each other on the nucleotide, and 93% on the amino acid level. Similarity and evolutionary conservation is particularly high in D1 and D2 domains by which NSF is assigned to the AAA-superfamily of ATPases [42].

For immunofluorescence localization studies we took into account that binding of NSF to fusogenic sites is transient and that it is released upon ATP hydrolysis [43] once a NSF/ α -SNAP complex has mediated rearrangement of SNAREs [32,44]. We therefore tried to localize NSF, by inhibiting its dissociation from fusogenic sites, by exposing carefully permeabilized *Paramecium* cells to ATP- γ -S and NEM. As to be expected, many fusion sites of the different membrane transport routes could so be labeled with fluorescent antibodies [41]. An exception are trichocyst docking sites, presumably because they are established and then remain in fully reactive, assembled form long before stimulation.

In *Paramecium*, a priming effect, rather than a direct role during membrane fusion, has been found for ATP [45]. The

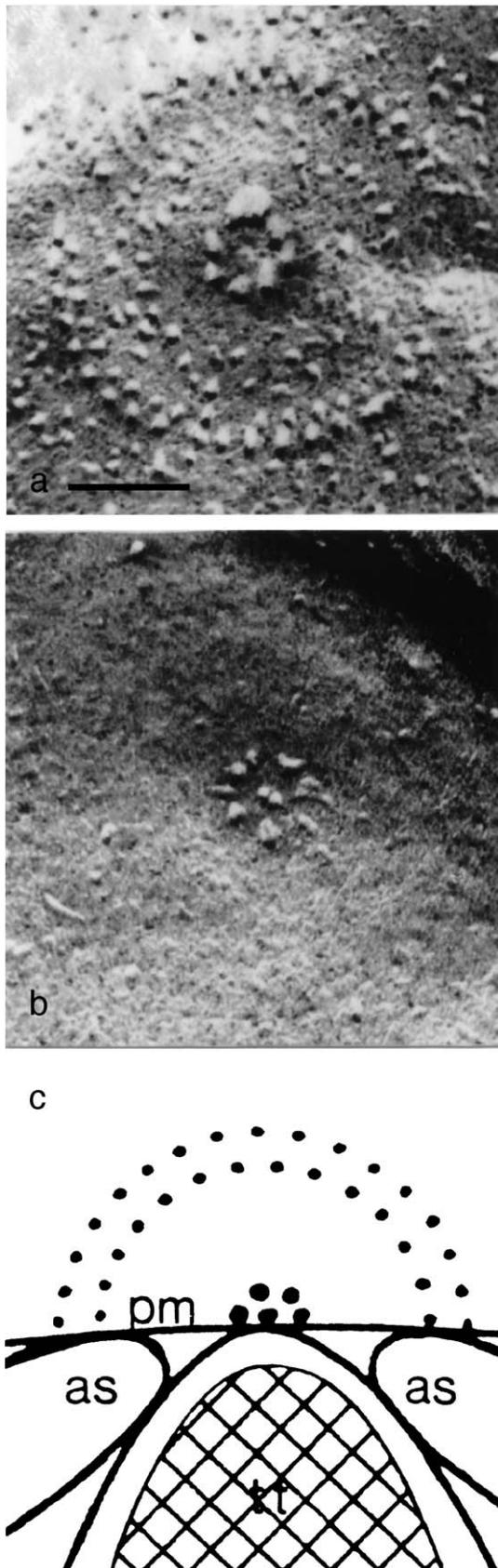


Fig. 2. Exocytosis sites in *Paramecium* have a distinct ultrastructure, allowing structure–function correlation. Trichocyst docking site in a non-stimulated *Paramecium* cell after fast freezing, followed by freeze-substitution and freeze-fracturing (Pt/C shadowing from bottom to top). Micrographs showing a 300-nm large double particle ring and a cluster of rosette particles in the center. (a) PF-face (“plasmatic fracture face” adjacent to the cytoplasm), (b) EF-fracture face (showing external face of cell membrane), (c) scheme indicating plasma membrane (pm), trichocyst tip (tt) with cross-hatched contents, and alveolar sacs (as). Bar=100 nm: from Knoll et al. [12].

implication of NSF in this system may perhaps, at least in part, bear on this phenomenon. Like in *Paramecium*, a role for ATP in priming, rather than in membrane fusion, is now generally accepted for most dense-core secretory systems, where it may encompass widely different aspects as discussed in Ref. [46].

Is NSF involved specifically in the assembly of trichocyst docking sites? With wild-type cells, it was not possible to address this question because of the multiple fusion processes “routinely” required to maintain a *Paramecium* cell alive. Based on an initiative by J. Cohen, the answer came from nd9 cells, a temperature-conditional mutant. When cultured at 18 °C, they are like wild-type cells; after culture at 28 °C, they no longer assemble “rosettes” and are unable to release any of their numerous trichocysts docked at the cell membrane [19]. When nd9 cells are shifted from 28 to 18 °C, “rosettes” could normally be assembled and exocytosis capacity restored within a few hours. When this is combined with homology-dependent gene silencing [47] of the *NSF* genes, we found that this structure/function repair no longer occurs [48]. This clearly implies the involvement of NSF in establishing functional trichocyst exocytosis sites.

Using antibodies against α -SNAP (kindly provided by A. Mayer, Tübingen) we recognized its presence on Western blots of *Paramecium* homogenates (unpublished observations). Following the general assumption that a NSF/ α -SNAP complex specifically serves the formation of appropriate SNARE arrangements before membrane docking and/or fusion can occur [32,49], our findings imply the occurrence of SNAREs in *Paramecium*. Although only a small portion of the *Paramecium* genome is identified, in the data base, we could find sequences homologous to synaptobrevin. The synaptobrevin genes cloned so far in *Paramecium* have up to 37% identity with synaptobrevin from some higher eukaryotes (unpublished observations) Further work is required to find and characterize the other types of SNARE proteins in *Paramecium*.

2.2. Time course and Ca^{2+} -binding components

Main Ca^{2+} -binding components discussed along the lines of dense-core vesicle docking are synaptotagmin, CaM and, from time to time revitalized, annexins [50].

The widely distributed Ca^{2+} -sensor, synaptotagmin, is not known from *Paramecium*. The following aspects led us to anticipate its presence in *Paramecium*. Synaptotagmin occurs not only in the fastest reacting, clear vesicle-type systems, but also in different dense-core vesicle systems, in isoforms with different Ca^{2+} affinities [51,52]. Comparison of the kinetics of dense-core vesicle release in higher eukaryotic cells [53] and in *Paramecium* [54] shows that the latter works much faster than any other system. The pores open with an apparent $t_{1/2}$ of ~ 56 ms (calculated for synchronous trichocyst release using quenched-flow/freeze-fracture, quantitative EM analysis of all sites in all cells analysed [12]). Pulses of

Ca^{2+} -activated currents, which accompany exocytosis of single trichocysts, are somewhat shorter, $t_{1/2}=21$ ms, and they pile up to longer-lasting events during massive trichocyst exocytosis [55]. At trichocyst exocytosis sites, local $[Ca^{2+}]$ was estimated to rise to $\sim 5 \mu M$ upon exocytosis stimulation [56,57], i.e. close to values reported for other dense-core vesicle systems [51,52,58,59]. From these aspects, it may be concluded that synaptotagmin or a Ca^{2+} sensor with similar kinetic properties will be found in *Paramecium* in the future.

So far, the only Ca^{2+} -binding proteins known to occur at trichocyst exocytosis sites are some annexin-related proteins and CaM. Though no annexins have been cloned in *Paramecium*, antibodies against a common sequence can differentially stain either trichocyst docking or some other exocytotic sites [60].

Occurrence of CaM at trichocyst docking sites has been shown stringently (Fig. 3), first by affinity labeling and immunolocalization [61], and then by genetic studies [62]. No “connecting material”, and hence no CaM, occurs at docking sites in nd mutants. Some strains with a point

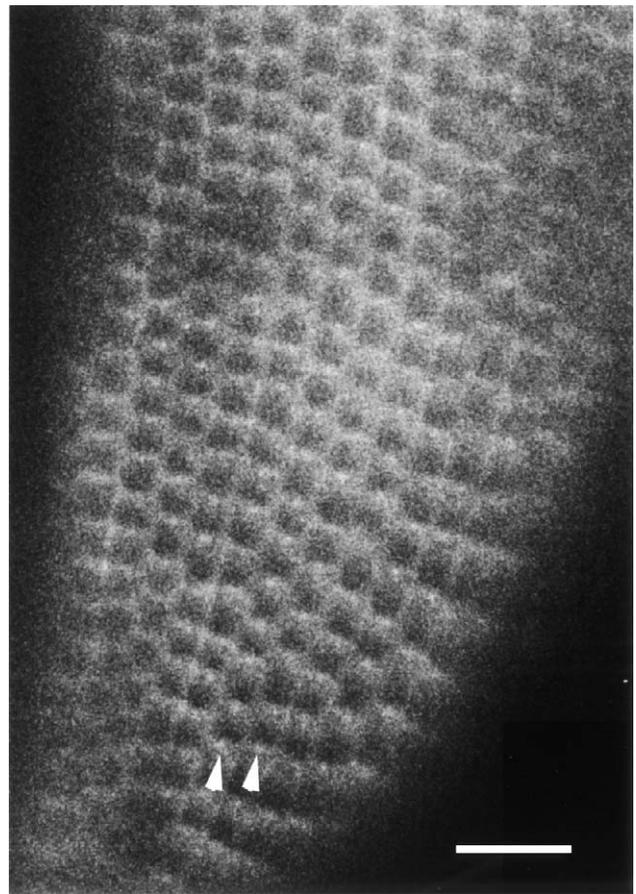


Fig. 3. CaM is bound to trichocyst exocytosis sites. *Paramecium* cell cortex, affinity labeling with fluorescently labeled endogenous CaM at $[Ca^{2+}] = 10^{-5}$ M. Labeling outlines kinetids and most strikingly docking sites of trichocysts located in the middle of “perpendicular ridges” between kinetids (e.g. along arrowheads). Note that trichocyst docking sites were also labeled with different antibody techniques. Bar = 3 μm ; from Momayezi et al. [61].

mutation in the *CaM* gene, *cam-*, are also unable to assemble functional docking sites with “rosettes” and “connecting material” between membranes, but can be repaired by transfection with the wild-type *CaM* gene [62]. Therefore, CaM may have a function during assembly and/or for maintenance of functional trichocyst docking sites. Further-

more, involvement of CaM in homotypic membrane fusion has been shown with yeast vacuoles [63].

Concerning exocytosis, quite recently, binding of CaM to synaptobrevin and a role in SNARE pin assembly have been reported in mammalian cells [64,65]. Further implications remain to be analysed in detail, particularly since Ca^{2+} sensed

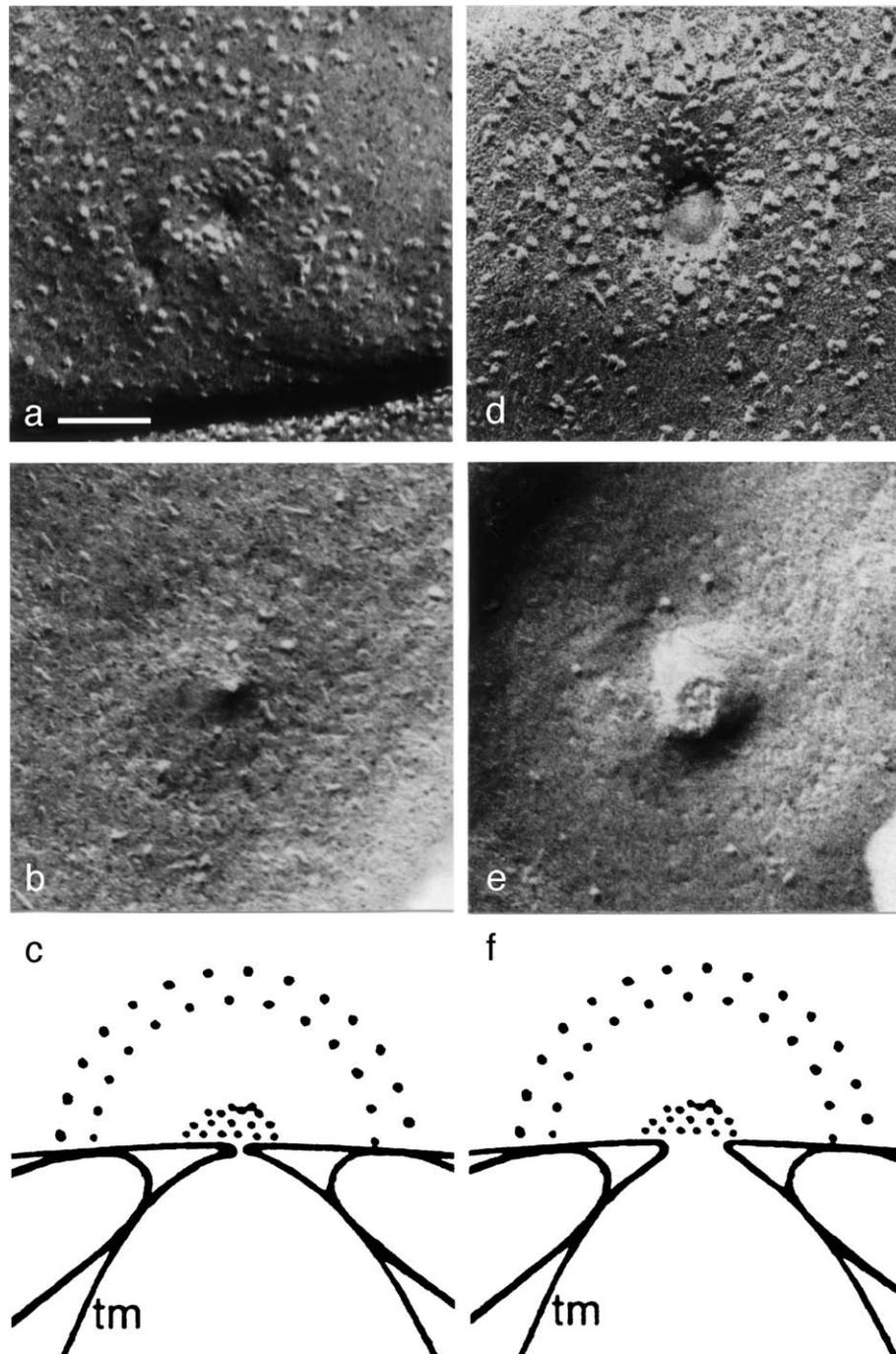


Fig. 4. Rapid freezing during synchronous exocytosis shows focal fusion and restructuring of exocytosis sites (dispersal of rosette subunits). Focal membrane fusion (a–c) shown in a freeze-fracture replica after ≤ 50 ms stimulation of trichocyst exocytosis. To be compared with resting state in Fig. 2. Note in the PF-face (a), disappearance of rosette particles and appearance of many more smaller particles around a focal fusion site (arrowhead), represented by a ~ 10 -nm large pit (recognized by opposite shadow direction) in the center of a funnel-like depression. (b) EF-face with corresponding particles (or pits instead of particles). (c) Corresponding scheme. (d–f) Later trigger stage, showing expanding fusion pore. For abbreviations, see Fig. 2. Shadowing from bottom to top. Bar = 100 nm; from Knoll et al. [12].

during stimulation by synaptotagmin may be transferred to syntaxin and SNAP-25, before they interact with synaptobrevin [66]. Synaptotagmin is reported to interact also with CaM [52]. Another aspect is the interaction of rab-type G-proteins and CaM at dense-core vesicle docking sites [67]. Some basal level of Ca^{2+} -binding at docking sites, before exocytosis stimulation, serves a priming effect [68], but whether this is mediated by CaM still remains to be elucidated in detail. We consider this possibility for the following reasons. Trichocyst docking could be reversed by “anti-CaM drugs” and by increased $[\text{Mg}^{2+}]$ in the medium [69]. Remarkably, Mg^{2+} can compete with Ca^{2+} in CaM [70] and may thus impede its function. Since these parameters are not additive in *Paramecium*, both may operate via the same target, presumably CaM. Spontaneous undocking of vesicles, as reported for hippocampus synapses [71] and chromaffin cells [72] has not been seen with *Paramecium* cells.

2.3. Additional components

Complementation cloning allowed for function repair, and identification of the respective genes, in a variety of mutants collected in the laboratory of Beisson [8]. This includes the *ND7* [73] and the *ND9* genes [74]. Neither one was known from higher eukaryotic cells. *ND6* is a 506-amino acid large protein type I integral membrane protein, with a highly charged cytosolic domain containing amphipathic and coiled-coil regions [73]. *ND9* contains *Armadillo*-like repeats [74]. Although this protein is also without precedent, *Armadillo*-like repeats are typical for binding GDP exchange factors, from *Dictyostelium* [75] to mammalian brain [76]. Later on, proteins with *Armadillo*-like repeats have also been considered for the regulation of secretory activity in yeast [77].

The *Paramecium* sequencing project reveals a variety of sequences related to rab-type monomeric G-proteins and regulatory proteins. Sequencing of the complete genes may help to substantiate the observation of numerous bands in GTP-overlay studies [78].

2.4. V_0 -ATPase subunits

Recently the group of Mayer [37,79,80] has presented evidence, based on work on homotypic fusion with isolated yeast vacuoles, that SNAREs may serve docking, while membrane fusion may be mediated by hexameric V_0 subunits of a V-ATPase. Matching hexameric V_0 proteolipid subunits (reminiscent of gap junctions) contained in the two membranes are assumed to occur before, and to be dispersed during membrane fusion, so that lipid molecules can diffuse between the subunits. This is also supported by the use of antibodies against the V_0 -associated a-subunit, which could inhibit homotypic membrane fusion (A. Mayer, personal communication). This would be a molecular equivalent of the membrane fusion model proposed by some electrophysiologists [81–84]. Interestingly, during synchronous tricho-

cyst exocytosis, we see structural changes in the cell membrane (Fig. 4), which would be largely compatible with such a model (for details, see below).

Some other electrophysiologists prefer this model for membrane fusions within the cell, yet the “lipid stalk model” for fusions on the cell membrane level [85]. Prerequisite for the model adapted by A. Mayer is the established occurrence of a fraction of V-ATPase molecules in inactive form, i.e. without catalytic V_1 headpiece. In fact, under the denomination “mediatophore” (before identification by cloning), V_0 proteolipid subunits were considered as mediating acetylcholine release [86–88].

To address this question in *Paramecium*, we have raised antibodies against the endogenous V_0 -associated a-subunit, which will be used for further identification and localization (Th. Wassmer, R. Kissmehl and H. Plattner, in prep.) Although *Paramecium* trichocysts are not acidic compartments [89] opposite to most dense-core vesicle systems [90], they may contain, nevertheless, V_0 subunits without H^+ -ATPase activity. Detailed analyses of any potential role in membrane fusion are in progress, probably also in other labs.

2.5. Conclusions

Two very similar *NSF* genes, *PtNSF1* and *PtNSF2* are known to exist in *Paramecium*. From this, we expect the occurrence of SNAREs. There is also some evidence for the presence of a synaptobrevin-like SNARE and of α -SNAP. CaM is a constitutive component of trichocyst docking sites. Mutation of CaM inhibits self-assembly, while inhibition of CaM causes their disassembly in wild-type cells. A synaptotagmin-like Ca^{2+} -sensor may be expected, although being unknown at present. Some genes, derived from some exocytotic mutations, like *ND7* and *ND9*, are novel and deserve interest in other systems. Sequences available for rab-type monomeric G-proteins and regulatory proteins remain to be used for cloning.

3. Polarity of trichocyst membranes pertinent to docking and fusion

Trichocysts are formed of a tip and a body part, each with different secretory protein arrangements, both under a common envelope. This structural polarity is paralleled by a functional polarity, as recognized by oriented saltatory docking, “nose” first, in an unusual plus-to-minus direction along microtubules, which emanate from ciliary basal bodies [91,92]. This “behaviour” is disturbed in some mutants, which then also impedes proper assembly of exocytosis sites [7]. This implies that components relevant for docking and membrane fusion must be properly arranged on the organelle membrane, i.e. in so far unexplored microdomains, for later fusion to occur.

Recently, we could induce membrane fusion at trichocyst exocytosis sites under conditions whereby content extrusion

is inhibited [69]. To be released by a decondensation process, paracrystalline contents of the trichocyst body must get in contact with Ca^{2+} from the outside medium [93]. If fusion is induced in the absence of outside Ca^{2+} , membranes can reseal, as visualized by the membrane dye FM1-43, and trichocysts, still with their contents inside and with resealed membranes, are then internalized for another round of docking and fusion. This implies that membrane fusion gives an earmark to the trichocyst membrane. Although time resolution of docking kinetics under these conditions are poor, our data indicate that assembly of a docking site may require the order of minutes, at most [69].

3.1. Conclusions

Trichocysts are polar structures with a polar arrangement of components relevant for docking and fusion. Exocytotic membrane fusion entails a signal for internalization.

4. Co-assembly of docking sites with components of Ca^{2+} -signaling (stores)

As mentioned, for exocytosis to occur, a local increase in $[\text{Ca}^{2+}]$ from ~ 65 nM at rest to ~ 5 μM upon stimulation is required [57]. This is facilitated by the co-assembly of exocytosis sites with cortical Ca^{2+} stores since Ca^{2+} signaling is mediated by mobilization of Ca^{2+} from alveolar sacs, superimposed by a “store-operated Ca^{2+} -influx” (SOC) [57,94,95]. Whether the distinct freeze-fracture particle arrays in this zone may contain components relevant for Ca^{2+} signaling remains open at this time. The positioning of trichocysts at the cell membrane, with closely surrounding alveolar sacs (Fig. 2), appears mandatory for two aspects, (i) for optimal Ca^{2+} signaling, and (ii) for trichocyst positioning, for the following reasons.

When alone the internal Ca^{2+} release component is activated, only up to $\sim 1/3$ of preformed docking sites undergo membrane fusion [55,96,97]. Under such circumstances, the membrane fusion machinery does not work optimally. This again implies the occurrence of a Ca^{2+} -sensor of adequate sensitivity, possibly synaptotagmin (see above).

Beyond this, alveolar sacs also serve as attachment sites for docking trichocysts [23]. Recall that in nd strains, trichocysts are attached only at this docking site I, while failure to assemble site II entails lack of exocytotic response. The situation where trichocysts are attached at the cell surface merely via docking site I (nd stains) can be reversed in different ways, (i) by treatment with cytochalasin B [23] and (ii) by some *Clostridium* toxins. Aspect (i) is poorly understood on a molecular level, particularly since any role of F-actin is unknown. Aspect (ii) has been probed by injecting light chains of different BoNT and TeTx isoforms into *Paramecium* (D. Vetter, H. Plattner, unpublished observations using toxins provided by H. Niemann). Interestingly,

only BoNT-A could remove trichocysts from the cell cortex selectively in nd-type cells. If one considers BoNT-A specific for SNAP-25 [98], this could mean its involvement in formation of docking site I, independent the assembly of fusogenic docking site II. Interestingly, a similar finding was reported with mammalian cells [99]. Once established, docking site II in *Paramecium* is no longer accessible to cleavage by the *Clostridium* toxins tested. These observations are in accordance with the reported resistance of mature docking/fusion sites to such toxins [100,101].

4.1. Conclusions

Co-assembly of trichocysts and surrounding alveolar sacs may serve not only efficient Ca^{2+} signaling, but also initial tethering of trichocysts to the cell cortex, with the possible involvement of specific SNARE components.

5. Possible mechanism of membrane fusion

As discussed above, fusogenic proteins currently discussed are mainly SNARE pins and V_0 subunits of V-ATPase, but some additional candidates may be envisaged as well [38]. Considering the ongoing debate on the elementary question on whether the forming fusion pore is lipidic or made of intrinsic proteins, or of dissociating protein subunits with progressively intercalating lipids, it is difficult to interpret the EM pictures we obtain on ongoing fusion at trichocyst exocytosis sites (Fig. 4).

Early on, there was some evidence presented that a specific lipid composition would be required for docking/fusion sites in *Paramecium* [20]. Evidence of lipid raft involvement in exocytosis has been substantiated with mammalian cells only quite recently [102,103]. Finally, in a more general discussion on membrane fusion, one should not neglect the question of whether homotypic fusion, such as between yeast vacuolar membranes, would be identical to that occurring during exocytotic membrane fusion.

As mentioned, in exocytosis-competent *Paramecium* strains, fusogenic sites display each about eight or nine rosette particles in a cluster. It is also noteworthy that in the trichocyst tip membrane, no equivalent structures are seen, while it forms a nipple protruding towards the cell membrane (Fig. 5). Such highly curved structures are known to be energetically favorable for fusion [104]. Considering the preparation-dependent partitioning of membrane particles [2], equivalent matching particles could still exist in the trichocyst tip membrane. However, its nipple-like protrusion towards the center of the fusogenic zone may accommodate only one or very few rosette equivalents, if they occur there at all. Their excess in the cell membrane would then represent multiple “ignition” sites. Alternatively, they may represent different molecular complexes. More scrutinized inspection reveals that rosettes do not, or not always, contain a central particle. This may change during

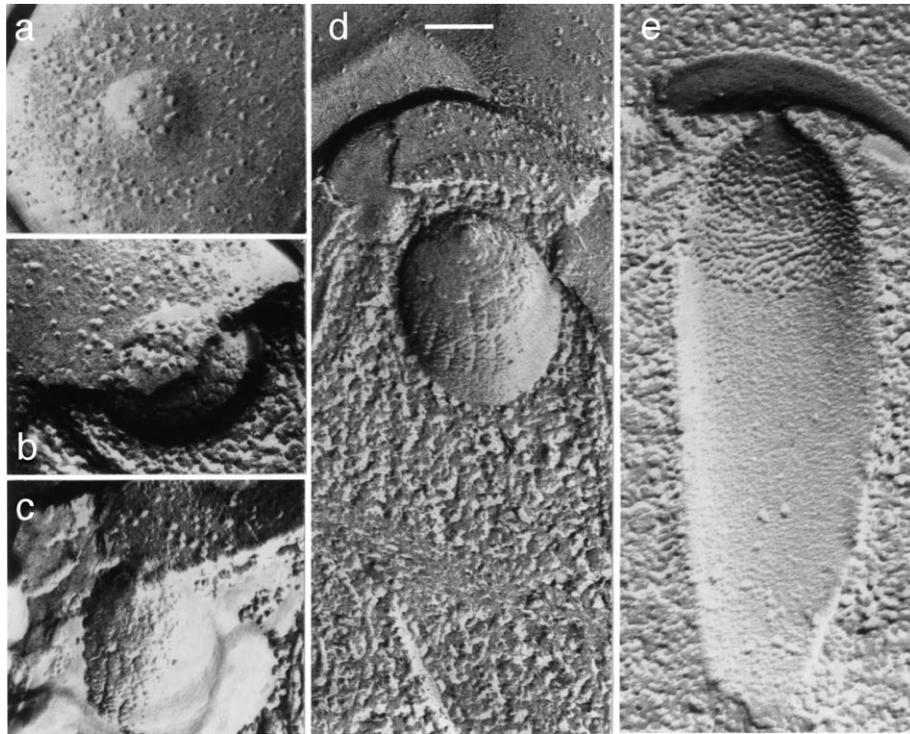


Fig. 5. Trichocyst docking sites in unstimulated *Paramecium* cells, viewed from different angles. Cells were freeze-fractured (Pt/C) after fixation with glutaraldehyde (to visualize more easily all particle populations). The series (a–e) proceeds from a vertical view on the PF-face of the cell membrane (a) to a lateral view of a docked trichocyst (e). Note occurrence of a ring with a rosette (a), a trichocyst tip visible right underneath the cell membrane (b–e), a “nipple” structure (d, e) without particles, and a more complex structure (not so relevant in the present context) somewhat below. Bar = 100 nm: from Plattner [2], including unpublished micrographs from K. Olbricht and H. Plattner.

stimulation, and matching complexes could be formed during fusion before they decay into subunits upon stimulation [12], as shown in Fig. 4.

Could both, SNAREs and V_0 subunits, account for the details seen during synchronous fusion of trichocyst membranes? Recall: upon exocytosis stimulation, we see a vanishing number of rosette particles, but an increasing number of smaller particles within the fusion domain delineated by a “ring” [105]. Particle counts suggest that large rosette particles may decay into six small subunits [12]. Could they correspond to the hexameric V_0 subunits? In fact, V_0 has a size of ~ 13 nm [106], which is identical to that of a rosette particle in freeze-fractures after fine-grain Ta/W replication [107]. This particle size is difficult to reconcile with the trimeric SNARE pins postulated in Ref. [108], each SNARE with one helical transmembrane domain [32,34] or a fatty acylation (SNAP-25). Based on estimations by Plattner and Zingsheim [109] or by Eskandari et al. [110], assuming 1.4 nm^2 per transmembrane helix, this would result in much smaller particles, invisible in freeze-fracture replicas. Still, SNAREs may be considered as candidates, as suggested in Refs. [34,36], if one assumes larger complexes. Isolated SNARE complexes, after Pt rotary shadowing, are indicated as 4-nm-thick bundles [111]. A number of such complexes would have to be assembled to yield a 13-nm large rosette particle, and eight to nine of them, equivalent to the number of rosette particles in one exocytotic site, would have to be

arranged. Undoubtedly, the number of SNAREs forming a pin—if it should become visible as a membrane particle—would have to contain many more SNARE molecules than the number reported and, if rosette particles were their equivalents, the number per exocytosis site would still have to be accordingly higher. In fact, Ca^{2+} (in the course of its priming effect mentioned above) can cause in vitro synaptotagmin to aggregate and to form still larger SNARE complexes [112]. The pore could then also be formed in the center of one such SNARE-protein complex/particle, perhaps the equivalent of a rosette particle. Furthermore, one has to consider that SNAREs may bind V_0 [79,113].

Alternatively, only the central particle (which is sometimes seen within a rosette) could be the only fusogenic structure. Strikingly, when we inhibited exocytosis (contents extrusion) by antibodies against (non-specified) cell surface components during simultaneous trichocyst exocytosis stimulation (Fig. 6), we obtained pictures showing focal membrane fusion without pore expansion [114]. In that case, intact rosette particles are seen to spread laterally, while a small pore is formed in the center of the preformed fusion zone. Very strikingly, the particulate structure in the center of the rosette in Fig. 6a looks hollow, but this is difficult to ascertain at the level of resolution achieved with Pt/C replicas [109]. Note under these conditions, the simultaneous occurrence in the cell membrane of a funnel-like depression towards the nipple-like expansion on the trichocyst tip, as discussed above.

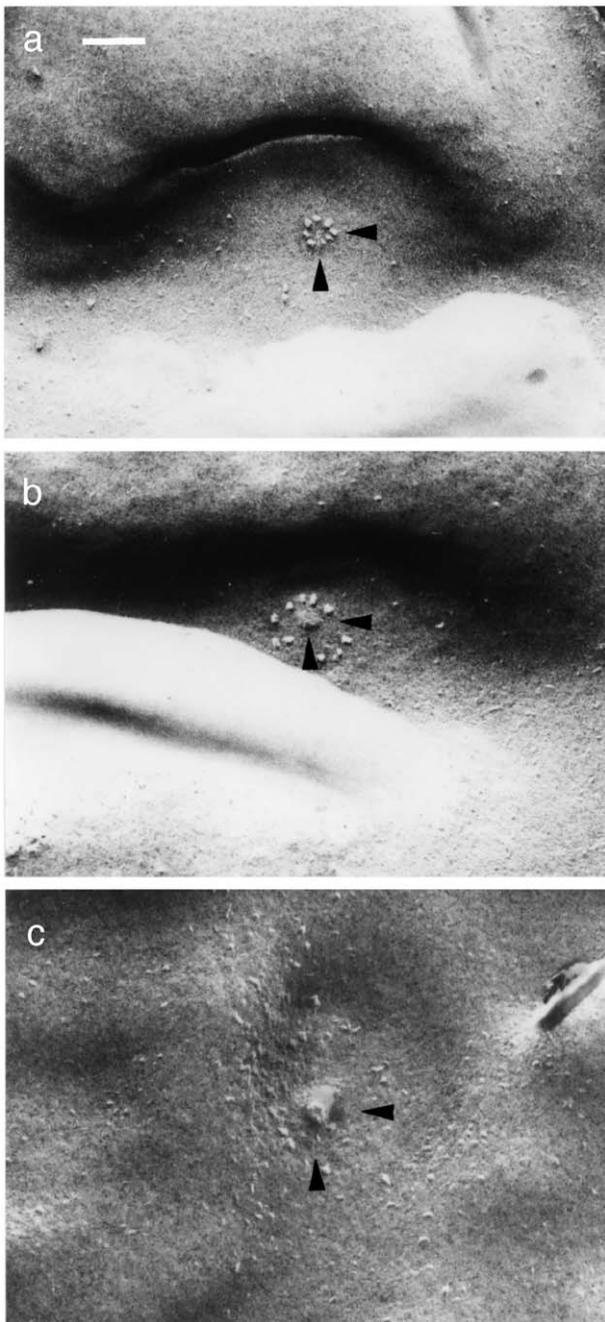


Fig. 6. When stimulated during inhibition by exogenous antibodies (against cell surface components), focal membrane fusion can occur transiently, without decay of rosette particles into subunits and pore expansion, but with lateral spread of intact rosette particles. Trichocyst docking sites (a–c) in an unfixed, fast-frozen *Paramecium* cell, freeze-fractured (Pt shadowing from bottom to top) after exocytosis stimulation in presence of (non-specified) antibodies against cell surface components. While ring particles are difficult to recognize due to the methodology used, this EF-face shows rosette particles in different states, with presumable fusions at the crossing point of arrowheads. (a) Presumable membrane fusion in the center of a rosette, (b) protruding fusion site within a group of intact rosette particles after further spreading, (c) slightly expanded fusion pore, represented by a hole on the depression and some further spread of intact rosette particles. Bars = 100 nm: from Momayezi et al. [114].

Recall that such “conformation” is considered energetically favorable to membrane fusion [104], as mentioned. The absence of fusion pore expansion in presence of antibodies may be due to inhibition of lateral spread of V_0 subunits, or alternatively to inhibition of the disassembly of SNAREs or of any other fusogenic protein. At this time, our data appears compatible with V_0 subunits as candidates for fusogenic proteins. However, considering the current uncertainty on the size of SNARE complexes, these or any other possible candidates could not be excluded at this time. Clearly, focal fusion occurs under conditions of simultaneous stimulation and inhibition; decay of one central particle is required for completing fusion/pore expansion. The low frequency of fusions without pore expansion, as we find it during simultaneous activation and inhibition [114], can be accounted for by the reversibility of small pore sizes, as shown with widely different exocytotic systems [115,116].

Among additional candidates possibly involved in membrane fusion are “secretory carrier membrane proteins” (SCAMPs). SCAMPs, 31–37-kDa large membrane-integrated proteins, were detected in dense-core vesicles of exocrine cells [117]. They are more widely distributed in intracellular membrane systems participating in membrane traffic, including the cell membrane [118], and in synaptic vesicles [119]. In part they co-localize with SNAP-23 and syntaxin and seem to play a role in a late step of exocytosis [120]. On the basis of the detailed molecular data on SCAMPs [119,121] on the one hand, and the steadily growing genomic information on *Paramecium* on the other, it should be possible to trace their occurrence and function in this system.

Is there a common mechanism for membrane fusion after all? Alone within one posterior pituitary nerve terminal, fusion pores formed by the two different types of vesicles have widely diverse dynamics according to patch-clamp analysis [122] and different molecular fusion machineries are postulated. One should also consider the question whether there may be any difference between homo- and heterotypic fusion, e.g. between isolated vacuoles and during exocytosis. Finally, the freeze-fracture particles occurring at trichocyst exocytosis sites may serve some other, so far unknown, functions within the fusogenic microdomain. Evidently, the question of the pore’s microanatomy has to await more detailed analysis.

Paramecium is also suitable to resolve the steps following PF-type membrane fusion (exocytosis), e.g. ensuing EF-type fusion (resealing). Notably, fusion during resealing is also of the focal type [54], with widely scattered rosette subunits formed during exocytotic membrane fusion. The short duration of exo-endocytosis coupling within 0.35 s [12,54] and occurrence of membrane-attached proteins may help to restrain membrane components from diffusing into the respective other membrane. Nevertheless, some glykokalyx components diffuse into the resealing trichocyst membrane [2] and one can also recognize, though quite rarely, transfer of trichocyst membrane components to the cell membrane upon resealing [123]. The open time during trichocyst ex-

pulsion is longer than with smaller dense-core vesicles [115] but resembles that of dense-core vesicles of beige mouse mast cells, also relatively large organelles [124].

5.1. Conclusions

Despite a clear-cut freeze-fracture morphology, i.e. conspicuous protein particle aggregates formed in the cell membrane after trichocyst docking specifically in exocytosis competent strains, it is difficult at this time to correlate these structural elements with the fusion mechanisms proposed in the literature. Increasing accessibility of genomic data lends additional support for work with *Paramecium* as a model system, and in the future, some crucial aspects can then be discussed in a new light.

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