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Trichocysts—*Paramecium's* Projectile-like Secretory Organelles

Reappraisal of their Biogenesis, Composition, Intracellular Transport, and Possible Functions

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ABSTRACT

This review summarizes biogenesis, composition, intracellular transport, and possible functions of trichocysts. Trichocyst release by *Paramecium* is the fastest dense core-secretory vesicle exocytosis known. This is enabled by the crystalline nature of the trichocyst "body" whose matrix proteins (tmp), upon contact with extracellular Ca^{2+}, undergo explosive recrystallization that propagates cooperatively throughout the organelle. Membrane fusion during stimulated trichocyst exocytosis involves Ca^{2+} mobilization from alveolar sacs and tightly coupled store-operated Ca^{2+}-influx, initiated by activation of ryanodine receptor-like Ca^{2+}-release channels. Particularly, aminoethyldextran perfectly mimics a physiological function of trichocysts, i.e. defense against predators, by vigorous, local trichocyst discharge. The tmp's contained in the main "body" of a trichocyst are arranged in a defined pattern, resulting in crossstriation, whose period expands upon expulsion. The second part of a trichocyst, the "tip", contains secretory lectins which diffuse upon discharge. Repulsion from predators may not be the only function of trichocysts. We consider ciliary reversal accompanying stimulated trichocyst exocytosis (also in mutants devoid of depolarization-activated Ca^{2+} channels) a second, automatically superimposed defense mechanism. A third defensive mechanism may be effectuated by the secretory lectins of the trichocyst tip; they may inhibit toxicyst exocytosis in *Dileptus* by crosslinking surface proteins (an effect mimicked in *Paramecium* by antibodies against cell surface components). Some of the proteins, body and tip, are glycosylated as visualized by binding of exogenous lectins. This reflects the biogenetic pathway, from the endoplasmic reticulum via the Golgi apparatus, which is also supported by details from molecular biology. There are fragile links connecting the matrix of a trichocyst with its membrane; these may signal the filling state, full or empty, before and after tmp release upon exocytosis, respectively. This is supported by experimentally produced "frustrated exocytosis", i.e. membrane fusion without contents release, followed by membrane resealing and entry in a new cycle of reattachment for stimulated exocytosis. There are some more puzzles to be solved: Considering the absence of any detectable Ca^{2+} and of acidity in the organelle, what causes the striking effects of silencing the genes of some specific Ca^{2+}-release channels and of subunits of the H^+-ATPase? What determines the inherent polarity of a trichocyst? What precisely causes the inability of trichocyst mutants to dock at the cell membrane? Many details now call for further experimental work to unravel more secrets about these fascinating organelles.

TRICHOCYSTS were first recognized by Allman (1855) and named according to the Greek words, τρίχός (hair) and κύστη (vesicle, cyst), in the first volume of the *Journal Cell Science*. Allman explained their vesicular appearance and position in the cell cortex and their release as elongate needle-like structures. It is thrilling to leaf through that first journal issue, with so many authors we still admire, and with *Paramecium* and its trichocysts in between, as a subject of early cell biology. Trichocysts were the first cytoplasmic organelles described, decades before mitochondria and chloroplasts.

Why care for trichocysts? Admittedly, one aspect is naive fascination by these explosive projectile-like secretory organelles that are best known from *Paramecium* the subject considered in this review. This motivation is supported by the recent accessibility of their secrets to molecular biology. Still there are a number of open questions that deserve experimental scrutiny. Central questions are: why does a cell invest in such an elaborate arsenal of trichocysts, what makes trichocysts the fastest reacting dense core-secretory organelles known (Plattner and Kissmehl 2003) and what enables them to serve as a highly efficient defense "weapon" (Harumoto and Miyake 1991)? On a broader scale, *Paramecium* currently comes again into the limelight also because of its recent appreciation of its ecological importance (Schmeller et al. 2014). In a broader view, trichocysts are just one form of dense core-secretory organelles occurring in a variety of taxons of protozoa and algae (Hausmann 1978; Hovasse and Mignot 1975; Kugrens et al. 1994; Rosati and Modeo 2003). Globally, they have been designated as extrusive organelles or extrusomes because of their impressive release mechanism, based on the highly ordered arrangement of their contents and their rearrangement upon stimulation. Examples range from algae, such as dinophytes and other taxons (Westermann et al. 2015), ciliates (Hausmann 2014) to parasitic Apicomplexa, such as *Toxoplasma* and *Plasmodium* (Cowman et al. 2012; Garcia et al. 2008) where they are indispensible for host cell penetration. Even these highly specialized forms of extrusomes, such as rhoptries, show important similarities to extrusomes of ciliates (Gubbels and Duraisingh 2012). Apart from some similarities, extrusomes of *Tetrahymena thermophila*, are different from trichocysts of *Paramecium tetraurelia*, as they release rather slowly mucous materials; see section "Ca^{2+}-binding proteins".

BASIC FEATURES OF TRICHOCYST STRUCTURE AND FUNCTION

This review concentrates on trichocysts of *Paramecium*, notably *P. tetraurelia*, whose basic structural and functional consequences are summarized in the scheme at the end of this review. Here, trichocysts contribute by almost 50% to total cell protein (Matt et al. 1978), as can be calculated from the compact, largely crystalline packing of their proteins and ~8% contribution to cell volume, whereas overall cell protein content is only ~10%. The large majority of trichocysts are docked at the cell membrane, ready for release, and, thus, heavily concentrated in the cell periphery (Fig. 1).

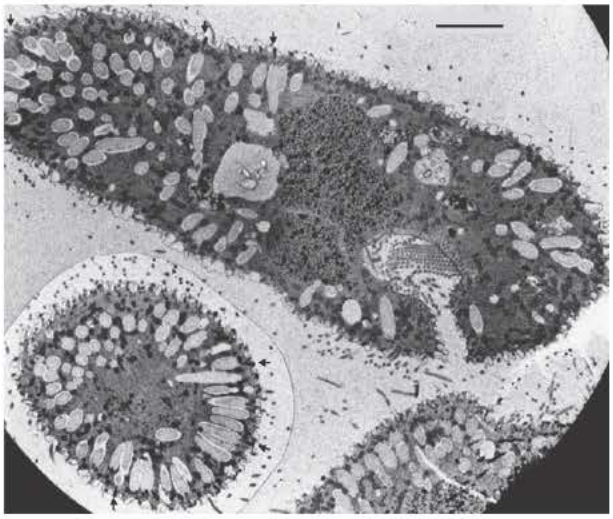

Figure 1 *Paramecium tetraurelia* wildtype cells in a montage of a longitudinal and a crosssection. This shows numerous trichocysts attached to the cell membrane (arrows) in longitudinally and crosssectioned cells. Scale bar 10 μm. H. Plattner (unpublished micrographs).

Structure and consequences for exocytosis performance

Trichocysts are unique for their polar construction, with a ~3 μm long and a ~2 μm wide "body" part and a ~2 μm long, much thinner "tip" part (Fig. 2) serving for the attachment at the cell membrane (Bannister 1972). The predominant mass of proteins contained in the trichocyst body is called the "trichocyst matrix proteins", tmp. There is a variable number of mature trichocysts attached to the cell membrane at predetermined, regularly spaced sites, ready for discharge (Adoutte 1988; Plattner et al. 1973, 1984, 1985a,b). Values reported range from ~1,000 to several thousand which is the actual number of potential docking sites in *P. tetraurelia* (Erxleben et al. 1997). Actual numbers also differ between the species mainly analyzed, i.e. *Paramecium multimicronucleatum* (Allen group, Honolulu), *Paramecium caudatum* (Hausmann group, Berlin), and *P. tetraurelia* (Beisson [Gif-sur-Yvette], Nelson [Madison] and our group in Konstanz). Otherwise, there is no remarkable difference between the species.

Figure 3 documents instantaneous release of trichocysts upon stimulation with the secretagogue aminoethyldextran, AED (Plattner et al. 1984); due to instantaneous expansion they present themselves as long needles. When early naturalists observed the vigorous expulsion of trichocysts by a *Paramecium* cell, this was instinctively interpreted as a defensive function (Maupas 1883). This is based on the capability of vigorous expansion of their body contents during stimulated exocytotic release upon contact with Ca^{2+} from the outside medium (Bilinski et al. 1981a; Schmitz and Zierold 1989) (Fig. 4).

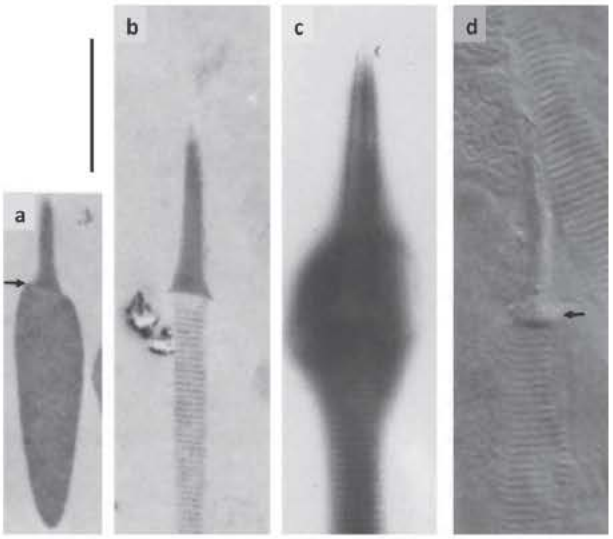

Figure 2 Trichocyst contents isolated from *Paramecium tetraurelia* wildtype. Trichocysts are shown (**a**) in condensed, (**b d**) in decon densed (discharged) form. (a, b) are ultrathin sections, (c) is a negative staining and (d) a freeze fracture example. Note the tip and the body part (separated by arrows), the latter with periodic crossbanding. Also note that (a) is not a strictly median section, so this trichocyst appears shorter than in reality. Scale bar 1 µm. From Bilinski et al. (1981a).

More than a century later, this was shown to be correct: Keeping *Paramecium* together with the predatory ciliate, *Dileptus margaritifer*, proved for the first time a predator-defense situation (Harumoto and Miyake 1991; Miyake et al. 1989). On this basis, it has been shown that the mechanism behind is not an overall, but a rather local release of trichocysts (Fig. 5a d) which keeps a predator at a distance by the severalfold stretching ("decondensation") of the bodies of locally discharging trichocysts (Knoll et al. 1991b). This can be perfectly mimicked by AED, as shown in Fig. 5e,f.

Trichocyst contents isolated in condensed and decondensed state, respectively, have also been subjected to deep temperature X-ray diffraction (Sperling et al. 1987) and similarly electron micrographs have been evaluated by laser diffractometry (Kersken et al. 1984). Electron microscope (EM) analysis of *Paramecium*'s trichocysts has shown that such stretching is paralleled by a change in the periodic crossbanding of the trichocyst body matrix (Hausmann 1978, 2014; Jakus and Hall 1946). This was thought to be enabled by a rearrangement of "trichynins" (Steers et al. 1969), a synonym for tmp, a collection of insoluble proteins of quite similar apparent molecular weight (MW) (Fig. 6a) and with different isoelectric points (pI) between 4.7 and 5.5 (Madeddu et al. 1995; Tindall et al. 1989). Together, they form a crystalline scaffold which can rapidly expand in vivo or in vitro (Bilinski et al. 1981a; Sperling et al. 1987). The tmp's are encoded by a multigene family of > 100 genes (Vayssié et al. 2001); meanwhile a total of 176 tmp genes have been annotated for *P. tetraurelia* (Arnaiz et al. 2010). The tmp proteins form dimers of 30 40 kDa from disulfide bond-linked

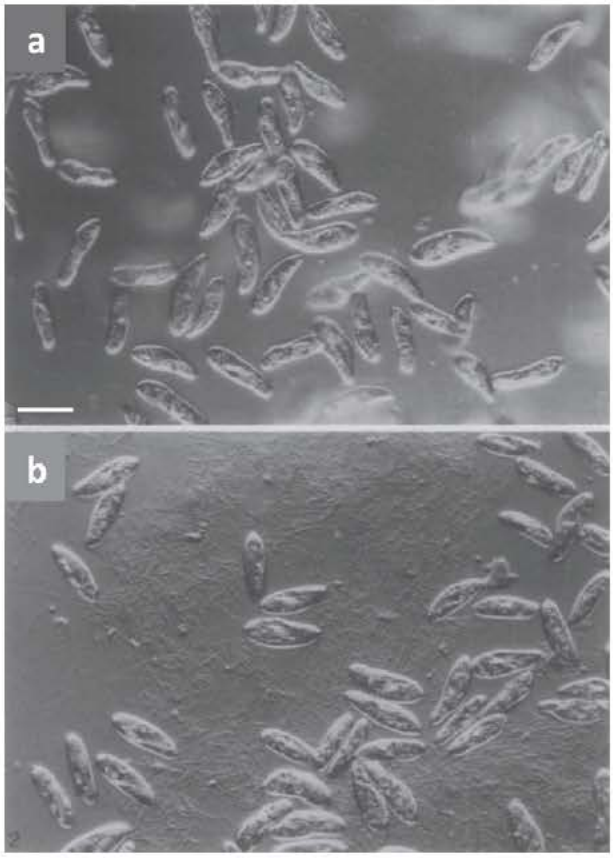

Figure 3 Live axenic *Paramecium tetraurelia* wildtype culture. Cells are shown (**a**) before stimulation, (**b**) immediately after stimulation of trichocyst exocytosis by AED. During expulsion, trichocysts explode to long needles. Scale bars 100 µm. From Plattner et al. (1984).

monomers of 15 20 kDa (Steers et al. 1969) a feature shared by secretory proteins from mucocysts (Maihle and Satir 1986). In *Paramecium*, the tmp's evidently contain a signal peptide for cotranslational sequestration (Arnaiz et al. 2010) and they are derived from large precursor proteins of 40 45 kDa by posttranslational cleavage (Adoutte et al. 1984; Gautier et al. 1994; Shih and Nelson 1991, 1992). See section "Biogenesis and ultrastructure". Beyond tmp's, also soluble proteins are successively released (Fig. 6a).

Synchronous exocytosis chance and challenge

Maupas (1883) noted an "explosion so instantaneous [at the cell's surface] and so fast that it was quite impossible to follow the transformation of the spindle-shaped rod into a fine needle" (cited after Gerritsen 2000). In fact, high speed kinematography allowed us to estimate the time required for decondensation as below 1 ms (Matt et al. 1978). Decondensation of tmp's is a kind of recrystallization, paralleled by an explosive ~eightfold stretching (Fig. 2 5) that occurs upon exposure of the organelle lumen to extracellular Ca^{2+}, $Ca^{2+}{}_{o}$, once an exocytotic

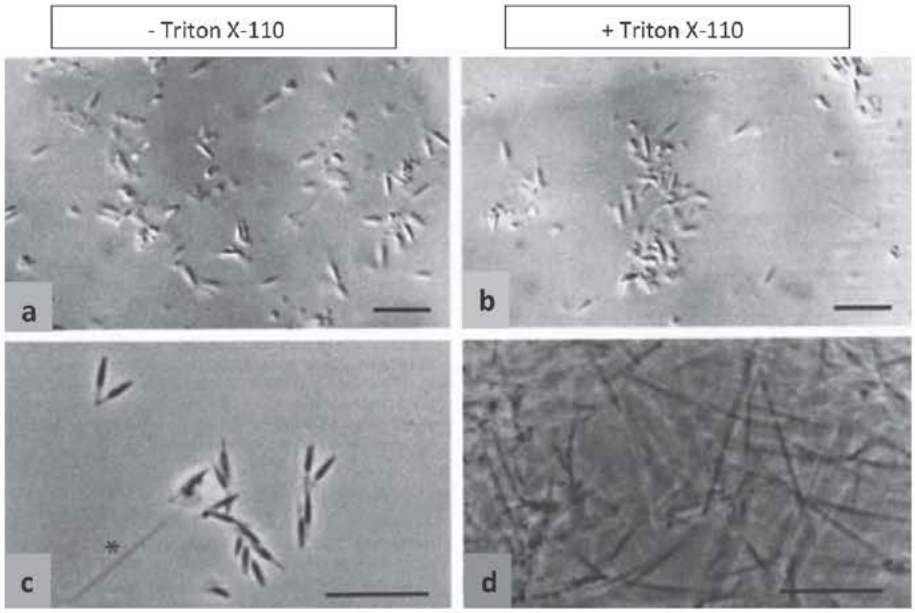

Figure 4 Trichocysts isolated with their membrane from a wildtype *Paramecium tetraurelia* culture. These images document decondensation by Ca^{2+} when membranes are leaky. (**a**, **c**) Without Triton X 100, (**b**, **d**) with Triton X 100 added. Upper panels (a, b) are without Ca^{2+}, lower panels (c,d) with 5 mM Ca^{2+} added. In (c) one trichocyst has "exploded" (asterisk) probably due to membrane leakiness. Scale bars 10 μm. From Glas Albrecht and Plattner (1990).

opening has formed (Bilinski et al. 1981a). Therefore, the rearrangement of the proteins during decondensation takes place in concert with exocytotic membrane fusion (Hausmann 2014; Kersken et al. 1984; Plattner et al. 1993; Sperling et al. 1987). The periodicity of crossbanding, as measured after different staining methods, either directly on EM micrographs or by their laser diffractometric analysis, results in a stretching factor of ~4 or 7.5, depending on which banding is considered (Kersken et al. 1984). In addition, longitudinal connections between densely stained crossbands can be recognized (Hausmann et al. 1972; Peterson et al. 1987b; Sperling et al. 1987), thus keeping the trichocyst matrix together. According to low temperature X-ray crystallography, for unexplained reasons, the period increases by only ~threefold (Sperling et al. 1987).

Considering the inconsistencies mentioned, it is still unknown how decondensation precisely takes place. What remains as an established fact is the role of Ca^{2+}-binding proteins found in the matrix which are involved in the decondensation process (section "Ca^{2+}-binding proteins"), as evidenced by mutants devoid of Ca^{2+}-binding to matrix proteins (Klauke et al. 1998). Interestingly, some "granule lattice proteins", Grl, in mucocysts contain a β/γ domain (Bowman et al. 2005a), that is mainly known from the mammalian eye lens. They bind Ca^{2+} (Mishra et al. 2016) and occur down to lower eukaryotes where they are stabilized by Ca^{2+} (Jaenicke and Slingsby 2001). Proteins with a β-crystallin fold structure are also deposited in the *Paramecium* database (Chan et al. 1999 and H. Plattner unpublished observ.). Evidently in *Paramecium*, the mature trichocyst tmp proteins are folded in an unstable configuration before they bind Ca^{2+} during expulsion; this allows transition to a stable conformation (Vayssié et al. 2000), just like a spring with a transition from high to low potential energy. The idea of the occurrence of some other Ca^{2+}-binding proteins, such as chromogranin A protein (Peterson et al. 1987a) and of calmodulin (Rauh and Nelson 1981), within the trichocyst has soon been abandoned by those groups who had propagated these ideas (Tindall et al. 1989); see "Additional trichocyst components".

Trichocysts can be released upon stimulation with a degree of synchrony that is unsurpassed by any dense core-secretory organelle system (Knoll et al. 1991a; Plattner et al. 1984, 1985a,b, 1993) when widely different systems, up to man, are compared (Kasai 1999; Plattner and Kissmehl 2003). To study membrane fusion during synchronous exocytosis with high temporal resolution, kinematography (6,000 frames/s, the maximum then available) has been applied to monitor the release of individual trichocysts (Matt et al. 1978). Standard light microscopy has been used for kinematographic recording of trichocyst docking and of the interaction of a *Paramecium* cell with the predatory ciliate, *Dileptus* (Knoll et al. 1991b). This was paralleled by visualization of accompanying Ca^{2+} fluorochrome signals (Klauke et al. 2000) and by whole cell-patch electrophysiology-recording of Ca^{2+}-activated currents accompanying the release of individual trichocysts (Erxleben et al. 1997). In most cases, fluorochrome analysis required dual wavelength recordings. This was difficult because of the mobility of *Paramecium* and because of

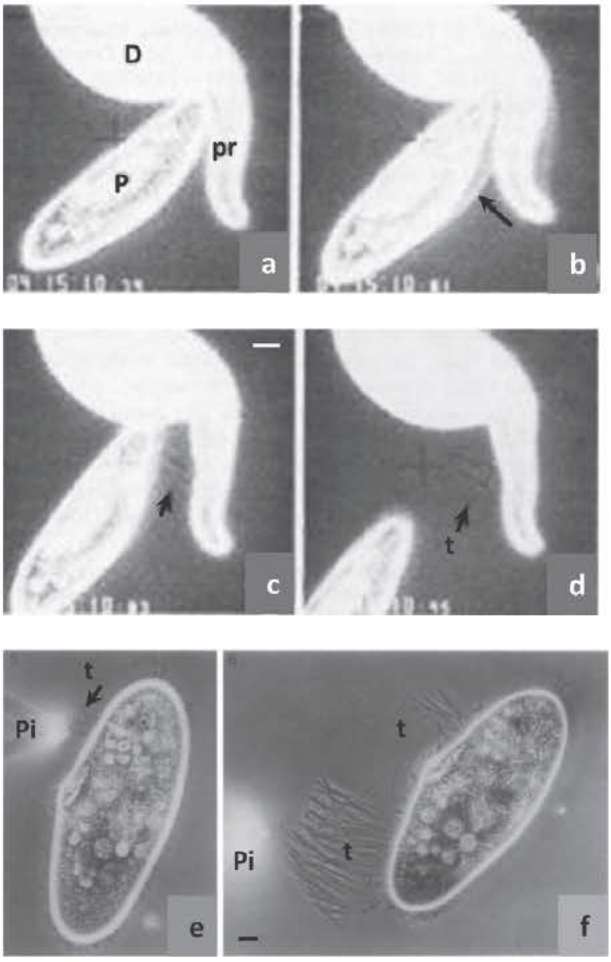

Figure 5 Time sequence series (**a d**) of a "dangerous encounter" of a *Paramecium tetraurelia* (P) wildtype cell with a predatory *Dileptus margaritifer* (D) cell. When a *Paramecium* cell unintentionally hits a *Dileptus* cell at its radar like rotating proboscis (pr), *Paramecium* immediately moves from the dangerous site (large arrow) as it locally discharges trichocysts (t, small arrows). Also note backward move ment in (d). (**e, f**) A *P. tetaurelia* wildtype cell locally triggered by AED application. Consecutive application was by a micropipette (Pi) at one (e) or two sites (f). Exocytosis achieved mimicks the situation during predator defense shown in (a d). Scale bars 10 µm. (a d) are from Knoll et al. (1991b), (e, f) from Plattner et al. (1984).

poor fluorochrome permeability. These problems were solved by fixing cells in the meniscus of a silicon oil-covered droplet of culture solution and by microinjection of the fluorochrome (Klauke and Plattner 1997). The calibrated values recorded reflect free (dissolved) intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, and its change upon stimulation. Real local values, e.g. at the very restricted exocytosis sites down to the nanometer-range, have been estimated by the inhibitory effects of Ca^{2+} chelators with different binding constant. Using more sophisticated microscopic equipment, with a CCD camera and videotaping, Iwadate and Kikuyama (2001) confirmed our findings.

Light microscopy has been frequently used for the localization of antigens by immuno-fluorescence. Labeled lectins were applied to see the distribution of glycosylation sites (Allen et al. 1988; Lüthe et al. 1986), and neoglycoproteins, as defined by Gabius et al. (1993), to visualize secretory lectins (Haacke-Bell and Plattner 1987), both at the light and EM level, using fluorochrome and gold conjugate labeling, respectively. Molecular data retrieved from the *P. tetraurelia* database have allowed for the prognostication of immunogenic sites, with the aim of producing antibodies for use at the light microscope and EM level. This has been complemented by gene silencing, to identify specific molecules relevant for defined steps of the secretory cycle (Ladenburger et al. 2009; Wassmer et al. 2005, 2006).

The capability of synchronous exocytosis of the entire arsenal of trichocysts has enabled the study of the upregulation of gene transcription. A transient and coordinate tenfold increase in mRNA encoding tmp's has thus been observed (Galvani and Sperling 2000). Altogether more than 400 genes are upregulated, the majority, but not all, being in direct context with trichocyst structure and function (Arnaiz et al. 2010). This is a unique chance in cell biology considering that, for instance, from adrenal medullary chromaffin cells only ~1% of the dense core vesicles can be released at a time upon stimulation (Plattner et al. 1997a). Prerequisite for exocytosis, also in *Paramecium*, is the availability of SNARE (soluble N-ethyl maleimide [NEM] sensitive attachment protein receptors) proteins (Plattner 2010a), of the SNARE chaperone, NSF (NEM-sensitive factor) and of a Ca^{2+} sensor (so far not specified in *Paramecium*), in conjunction with rapid cytosolic Ca^{2+} signaling (Plattner 2014a).

Thus, *Paramecium's* battery of trichocysts and their explosive discharge presents itself as a chance, and simultaneously as an experimental challenge. Notably, the role of luminal Ca^{2+}-binding proteins and of lectins, as well as of matrix-membrane links (Momayezi et al. 1993) remains to be scrutinized. Much can be learned about trichocysts from comparison with *Tetrahymena's* mucocysts, since these organelles, although different in many regards, are more easily amenable to molecular biology and also share some important constituents and properties with *Paramecium's* trichocysts (Briguglio et al. 2013; Elde et al. 2007). Similarities concern biogenesis (section "Biogenesis and ultrastructure"), dissimilarities are seen in release kinetics and function (section "Trichocyst function").

Secretory mutants another chance for scrutiny of the secretory cycle

A series of mutants has allowed to point out a wide range of genes/proteins somehow involved in biogenesis, docking and release of trichocysts in *P. tetraurelia* according to physiological and structural analyses (Froissard et al. 2004; Pouphile et al. 1986; Vayssié et al. 2000). Such proteins are beyond the standard repertoire, like GTPases and SNARE proteins, although the majority of them have not been identified at a molecular level. During biogenesis, processing of tmp precursors by limited proteolysis is

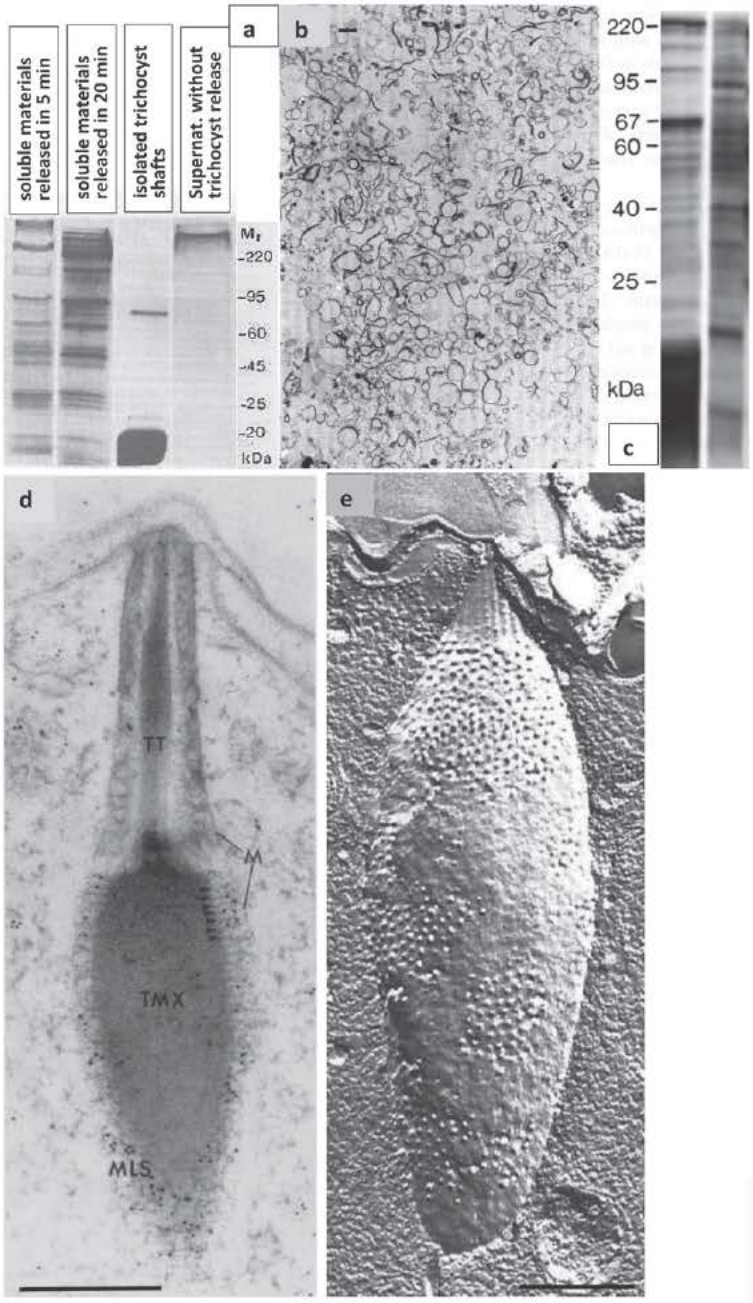

Figure 6 Some essential characteristics of trichocyst contents and membranes. (**a**) Soluble and insoluble proteins of trichocysts, after isolation from *Paramecium tetraurelia* wildtype cells. Sodium dodecylsulfate polyacrylamide gel electrophoresis from samples reduced with mercap toethanol; gels stained with Coomassie Blue. Soluble material released within 5 and 20 min, respectively, insoluble trichocyst shafts and control supernatant without trichocysts. Note increasing release of soluble proteins which widely differ in molecular mass, M_r, and which are different from the crystalline shaft material; this is of the typical low molecular size. (**b**) Electron micrograph obtained from *P. tetraurelia* trichocyst mem branes isolated from a pure trichocyst fraction by osmotic shock and filtration on a sieveplate. (**c**) Electrophoresis samples, with mercaptoethanol, obtained from isolated trichocysts, subjected to biotinylation. *Left lane*: Processing by Coomassie blue staining, *right lane*: processing by labeling with streptavidin horseradish peroxidase. Both lanes obtained from biotinylated trichocysts. Molecular size markers are indicated in kDa units. Note complexity of the membrane protein inventory. (**d**) Electron micrograph showing periodically arranged "connecting material" (MLS) located between the trichocyst matrix (TMX) and the membrane (M) in a trichocyst in situ and immuno gold labeled. Preparation: *P. tetraurelia* wildtype cell rapidly frozen and subjected to freeze substitution fixation; ultrathin section incubated with a monoclonal antibody, followed by protein A gold conjugate, 10 nm. TT trichocyst tip. (**e**) Freeze fracture of a *P. tetraurelia* wildtype cell exposed to filipin after slight fixation. Note the formation of large particles, as they are considered typical of sterol filipin complexes. Scale bars 10 μm (b), 0.5 μm (d,e). (a) is from Glas Albrecht et al. (1990), (b, c) from Glas Albrecht et al. (1992), (d) from Momayezi et al. (1993), (e) from K. Olbricht and H. Plattner (unpublished micrograph).

crucial to achieve proper (ultra)structure as well as docking and release competence (Gautier et al. 1994).

In *Paramecium*, secretory mutants, derived from the wildtype (strain *7S* and related) are available from *P. tetraurelia*. Mutants most frequently used, spontaneous or chemically induced, are called *nd* (nondischarge), *tl* (trichless), *tam* (derived from "trichocyst defect and amacronucleate cells") etc. The *pawn* mutant *d4-500r* was also used for some controls, as it lacks voltage-dependent Ca^{2+}-channels in cilia, for analyzing in parallel ciliary reversal and stimulated exocytosis (Husser et al. 2004). From *P. caudatum*, strain *tnd1* ("trichocyst nondischarge", Watanabe and Haga 1996) allowed us to study (de)coupling of trichocyst contents discharge from membrane fusion (Klauke et al. 1998).

As documented in this review, the usefulness of secretory mutants has been frequently appreciated. This holds for all steps of the secretory cycle, from organelle biogenesis to exocytosis and exocytosis-coupled endocytosis. Secretory mutants, mainly from the Beisson group enabled the scrutiny of details relevant for all of the different steps of the secretory cycle. Considering the impact of this work on a general understanding of dense core-secretory vesicle biogenesis and function, this aspect is now briefly summarized.

After translation, trichocyst secretory contents have to be processed by limited proteolytic cleavage. Prototypes of such mutation in *P. tetraurelia* is strain *tl* (Pollack 1974). Such cleavage is in accordance with metazoan cells up to mammals (Orci et al. 1987). Failure of proteolytic cleavage impedes formation of the classical trichocyst morphology, with tip and body, and of a crystalline matrix. Rather, in *tl* cells, secretory vesicles do not develop beyond precursor vesicles which are rather small and inconspicuous, with amorphous contents, and unable to dock at the cell membrane (Beisson et al. 1976; Pollack 1974). Such precursor vesicles are released by constitutive exocytosis at undefined sites (Gautier et al. 1994).

Mutations type *tam* are very heterogenous and pleiotropic in their defects. The *tam* mutations are preferably inhibited in docking (Beisson et al. 1976; Gautier et al. 1994; Lefort-Tran et al. 1981; Pouphile et al. 1986; Vayssié et al. 2000). More frequently used strain *tam38*, for example, accumulates nonmature, relatively large secretory vesicles, devoid of the spindle shape body and a tip, as would be typical of normal trichocysts. Other strains, also with malformed, abortive, unattached trichocysts, and also without a tip, are football (*ft*), *rug*, stubby (*st*) and pointless (*pt*); for these less used mutants, see Vayssié et al. (2000). Their docking capability is also defective.

Trichocysts of other *tam* strains, type *tam1*, *tam6*, *tam8*, and *tam 11* appear almost normal, but most of them are not transported (Vayssié et al. 2000). Effects are often pleiotropic and may also affect macronuclear division. The common denominator to this and to trichocyst immobility may reside in aberrant deployment of the microtubulae apparatus (Cohen et al. 1980) or attachment to them. Strains *tam8* and *tam38* have been selected to scrutinize acquirement of docking-competence (Beisson et al. 1976; Lefort-Tran et al. 1981; Pouphile et al. 1986; Vayssié et al. 2000).

Experimentally most important are strains *nd*, including *nd6* and *nd7*, and particularly also the temperature-sensitive strain *nd9* (Beisson et al. 1976). Cells type *nd9* cultivated at 18 °C are able of exocytosis performance, in contrast to aliquots cultivated at a nonpermissive temperature of 28 °C, although cells from permissive and nonpermissive temperatures, respectively, can all dock their normal-shaped trichocysts (Beisson et al. 1976). Morphologically, *nd9*-18 °C and *nd9*-28 °C cells differ by the presence or absence of "connecting material" between trichocyst and cell membrane (Beisson et al. 1980; Plattner et al. 1980).

Strain *nd9* cells have been used to scrutinize the role of SNARE protein assembly by interaction of the SNARE chaperone, NSF, for acquirement of exocytotic membrane fusion capacity (Kissmehl et al. 2002). In these experiments, *NSF* gene silencing was combined with transition from nonpermissive to permissive temperature. This study showed, by freeze-fracture appearance, the transformation of preformed trichocyst docking sites at unoccupied sites to occupied, mature exocytosis sites ("oval" → "ring" transformation). For details, see section "Exocytotic membrane fusion". This gave us a rare chance to verify the involvement of NSF in SNARE assembly, i.e. during maturation of exocytosis sites. This is in contrast to the more general assumption of a role of NSF in SNARE disassembly, as outlined in more detail elsewhere (Plattner 2010b). The *nd9* protein contains armadillo repeats (Froissard et al. 2001) and, thus, has brought into play proteins that have not been envisaged in any more detail up to now.

What makes trichocysts a special type of secretory organelles?

Trichocysts are very special secretory organelles because of several reasons. First, crystallinity of their contents is an unusual feature. Trichocysts contain up to ~50% of the cell protein (Bilinski et al. 1981b; Matt et al. 1978) in crystalline form with maximal density packing. In vertebrates, crystallinity of secretory products is not the rule, although it also occurs. Examples range from amphibian yolk granules (Lange 1985) to hormone storing vesicles in mammalian neuroendocrine cells (Arrandale and Dannies 1994; Han et al. 1999). Second, the main secretory components of trichocysts are a collection of quite similar proteins encoded by numerous paralogs, as stated above (Arnaiz et al. 2010; Madeddu et al. 1995). This serves for gene amplification and the resulting number of paralogs, or ohnologs (when derived from whole genome duplications), is even much larger than that of other genes in *Paramecium* (Ladenburger and Plattner 2011; Plattner 2010b); it even exceeds the amazing number of K^+-channel encoding genes (Haynes et al. 2003). It, thus, recalls storage proteins of corn, *Zea mays*, called zeins a paradigm for such amplification effects (Song et al. 2001; Wilson and Larkins 1984); their large α-helical configuration (Momany et al. 2006) also recalls the major trichocyst proteins, tmp's (Gautier et al.

1996). Third, trichocysts display an unusual structural and functional polarity, as their bipartite composition with a "body" and a "tip" part (Bannister 1972) is unusual, the tip mediating docking at the cell membrane (Beisson et al. 1976; Pouphile et al. 1986). Fourth, the high speed of stimulated trichocyst release, which is the fastest known dense core-secretory organelle exocytosis process, as well as its synchrony are without precedent (Knoll et al. 1991a; Plattner 2014a; Plattner and Kissmehl 2003). Fifth, also a mechanical, defensive function (Harumoto and Miyake 1991), driven by luminal Ca^{2+} binding, is an unusual secretory function (Bilinski et al. 1981a; Klauke et al. 1998). Sixth, release of lectins contained in secretory vesicles is another rare observation, occurring in few cells, e.g. in chicken Goblet cells (Beltrame et al. 2015; Beyer and Barondes 1982). Normally only stationary lectins, integrated in the early secretory pathway and serving for protein folding control, belong to the standard inventory of eukaryotic cells (Hauri et al. 2002).

However, there are also many features in common with many other secretory systems, such as proteolytic processing and glycosylation of contents as well as requirement of SNAREs for docking and of a cytosolic Ca^{2+} signal for membrane fusion to occur (Plattner 2010b, 2014a). Focal ("point") fusion, seen during trichocyst release, was for some time at odds with EM analyses of other systems, but finally became a standard model for exocytotic membrane fusion, as reviewed recently (Plattner 2014b). To exploit the unsurpassed degree of dense core-vesicle (trichocyst) exocytosis, EM analysis was often combined with rapid freezing (cryofixation), eventually in combination with a newly developed quenched-flow apparatus (Knoll et al. 1991a). For further processing, freeze-substitution or freeze-fracture analysis was also applied. Quenched-flow and freeze-substitution under conditions allowing for retention of Ca^{2+} at its specific intracellular sites, also during stimulation-induced Ca^{2+} redistribution, was combined with analytical EM methods. Besides electron spectroscopic imaging (ESI), this included calibrated energy-dispersive X-ray microanalysis (EDX), combined with scanning transmission EM imaging. Both, ESI and EDX visualize total Ca^{2+} (free and bound) and, thus, are appropriate to register calcium in stores and its change upon stimulation. Another option was immuno-EM localization of sensitive antigens, eventually after rapid freezing (cryofixation) and freeze-substitution (Momayezi et al. 1993).

BIOGENESIS AND ULTRASTRUCTURE IN A CORRELATIVE VIEW

Trichocyst precursor structures are believed to originate from the endoplasmic reticulum, to pass through the Golgi apparatus, and to undergo a maturation process (pretrichocysts) before mature trichocysts are delivered to the cell membrane. This has been documented by Garreau De Loubresse (1993) when she followed trichocyst biogenesis, using antibodies against trichocyst proteins, in a time-sequence study after depleting paramecia of trichocysts. Similar details have been reported for *Pseudomicrothorax dubius* (Peck et al. 1993). In a transcriptome analysis after depleting *Paramecium* cells of trichocysts by AED stimulation (Fig. 3), followed by synchronous de novo biogenesis, 118 out of a total of 176 tmp genes have been upregulated, including proteins with signal peptide sequences, as required for cotranslational sequestration (Arnaiz et al. 2010). This is a transient response somehow assigned to the ratio of occupied and unoccupied trichocyst docking sites (Galvani and Sperling 2000). This regulatory aspect is of basic interest for future work in cell biology insofar as one now can ask for the unknown molecular signals beyond Ca^{2+} that keep the balance between actually available and finally required numbers of a specific organelle.

Some trichocyst content proteins are proteolytically trimmed. In the section "Glycoproteins and secretory lectins", lectin labeling indicative of core and peripheral glycosylation will be discussed. Also, during maturation, a tip structure is differentiated, so that the spindle-shaped organelle looks bipartite, with inherent polarity.

Interestingly, a sufficient basal cytosolic Ca^{2+} level is required for early biogenetic stages (section "Ca^{2+}-binding proteins"), in contrast to later stages where luminal Ca^{2+} would compromise normal function by causing intracellular decondensation (Bilinski et al. 1981a). Mature trichocysts are transported in a saltatory manner (Aufderheide 1978a) to the cell membrane where they are docked, thus becoming available for exocytotic release upon stimulation. Multiple mutants are available for many of these individual steps, frequently with aberrant morphology (Gogendeau et al. 2005; Pouphile et al. 1986; Vayssié et al. 2000).

Early trichocyst precursors contain a growing mass of electron dense secretory materials. Such pretrichocysts elongate, while their luminal space is successively filled with crystallizing secretory materials (Garreau De Loubresse 1993). With careful preparation methods, i.e. fast freezing followed by freeze-substitution, delicate links become visible between the trichocyst matrix and the membrane (Momayezi et al. 1993); see Fig. 6d and also see section "Trichocyst membrane", below. These links normally break and then can contribute to the "beaded" or "meshlike sheath". This is attached to the trichocyst matrix, yet without contact to the membrane (Anderer and Hausmann 1977; Bannister 1972; Fok et al. 1988). These matrix-membrane connections are stained by monoclonal antibodies, combined with peroxidase label; in the EM, these antibodies also stain the Golgi apparatus (Momayezi et al. 1993). Such labeling is less evident with the use of section labeling using gold conjugates (Vayssié et al. 2001). On Western blots, our monoclonal antibodies recognize bands about twice as large as those stained in the work by Fok et al. (1988). Therefore, they could represent a dimer/monomer situation, if not omission and application, respectively, of reducing agents were in adverse. Disregarding this uncertainty, one can assign a novel identity to the mesh-like structure as it connects matrix contents and membranes in the trichocyst body.

Other hints supporting passage through the Golgi come from glycosylation studies with *P. tetraurelia* and

molecular biology studies with *T. thermophila* mucocysts. The occurrence of glycosylation of some of the tmp's has been ascertained by using labeled lectins (Allen et al. 1988; Glas-Albrecht et al. 1990; Lüthe et al. 1986). This, together with the signal recognition peptide (Arnaiz et al. 2010), posttranslational cleavage (Adoutte et al. 1984; Gautier et al. 1994; Shih and Nelson 1991, 1992) and binding of appropriate lectin (section "Glycoproteins and secretory lectins") is compatible with a classical biogenetic pathway for trichocysts, from endoplasmic reticulum on to mature secretory organelles. Moreover, secretory lectins were seen to be contained in the peripheral part of the tip from where they diffuse upon discharge (Haacke-Bell and Plattner 1987). See section "Glycoproteins and secretory lectins".

Furthermore, recent analysis discovered that 23 genes associated with the transport from the endoplasmic reticulum to Golgi vesicles are upregulated when cells are induced to synthesize a new set of trichocysts (Arnaiz et al. 2010). Accordingly, the membrane of trichocysts also exhibits a rather complex protein profile, as shown in biotinylation studies (Fig. 6c). Vesicles trafficking from the endoplasmic reticulum to the Golgi show the usual smooth coat, made of COPs (Rothman 2014) also in *Paramecium* (Garreau De Loubresse 1993). The type of vesicles budding from the Golgi apparatus, including smooth and bristle-coated vesicles, is not very clear (Garreau De Loubresse 1993; Vayssié et al. 2001), particularly since some of the latter contain acid phosphatase activity and, thus, are involved in the biogenesis of lysosomes (Fok et al. 1984). However, after stimulation of massive trichocyst release by AED, not only more smooth vesicles, but also more bristle-coated vesicles appear at the trans-Golgi side (Garreau De Loubresse 1993). Their involvement in pretrichocyst formation is supported by ultrastructural evidence from *P. dubius* where vesicles with clear and dense contents fuse to finally form mature trichocysts (Peck et al. 1993). Whether bristle-coated vesicles would also transport lysosome-like constituents to trichocysts (as outlined below) remains unclear.

From the Turkewitz group (Chicago), we know much more about molecular corollaries of the biogenesis of mucocysts than we know about trichocysts. Only a few crucial aspects are tentatively discussed here with a side glance on *Paramecium*'s trichocysts. It is important to note that *Tetrahymena* mucocysts and *Paramecium* trichocysts share several molecular similarities and the simplest hypothesis is that they both derive from a secretory granule present in a common ancestor. Evidence for this includes the fact that genes encoding either membrane or luminal proteins of trichocysts or mucocysts, in *P. tetraurelia* and *T. thermophila*, respectively, generally have clear orthologs in the other species. On this basis, it is reasonable to expect that mucocysts and trichocysts rely to a large extent on shared mechanisms for their biogenesis, although there will be differences in several details, as can be recognized by comparing the papers by Vayssié et al. (2001) and Bradshaw et al. (2003). One morphological difference is that trichocysts have a clear tip structure that is absent in mucocysts. However, at least one abundant mucocyst luminal protein, Grt1, shows a highly polarized distribution, concentrated at the mucocyst end that corresponds functionally to the trichocyst tip, i.e. the end that docks at sites of exocytosis (Bowman et al. 2005a,b).

The most suggestive finding regarding mucocyst biogenesis is that it relies on a receptor in the VPS10 (Vacuolar Protein Sorting)/sortilin family (Briguglio et al. 2013). In general, sortilins appear to be a family of evolutionarily old receptors for transporting proteins to lysosome-related organelles (Canuel et al. 2009), such as budding yeast vacuoles or lysosome-related secretory organelles in *Toxoplasma* (Ngô et al. 2004; Sloves et al. 2012). Specifically, the role of Sor4p suggests that mucocysts also rely on lysosome-related mechanisms for their synthesis. The expansion of the Sor family occurred prior to the split between *T. thermophila* and *P. tetraurelia* (A. Turkewitz, personal communications). Thus, the phylogenetic analysis suggests that the *P. tetraurelia SOR4* ortholog is likely to be active in trichocyst biogenesis. Whether additional genes and mechanisms underlying lysosome-related organelles are also pertinent to mucocysts/trichocysts remains to be investigated. One interesting question is whether biogenesis of secretory organelles in ciliates includes an endocytotic component, as has been postulated in *Toxoplasma* (Ngô et al. 2004). At present, this has never been observed for trichocysts (H. Plattner, unpublished observ.). Importantly, the analysis of *SOR4* knockout cells suggested that more than one mechanism is involved in luminal protein targeting to mucocysts, since the delivery of Grl proteins was *SOR4* independent (Briguglio et al. 2013). It had previously been shown that the Grl proteins tended to form large aggregates in the secretory pathway, while the Grt proteins showed no tendency to aggregate (Rahaman et al. 2009). Thus, one possibility is that the Grl proteins are sorted on the basis of coaggregation, while sorting of Grt proteins depends on a classical receptor.

For mucocysts as for trichocysts, evidence supports the transport of proteins through the classical secretory pathway. The specific steps involved likely include orthologs of the same genes that are upregulated during trichocyst biogenesis; some 27 genes are indicative of "signal recognition particle-dependent cotranslational protein targeting to membrane" (Arnaiz et al. 2010). The most abundant mucocyst cargo proteins form heterooligomers in the endoplasmic reticulum (Cowan et al. 2005). Following transport through, and exit from the Golgi apparatus, mucocyst cargo proteins, like those of trichocysts (Adoutte et al. 1984; Madeddu et al. 1995), undergo obligatory proteolytic processing during a maturation stage (Collins and Wilhelm 1981; Ding et al. 1991; Turkewitz et al. 1991; Verbsky and Turkewitz 1998). The processing recognition sites are as yet poorly defined, but processing in maturing mucocysts showed surprising independence of local amino acid sequence and therefore might be based primarily on accessibility (Bradshaw et al. 2003). That possibility had already been raised in the context of a model for tmp protein structure in *Paramecium* (Gautier et al. 1996). It should be noted, however, that tmp and

Grl proteins, in *Paramecium* and *Tetrahymena*, respectively, are not closely related, though they are rich in predicted short α-helices and share very short amino acid motifs near their respective proteolytic processing sites (Verbsky and Turkewitz 1998). The *Tetrahymena* genome also encodes genes that are much closer homologs to tmp's (A. Turkewitz, unpubl. observ.), but these have not been characterized as yet.

Which mechanisms may govern biogenesis of trichocysts? Considering the biogenesis of the trichocyst body, one first sees vesicles involved in the formation of pretrichocysts containing amorphous electron dense contents. Two types of vesicles deliver immunologically different tmx proteins to the same precursor organelle (Vayssié et al. 2001) where different gold-labeled antibodies localize to the core and the peripheral part, respectively (Garreau De Loubresse 1993; Hausmann et al. 1988; Shih and Nelson 1991; Vayssié et al. 2001). The trichocyst core then condenses increasingly to highest packing density in a crystalline state. Again, similar observations are reported from *P. dubius* (Peck et al. 1993) and, thus, may be a general rule. That crystallization fails in docking-defective *Paramecium* mutants underscores its relevance for biogenesis and maturation to docking-competence (Pouphile et al. 1986; Vayssié et al. 2000, 2001). May there operate a mechanism similar to that in metazoans? Here, principles of dense core-secretory vesicle formation encompass different aspects: First, intraorganellar Ca^{2+} can cause aggregation of some peptide hormones (Canaff et al. 1996). However, we did not get aware of any Ca^{2+} enrichment in trichocysts above detection level in EDX analyses (Hardt and Plattner 2000; Schmitz and Zierold 1989). However, low intraorganellar $[Ca^{2+}]$, though undetectable, may be required for trichocyst biogenesis. This can be postulated not only from the stabilizing effect of Ca^{2+} on β-crystallin proteins in the trichocyst matrix (see "Basic features of trichocyst biogenesis"), but also from the reduced number of trichocysts at low $[Ca^{2+}]_o$ (Ladenburger et al. 2009) to which $[Ca^{2+}]_i$ is rather tightly coupled in *Paramecium* (Plattner 2014a). Second, aggregation of different protein components within the trans-Golgi network and subsequent budding into different transport vesicles occurs in some systems (Sobota et al. 2006). Evidently, this applies to tmx proteins as they are delivered in different vesicles (Vayssié et al. 2001). Third, in mammalian cells, acidification can cause aggregation of specific membrane proteins, e.g. a carboxypeptidase E, with proteohormones at acidic pH (Rindler 1998). Acidification of the vesicle lumen is also required, e.g. for proinsulin to insulin transformation (Orci et al. 1987). This is different in pretrichocysts which are not acidic (Garreau De Loubresse et al. 1994), as outlined below. In *T. thermophila*, a carboxypeptidase (requiring detailed enzymatic characterization) is coexpressed with Grl mucocyst components (Kumar et al. 2014). This, in conjunction with endopeptidases, was assumed to be possibly relevant for core maturation (Verbsky and Turkewitz 1998), although in a later study a knock-out strain displayed a wildtype secretory response (Kumar et al. 2014). Considering the occurrence of carboxypeptidases in the *P. tetraurelia* database (while keeping in mind important deviations between trichocysts and mucocysts), all these components known from mucocysts now call for analysis in *Paramecium*. The aim is to pinpoint factors potentially controlling trichocyst protein processing. Fourth, the connection of some specific proteins of the secretory contents to some specific membrane components is a sorting principle (Dikeakos and Reudelhuber 2007), thus recalling the matrix-membrane connections seen in *Paramecium* by immuno-EM (Momayezi et al. 1993) (see "Biogenesis and ultrastructure"). In conclusion, several basic mechanisms may contribute to the biogenesis of trichocysts in *Paramecium* cells. However, a number of additional aspects discussed in the literature for higher eukaryotes (Gondré-Lewis et al. 2012; Weisz and Rodriguez-Boulan 2009) also remain to be investigated in ciliates.

Just like with *Paramecium* trichocysts (Pouphile et al. 1986; Vayssié et al. 2000), transformation of amorphous matrix contents into a crystalline-like core in *Tetrahymena* mucocysts is paralleled by exocytosis competence (Bowman et al. 2005b). Yet work with *Tetrahymena* suggests that the situation is more delicate: While this transformation is normally achieved by the activity of an aspartyl cathepsin (Kumar et al. 2014), mutation in a cysteine cathepsin 4, ΔCthp, inhibits decondensation of the mucocysts in despite of their endowment with a crystalline core (Kumar et al. 2015). No such details are known from trichocysts as yet.

In *Paramecium*'s trichocysts, the pro-Grl1p to Grl1p transition known from mucocysts (Verbsky and Turkewitz 1998) may have its equivalent in the cleavage of tmp's. Possibly depending on this process, the trichocyst tip may undergo self-assembly. All this is mandatory for acquiring docking-competence also by trichocysts (Madeddu et al. 1994). As mentioned, in *Tetrahymena* this includes unilateral assembly of one of the non-Grl (granule lattice) proteins and of Grt1p (Bowman et al. 2005b) and is mirrored by the polar assembly of a well-defined trichocyst tip in *Paramecium* (Pouphile et al. 1986). Mislocation of structural elements at the flanks of the matrix, rather than in the tip, in some secretory mutants (*tam* and some *nd* strains) of *P. tetraurelia* (Pouphile et al. 1986), indicates mutual biogenetic interdependency between the two structures, matrix and tip. Mislocation causes incompetence for intracellular transport and release.

For trichocysts, more details of biogenesis still have to be substantiated at the molecular level. For instance, the tip contains a central core with periodic banding (which, however, does not expand during exocytosis), flanked by robust electron dense structures, and a most peripheral part. The latter is made of fluffy material containing secretory lectins (Haacke-Bell and Plattner 1987); see section "Glycoproteins and secretory lectins".

Neither trichocysts in statu nascendi (Garreau De Loubresse et al. 1994), nor the mature organelles (Lumpert et al. 1992; Wassmer et al. 2009) show any sign of

acidification quite in contrast to many other secretory organelles up to mammals (Forgac 2007; Mellman 1992). Also, the presence of free luminal Ca^{2+} in trichocysts is unlikely, as Ca^{2+} would cause decondensation (stretching) of the matrix, at least in mature trichocysts (Bilinski et al. 1981a). In contrast, many types of secretory vesicles contain Ca^{2+} in concentrations well detectable by EDX (Hay 2007; Nicaise et al. 1992). How can this be reconciled with the requirement of Ca^{2+} and acidic pH by secretory proprotein cleaving proteases (Davidson et al. 1988)? Use of the H^+/Ca^{2+} antiporter monensin, routinely used to break down luminal acidification (Mollenhauer et al. 1990), would be advised. Its additional activity as a Na^+/Ca^{2+} antiporter would also take into account the high Na^+ and low Ca^{2+} content measured in cryosections of trichocysts by EDX (Schmitz and Zierold 1989). In *Tetrahymena* monensin inhibits processing of the large mucocyst precursor proteins to the small-sized secretory material (Ding et al. 1991). The efficiency with *Paramecium* trichocysts was less convincing, as it worked less with the wildtype, but preferably with the *tam38* strain (Adoutte et al. 1984; Garreau De Loubresse 1993). This mutant does not process the contents of its trichocysts which display aberrant ultrastructure and which are not docked. (However, the *tam38* mutation entails pleiotropic effects; Ruiz et al. 1976). Ca^{2+} may be relatively higher in early stages of trichocyst processing and then kept considerably lower, i.e. below detection limit in EDX.

In summary, there are open questions concerning the conversion of pretrichocyst to mature trichocyst contents. In the mature organelle, the tmp proteins are arranged in a regular, crystalline pattern, causing crossbanding in the EM and allowing for explosive recrystallization upon release. There are hints as to the importance of luminal Ca^{2+} at early stages of biogenesis. A mechanism to keep Ca^{2+} low in mature trichocysts is discussed in the section "Are trichocysts acidic compartments?".

TRICHOCYST DOCKING

Intracellular transport

In *Paramecium*, mature trichocysts undergo cyclosis, i.e. they travel through subcortical regions in a steady flow with a velocity of several $\mu m \times s^{-1}$ (Sikora 1981) until they suddenly seem to be caught like by an invisible "hand". Then, they approach in a saltatory manner, tip first, a docking site at the cell membrane (Aufderheide 1978a,b). Thereby, trichocysts are guided by the very same "hand". This is a microtubule emanating from a nearby ciliary basal body (Glas-Albrecht et al. 1991; Plattner et al. 1982) which always is situated ~0.5 to 1 μm from a docking site proper. As mentioned, in wildtype cells, the organelles display inherent polarity, in contrast to some mutants (Pouphile et al. 1986). This polarity is prerequisite for migration in plus-to-minus direction along a guiding microtubule. Similar behavior was found later on also in some mammalian canine kidney epithelial cells (Bacallão et al. 1989; Buendia et al. 1990) and in T-lymphocytes (Griffiths et al. 2010). In most metazoan cells, however, dense core-secretory organelles are transported along microtubules in minus-to-plus direction (Kelly 1990) because in these cells microtubules emanate from the cytocenter/centrosome. Interestingly, the usual minus → plus directionality is maintained when chromaffin granules, isolated from bovine adrenal medulla, are microinjected into *Paramecium* cells; here they aim toward the plus end, as they would in their cells of origin, but in *Paramecium* this leads them away from the trichocyst docking sites at the cell periphery (Glas-Albrecht et al. 1991).

Docking at the cell membrane

After arriving at the cell membrane, a trichocyst sways for a short time (Aufderheide 1978a,b); thereby a "plug" of proteins occurring at unoccupied docking sites is transformed into an electron dense coating over the trichocyst tip (Pouphile et al. 1986). This includes diffuse materials connecting trichocyst tip and cell membrane which, therefore, has been called "connecting material". At this site, membrane-integrated proteins, such as SNAREs and Ca^{2+}-sensitive proteins, become assembled, as discussed below. If such assembly fails, docking does not entail competence for exocytotic membrane fusion. This has been evidenced with the temperature-sensitive mutant, *nd9* (Beisson et al. 1980; Froissard et al. 2001), by silencing the SNARE-specific chaperone, NSF, during transition from the nonpermissive to the permissive temperature (Froissard et al. 2002). All this corresponds to the general requirement of membrane-to-membrane docking and fusion (Rothman 2014; Südhof 2014).

For docking to occur, one has to postulate the interference of small GTP-binding proteins, i.e. GTPases, as to be expected from other systems and as previously discussed (Plattner 2015c). When a battery of GTPases was expressed as green fluorescent protein (GFP) fusion proteins, some labeling near mucocyst docking sites has been observed, but no labeling of mucocysts proper when living *Tetrahymena* cells were analyzed (Bright et al. 2010), possibly because in vivo association of GTPases is transient. The postulate of GTPase involvement is supported by ^{32}P-GTP labeling applied to overlays prepared from electrophoresis gels from wildtype *P. tetraurelia* trichocysts and its absence from some secretory mutant trichocysts (Peterson 1991).

Trichocyst membrane

Cell fractionation methods have been developed to isolate intact trichocysts with their membrane envelope (Glas-Albrecht et al. 1992), or of their contents in condensed or decondensed state (Bilinski et al. 1981b; Matt et al. 1978). A method has been developed to separate trichocyst membranes from contents in purified form (Glas-Albrecht et al. 1992); Fig. 6b,c. This has been combined with enzymatic measurements (to monitor purity of fractions; Bilinski et al. 1981b), with sodium dodecylsulfate polyacrylamide gel-

electrophoresis (SDS-PAGE) under nonreducing or reducing conditions, with Western blots using antibodies against trichocyst components (Bittermann et al. 1992; Glas-Albrecht et al. 1990, 1992) and with application of exogenous lectins to analyze glycosylation (Allen et al. 1988; Glas-Albrecht et al. 1990; Lüthe et al. 1986).

Trichocyst membranes have a relatively high proportion of lipid. A spectrum of membrane proteins, as determined by biotinylation and streptavidin-peroxidase labeling (Fig. 6c), ranges from 14 to > 105 kDa. (This will also include some proteins of the luminal side since leakiness of a few trichocysts cannot be excluded.)

The trichocyst membrane contains 3-hydroxysterols, as visualized in freeze-fractures where filipin binding sites were visualized (K. Olbricht and H. Plattner, unpublished observ.). As *Paramecium* cells are auxotrophic for such sterols (Whitaker and Nelson 1987), they either have to be provided in pure form or as in our filipin experiments in a salad decoction. We exposed cells to filipin, a polyene-macrolide compound selectively binding to 3-OH-sterols (Gimpl and Gehrig-Burger 2007). Filipin was known already to bind to membrane components of *Paramecium* (Kaneshiro et al. 1983). In freeze-fractures, filipin forms the typical wart-like structures in the trichocyst membranes (Fig. 6e). These structures resemble those found after filipin exposure in various membranes of *Tetrahymena* (mucocysts not having been analyzed; Sekiya et al. 1979). Labeling of trichocyst membranes is compatible with enrichment of binding sites along the secretory pathway up to secretory organelle membranes in higher eukaryotes (Hannich et al. 2011), from yeast (Klemm et al. 2009) to mammals (Kim et al. 2006). Here, carboxypeptidase localization and its contribution to directing secretory protein substrates to the stimulated secretory pathway depend on cholesterol-rich domains in the organelle membrane (Dhanvantari and Loh 2000). However, considering the aberrant sterol pathway in ciliates (Tomazic et al. 2014) specification of actual binding sites remains another aspect for further scrutiny.

Immuno-peroxidase EM labeling in situ revealed calmodulin along the cytosolic face of trichocyst membranes where it covers the entire trichocyst "body" part, but labeling was particularly strong over the tip part at, and near docking sites (Momayezi et al. 1986). As outlined in the section "Exocytotic membrane fusion", this is in line with the established regulatory role of calmodulin in SNARE assembly at the docking sites of secretory vesicles. The local SNARE is likely *Pt*Syb5, as derived from knockout experiments combined with immuno-gold EM labeling (Schilde et al. 2010).

Silencing in various H^+-ATPase subunits resulted in a significant reduction of the number of docked trichocysts, although in the trichocyst membrane H^+-ATPase components become visible only when expressed as GFP fusion proteins (Wassmer et al. 2005, 2006). Intriguingly, silencing not only of some subunits of the H^+-ATPase (Fig. 7), but also of a Ca^{2+}-release channel type inositol 1,4,5-trisphosphate receptor ($InsP_3R$) (Ladenburger et al. 2009) inhibits trichocyst biogenesis (Fig. 8), in contrast to a ryanodine receptor-like channel. This is surprising since neither acidification nor Ca^{2+}-release channels are recognized in the trichocyst membrane (Lumpert et al. 1992, Ladenburger and Plattner 2011). By analysis of genes upregulated in consequence of massive mucocyst exocytosis in *Tetrahymena*, sequences of a K^+-dependent Na^+/Ca^{2+} exchanger have been found (Haddad et al. 2002). Although its localization is not known, one may speculate for *Paramecium* that, in the absence of a Ca^{2+}-pump, this could account for the clearing of Ca^{2+} from the lumen of trichocysts. As an alternative, H^+/Ca^{2+} exchange, as outlined in the section "Are trichocysts acidic compartments?", may be envisaged in future work. Rapid consumption of the ΔH^+ would explain the absence of any visible acidification.

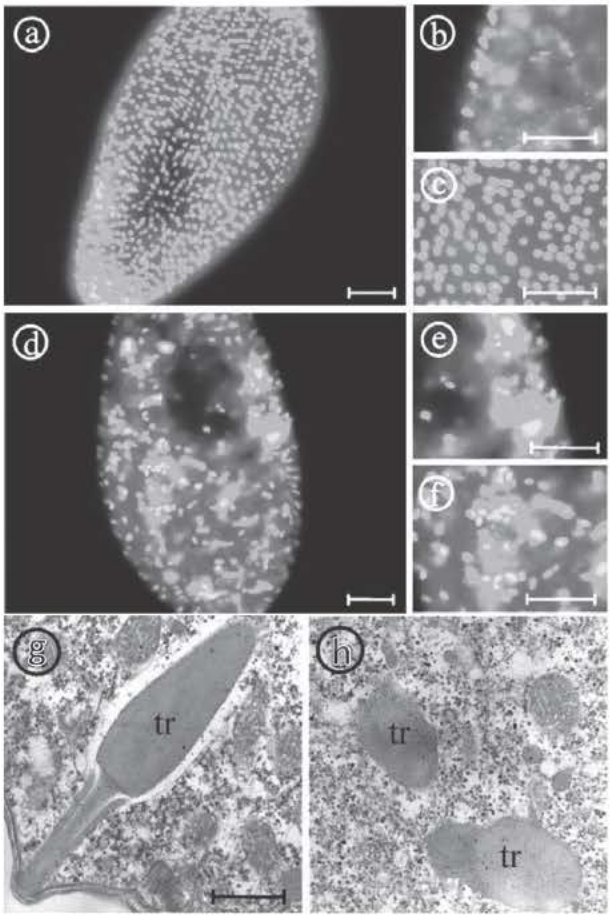

Figure 7 H^+ ATPase c subunits are required for trichocyst biogenesis in *Paramecium tetraurelia* wildtype cells. (**a f**) Cells transformed with trichocyst matrix protein, TMP1b, fused to green fluorescent protein. (a c) Control cells, without c subunit silencing, (d, f) cells after silencing of all c susbunits. Note considerable distortion and reduction of the number of trichocysts after c subunit silencing. (**g**) Electron micrograph obtained with unsilenced cells and (**h**) after H^+ ATPAse c subunit silencing, respectively. Note in (h) heavily distorted trichocysts (tr) in the cytoplasm after silencing. Scale bars 10 μm (a f), 1 μm (g, h). From Wassmer et al. (2005).

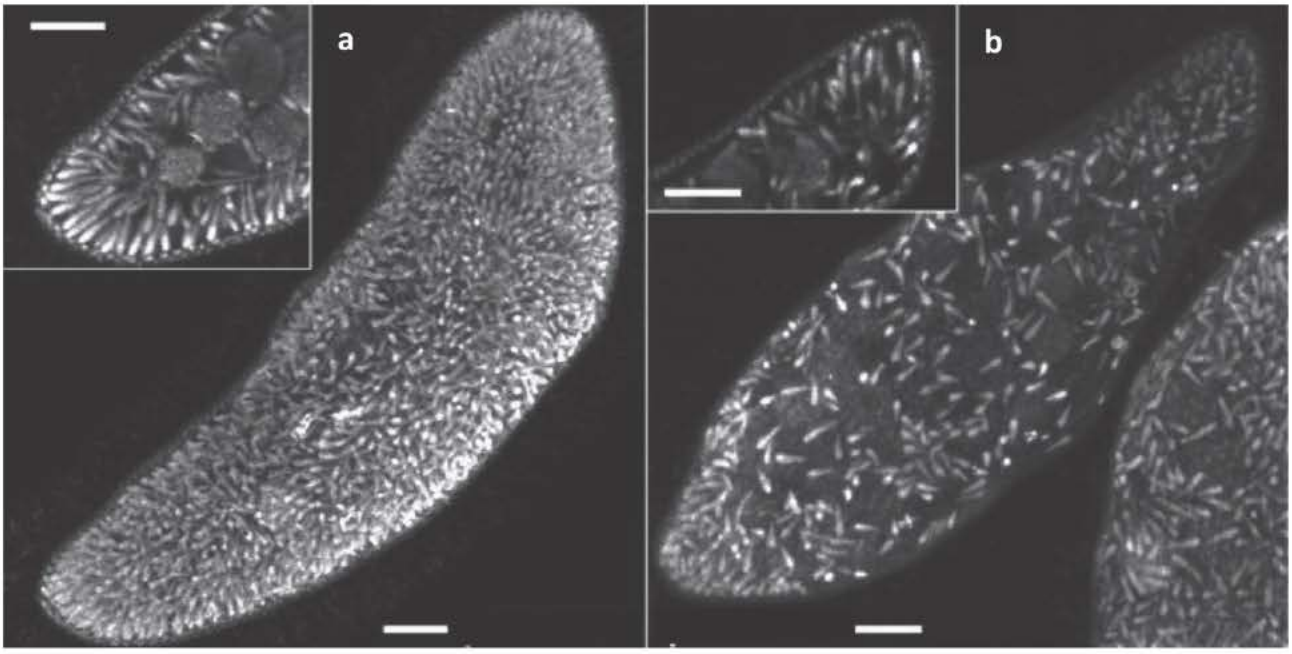

Figure 8 Relevance of Ca^{2+} release channels, type CRC II/$InsP_3R$ for trichocyst biogenesis in *Paramecium tetraurelia* wildtype cells. Trichocysts labeled with an antibody against trichocyst matrix protein, TMP4 (kindly provided by Linda Sperling, CNRS, Gif sur Yvette). (**a**) Silencing of CRC IV/RyR LP by the construct pPD C1C2, which has no effect on appearance and abundance of trichocysts. (**b**) Heavy reduction and distortion of trichocysts after silencing of CRC II/$InsP_3R$. Insets are enlargements from median optical sections. Scale bars 10 μm. From Ladenburger et al. (2009).

Some trichocyst membrane proteins are glycosylated (Glas-Albrecht et al. 1990). As mentioned, monoclonal antibodies can recognize delicate links between trichocyst contents (matrix) and the membrane (Momayezi et al. 1993); see Fig. 6d and section "Biogenesis and ultrastructure"). On Western blots, this antibody stained a band of ~60 kDa in membranes and soluble materials from trichocysts, as well as a band of ~70 kDa in membranes and whole trichocysts. The proteins under consideration are, therefore, different from the main secretory proteins. Such links between secretory contents and secretory vesicle membrane are known from mouse corticotrope cell-derived AtT-20 cells (Hosaka et al. 2004) and from other hormone producing cells (Dikeakos and Reudelhuber 2007) where they are thought to be engaged in sorting during organelle biogenesis. Matrix-membrane links in trichocysts may as well act as a signal indicating whether a trichocyst is full or empty and, thus, contribute to exo-endocytosis coupling, as discussed in section "Exocytotic membrane fusion".

TRICHOCYST MATRIX

Biochemical methods applied were as outlined above for trichocyst membranes. In addition, Ca^{2+}-binding as well as binding of lectins (for glycosylation assays) and of synthetic glycoproteins (neoglycoproteins, for monitoring secretory lectins) has been investigated.

Ca^{2+}-binding proteins

Data obtained by EDX analysis show the absence of Ca^{2+} signals in resting trichocysts (Hardt and Plattner 2000) (though sensitivity for detectability is limited). As mentioned, trichocysts decondense explosively by about sevenfold, as soon as the extracellular medium, which usually contains (sub)millimolar Ca^{2+}, gets access to trichocyst contents through the exocytotic opening (Bilinski et al. 1981a; Schmitz and Zierold 1989).

The following observations with *P. tetraurelia* support the concept of matrix contents decondensation as a distinct step in trichocyst exocytosis. First, decondensation is achieved in vitro, when Ca^{2+} is added to demembranated trichocysts (Bilinski et al. 1981a); Fig. 4. Second, in situ, decondensation can be inhibited by chelating $[Ca^{2+}]_o$ to low levels during induction of membrane fusion by dibucaine, an agent known to mobilize intracellular Ca^{2+} by desorption from intracellular binding sites (Matt and Plattner 1983). Third, the same is achieved with established secretagogues when $[Ca^{2+}]_o$ is chelated to ~30 nM, i.e. close to resting levels of $[Ca^{2+}]_i$ (Plattner et al. 1997b). Fourth, a *P. caudatum* mutant type *tnd1*, though performing membrane fusion upon stimulation, is unable to release its trichocysts (Fig. 9). This mutant does not bind Ca^{2+} to its tmp (Klauke et al. 1998), as to be discussed in more detail below. Fifth, in the extreme, when membrane fusion is induced, at low $[Ca^{2+}]_o$, membranes are to be resealed, followed by detachment of

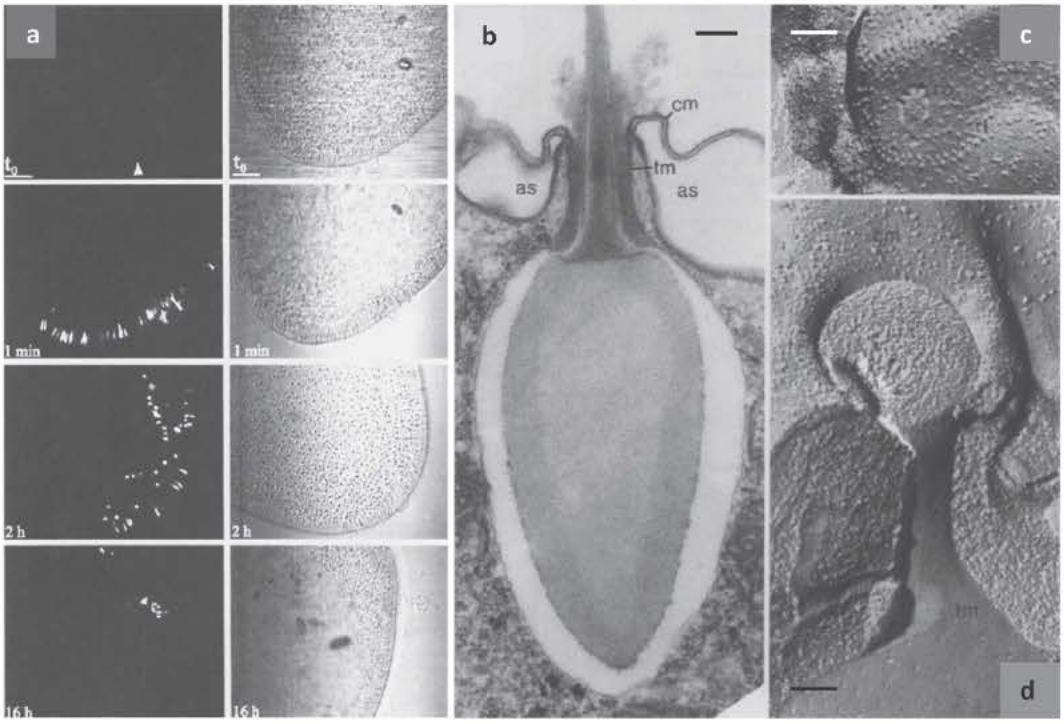

Figure 9 Membrane fusion, in the absence of trichocyst release, during stimulation of the *Paramecium caudatum tnd1* (trichocyst nondischarge) mutant by AED. In (**a**) membrane fusion is shown by F2 fluorescein isothiocyanate (FITC) labeling, added before AED stimulation (t_0), as well as 1 min, 2 and 16 h after stimulation; left: fluorescence, right: phase contrast. Note labeling of trichocysts already during short times of exocytosis stimulation, followed by increasing detachment and intracellular clumping of labeled trichocysts. (b d) Electron microscopic demonstration of membrane fusion, in the absence of trichocyst contents release, in the *P. caudatum tnd1* mutant. (**b**) Ultratin median section of an AED stimu lated cell. (**c**) Freeze fracture of a docking site before stimulation, displaying in the cell membrane a normal preformed trichocyst docking/release site encircled by a ring (ri) of intramembranous particles and a central particle rosette (ro) indicative of fusion capacity. (**d**) Freeze fracture of an exocytosis site activated by AED, showing membrane fusion and retention of the trichocyst contents. as alveolar sacs, cm cell membrane, ro "rosette" intramembranous particle aggregate indicative of exocytosis competence, tm trichocyst membrane. Scale bars 10 μm (a), 0.2 μm (b), 0.1 μm (c, d). (a d) are from Klauke et al. (1998).

trichocysts for a new secretory cycle a process called "frustrated exocytosis" (Klauke and Plattner 2000); see Fig. 10 and section "Exocytotic membrane fusion", below.

Which trichocyst content proteins are involved in the reaction to Ca^{2+}? In *P. caudatum* wildtype cells, SDS-PAGE gels analyzed by the $^{45}Ca^{2+}$ overlay technique resulted in very strong bands of 165 190 kDa and 15 19 kDa, as well as bands of medium density of 26 and 45 kDa, complemented by weak bands of 20, 24, 35, 39, and 155 kDa (Klauke et al. 1998). This likely includes some tmp's, considering an excess of negatively charged amino acids in the matrix proteins, pI 4.7 5.5 (Madeddu et al. 1995; Tindall et al. 1989). This may be crucial for Ca^{2+} binding during explosive trichocyst discharge. In the *tnd1* mutant, unable to eject its trichocysts after membrane fusion, most of these Ca^{2+}-binding bands were not detectable; only the 15 19 kDa region exhibited some weak $^{45}Ca^{2+}$ binding (Klauke et al. 1998). After silver staining, the 165 190 kDa proteins were not detectable, neither in wildtype nor in *tnd1* trichocysts. Whereas in the wildtype, the 15 19 kDa bands by far dominated over other bands, in *tnd1* cells the 45 kDa band was dominant. All this suggests that the smaller derivatives formed in the wildtype from the tmp precursor (45 kDa) most likely account for the $^{45}Ca^{2+}$ binding observed.

In conclusion, mature tmp's (15 19 kDa) can be considered as the predominant part of the Ca^{2+}-binding protein inventory which is incompletely produced in the *P. caudatum tnd1* mutant. (Furthermore, for unknown reasons the highest MW bands of 165 to 190 kDa are not visible after silver staining and they bind $^{45}Ca^{2+}$ only in the wildtype cells of *P. caudatum*.) Remarkably, Ca^{2+} precipitates tmp precursors (Shih and Nelson 1992). In mature trichocysts, mature tmp's of 15 19 kDa can perform conformational change. Depending on regularly distributed α-helical motifs in tmp proteins (Gautier et al. 1996) and Ca^{2+}-sensitivity of β-crystallin (see section "Biogenesis and ultrastructure"), respectively, conformational change of matrix proteins by Ca^{2+} binding appears the most likely way enabling the decondensation process. Remarkably, also the mature main secretory product of *Tetrahymena* binds $^{45}Ca^{2+}$ (Turkewitz et al. 1991).

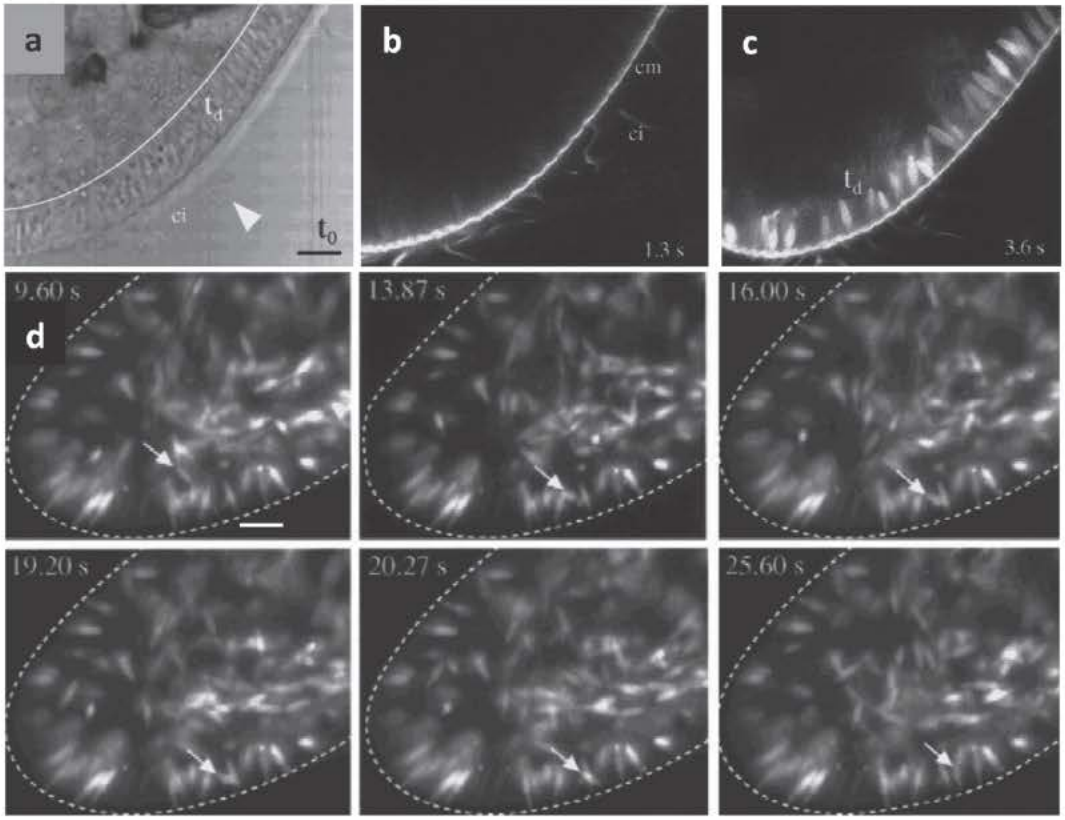

Figure 10 *Paramecium tetraurelia* wildtype cell exposed to 10 μM FM1 43 at low $[Ca^{2+}]_o$ 30 nM, showing "frustrated exocytosis". (**a**) Phase contrast. (**b**) FM1 43 causes labeling of the cell membrane within 1.3 s. (**c**) Within 3.6 s, fusion of the trichocyst membrane with the cell membrane occurs without any further stimulation. Note that the fused trichocyst membranes are stained, but that no contents are released ("frustrated exocytosis"). (**d**) Time sequence (9.60, 13.87, 16.00, 19.20, 20.27, and 25.60 s) analysis showing recycling of trichocysts after "frustrated exocytosis" and membrane resealing. *Paramecium tetraurelia* wildtype cell exposed to 10 μM FM1 43 at low $[Ca^{2+}]_o$ 30 nM, followed by 5 min exposure to 3 mM Mg^{2+} to allow for membrane resealing, followed by detachment and reattachment of labeled trichocysts in a medium supplemented with 90 μM Ca^{2+} + 100 μM Mg^{2+}. The reattachment phase of a trichocyst labeled by arrow is shown between 9.6 and 25.6 s. Scale bars 5 μm. From Klauke and Plattner (2000).

As mentioned, luminal Ca^{2+}-binding may play a role during biogenesis, e.g. for sorting contents and making them compact (assuming Ca^{2+} content would be too low for detectability by EDX; Hardt and Plattner 2000). If so, this could explain that experimental $InsP_3R$ downregulation disturbs trichocyst biogenesis (Ladenburger et al. 2009; see section "Trichocyst membrane"). Although this has not been analyzed in detail, support comes from reassembly studies with dissociated trichocyst matrix components which is stimulated by Ca^{2+} (Peterson et al. 1987b).

During exocytosis, Ca^{2+} binding causes rapid recrystallization of the matrix and quasi-explosive ejection of the secretory contents by decondensation this ultrafast contents release apparently being a privilege of *Paramecium* (Plattner and Kissmehl 2003). One may imagine a cooperative effect propagating itself throughout the trichocyst matrix once Ca^{2+} binding has started after formation of an exocytotic pore. The overall time required for comprehensive exocytosis (all trichocysts in all cells of the population analyzed by quenched-flow) is ≤80 ms (Knoll et al. 1991a). In contrast, 20 s are registered for a train of stimulated mucocyst release (Kumar et al. 2014). Evidently, requirements are different for predator defense by trichocyst expulsion and for mucocyst release in the course of capsule formation for encystment, respectively. The difference may reside in the specific arrangement of matrix proteins and their kinetics of Ca^{2+} binding. According to microkinematographic analyses, individual trichocyst discharge events required $< 10^2$times shorter times, i.e. < 1 ms (Matt et al. 1978), than the overall response in a cell (Knoll et al. 1991a).

Considering on the one hand that well over 100 genes encode a collection of quite similar tmp precursors in *P. tetraurelia* (Arnaiz et al. 2010; Madeddu et al. 1994, 1995) and on the other hand the pleiotropic changes in $^{45}Ca^{2+}$-binding bands observed with trichocysts of the *P. caudatum* mutant, *tnd1*, the mutation is unlikely to concern directly tmp-encoding genes. Which mutation can be envisaged? One possibility is a mutation in a protein involved in targeting or processing of all $^{45}Ca^{2+}$-binding secretory proteins, be they sortilin, carboxypeptidase or cathepsin (section "Biogenesis and ultrastructure"). This

is supported by the difference in precursor/mature tmp ratio in wildtype and mutant trichocysts of *Paramecium* (Vayssié et al. 2000). Hypothetically, this could also include some posttranslational modification processes, such as masking of Ca^{2+}-binding sites by amidation of acidic amino acid, considering that tmp's contain an excess of glutamic and aspartic acid (Gautier et al. 1996; Steers et al. 1969). The answer remains to be found.

Glycoproteins and secretory lectins

Soluble and insoluble secretory components of trichocyst contents have been studied. Surface biotinylation, combined with streptavidin-peroxidase labeling has been applied to intact trichocysts isolated by a newly developed method (Glas-Albrecht and Plattner 1990), to visualize the rich profile of trichocyst membrane proteins (Glas-Albrecht et al. 1992). Integrity of the membranes was checked by the absence of decondensation in response to added Ca^{2+} (Glas-Albrecht and Plattner 1990; Lima et al. 1989).

Some of the soluble trichocyst proteins of *P. tetraurelia*, bands of 76, 58, and 56 kDa, as well as some of the insoluble bands of 35, 31, and 14 kDa, bind a concanavalin A-(ConA-) peroxidase conjugate and, thus, represent secretory glycoproteins (Glas-Albrecht et al. 1990). These and some more bands have previously been detected with trichocysts isolated without their membranes, all in the range of 25 to 32.5, 36.5 to 68, 130 and 140 kDa, all labeling being inhibited by α-methylmannoside (Lüthe et al. 1986). In situ concanavalin A-FITC-(fluorescein isothiocyanate) labeled predominantly the trichocyst body, whereas *Ricinus communis* agglutinin II-(RCAII-)FITC-labeled more intensely the trichocyst tip (Fig. 11), as did RCAII-gold at the EM level (Lüthe et al. 1986). These lectins bind to α-D-mannose as well as to α-D-glucose (ConA) and to β-D-galactose as well as to β-D-galactosamine residues (RCAII), respectively. The list has been expanded for some additional lectins recognizing N-acetyl-D-glucosamine and L-fucose which stain the trichocyst tip (Allen et al. 1988). In summary, a collection of trichocyst proteins are glycosylated. Recognition by the lectins of mannose and of fucose residues, representative for core and peripheral glycosylation (at least in mammalian cells), respectively, gives some more support to a classical biogenetic pathway for trichocysts via endoplasmic reticulum and Golgi apparatus. Remarkably, staining of trichocyst tips coincides with the presence of secretory lectins in this part of the organelle (Haacke-Bell and Plattner 1987), as discussed below.

Partial glycosylation of some secretory proteins is also reported from *Tetrahymena*'s mucocysts (Attanoos and Allen 1987; Wolfe 1988), although not all of the lectin-labeled, constitutively secreted material can be strictly assigned to mucocysts (Becker and Rüsing 2003). In *Tetrahymena*, glycoproteins from mucocysts are assigned a role in capsule formation (Gutiérrez and Orias 1992; Turkewitz et al. 2000). Thus, glycosylation of extrusome contents seems to be the rule.

Similar to *Paramecium*'s trichocysts, those of *P. dubius* contain proteins of 15 22 and 28 32 kDa, which are derived from medium-sized precursors of 41 47 kDa (Eperon and Peck 1988; Eperon et al. 1993). Under reducing conditions, glycosylated bands of 30 32 kDa and up to 48 kDa have been found in the trichocysts of *P. dubius* (Eperon and Peck 1988). This includes molecules of the size characteristic of the main secretory proteins and, thus, supports the concept that some main matrix proteins are glycosylated also in *P. dubius* trichocysts. Globally, molecular size and charge are so similar between trichocysts from *P. dubius* and *P. tetraurelia* and *P. caudatum* that antisera against *P. dubius* trichocyst components crossreact with the other species (Eperon and Peck 1993).

By application of FITC- or gold-labeled artificial neoglycoproteins, e.g. artificially glycosylated bovine serum albumin, in vivo during trichocyst discharge, secretory lectins have been detected in the trichocyst tips of *P. tetraurelia* (Haacke-Bell and Plattner 1987); Fig. 11. The neoglycoproteins used recognize galactose, mannose and dextran. They are localized in the nonstructured outer layer of the trichocyst tip from where they rapidly diffuse upon exocytosis. Possible functional implications, including aggregation of bacteria (Fig. 11e), are discussed in the section "Trichocyst function".

May cyst formation have been a precursor function?

There is no serious work claiming cyst formation by *Paramecium*. Yet a side glance on its close relative, *Tetrahymena*, which is phylogenetically much older (Aury et al. 2006), appears appropriate to indicate a possibly older dedication of secretory glycoproteins and lectins in these ciliates. In *Tetrahymena*, mucocysts serve for the formation of cryptobiotic states, i.e. the cysts (Gutiérrez and Orias 1992). This can explain why mucocyst release is much slower than that of trichocysts. The mucocyst tip protein, Grt1p, is thought to serve for an adhesive function (and, thus, for a potential lectin function), as contents of Δ*GRT1* and Δ*GRT2* cells are much less adhesive than aliquots from the wildtype (Rahaman et al. 2009). Another soluble protein released from mucocysts is Igr1p (Haddad et al. 2002). These proteins have not yet been analyzed in more detail with regard to potential glycosylation and/or any lectin function. Using antibodies against a sponge lectin, a 36 kDa band was stained in *T. pyriformis* mucocysts (Kovács et al. 1997). Glycosylation of some mucocyst components may enable flocculation and crosslinking of components forming the cyst capsule. Since flocculation in water treatment systems is caused by ciliates, including *Tetrahymena* (Arregui et al. 2007), any potential glycosylation residues and secretory lectins in extrusomes may turn out to be also of practical relevance, and this may also apply to *Paramecium*. Here, soluble trichocyst proteins are also responsible for the clumping of bacteria (see "Effects of trichocyst contents on bacteria" and Fig. 11e).

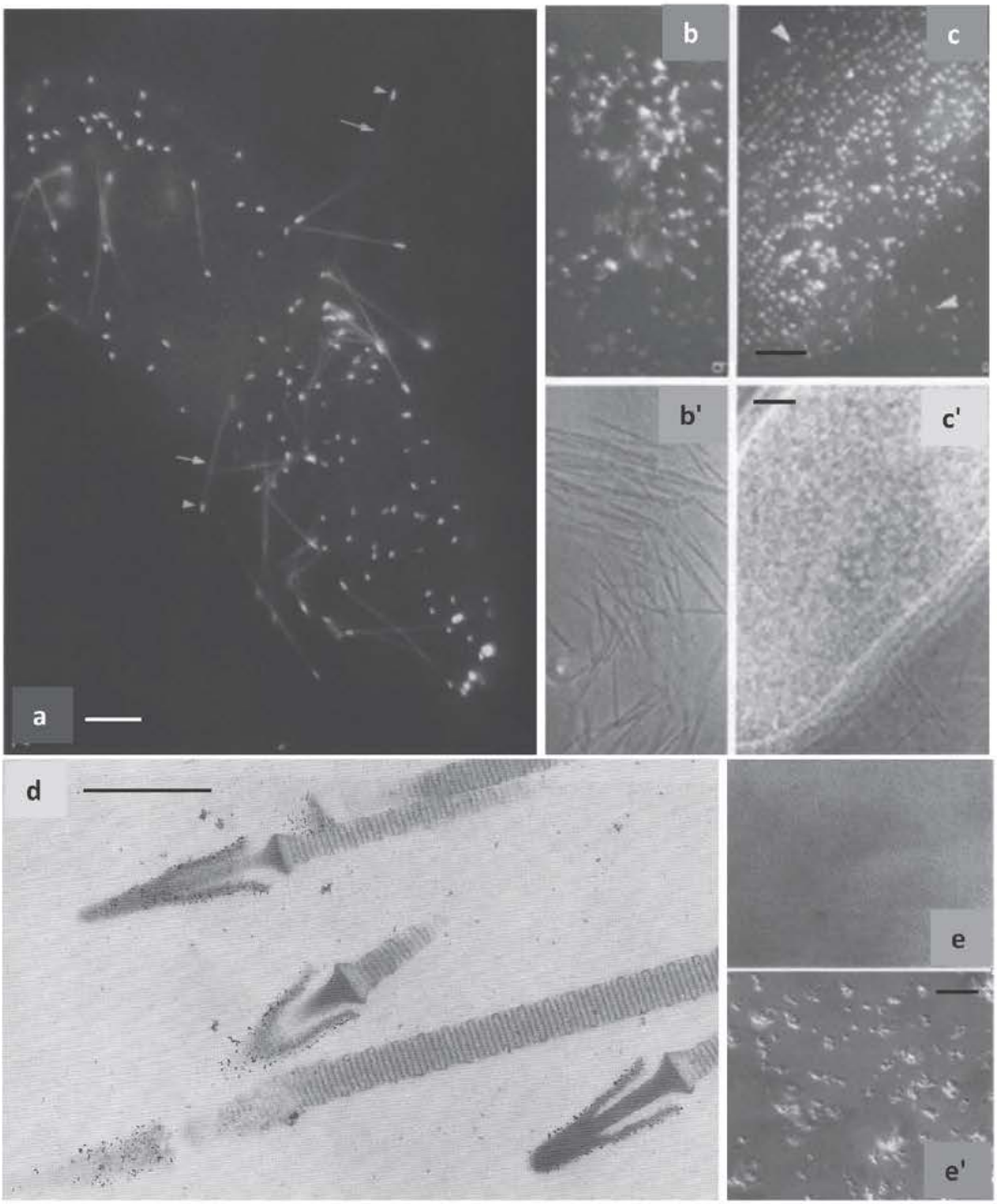

Figure 11 Secretory glycoproteins and lectins in *Paramecium tetraurelia* trichocysts. (**a**) Glycoprotein labeling of trichocyst contents in situ and after discharge by the *Ricinus communis* lectin, RCAII FITC (appropriate for staining mannosyl and glucosyl residues). Note intense labeling of the trichocyst tip (arrowheads) and less labeling of the trichocyst matrix, i.e. the shaft (arrows). (**b**,**b′**, **c**,**c′**) Gal BSA FITC (galactose coupled to FITC labeled bovine serum albumin) labeling of an endogenous lectin, in trichocysts after (b) and before stimulated exocytosis (c), respectively, together with the corresponding phase contrast images (b′, c′). Note labeling of trichocyst tips. (**d**) EM analysis of discharged trichocysts after Gal BSA gold labeling of an endogenous secretory lectin which is restricted to the fluffy outer layer of the trichocyst tip (arrowheads). (**e**, **e′**) Soluble proteins collected from isolated *P. tetraurelia* wildtype trichocysts clump bacteria, (e) without, (e′) with soluble trichocyst proteins added to *Enterobacter aerogenes* food bacteria. Scale bars 10 μm (a c, e; a′ c′, e′), 1 μm (d). (a) is from Lüthe et al. (1986), (b, c, e) from Haacke Bell et al. (1990), (d) from Haacke Bell and Plattner (1987).

ADDITIONAL TRICHOCYST COMPONENTS AND LUMINAL PH

In immuno-localization studies, several foreign proteins have unexpectedly been seen associated with the trichocyst and mucocyst matrix. An example is the serotype antigen, SerH in *T. thermophila* (Bolivar and Guiard-Maffia 1989), thus suggesting secretion via the dense core-vesicle pathway. This is unlikely considering secretion of such proteins via clear vesicles in *P. tetraurelia* under conditions avoiding antigen redistribution (Flötenmeyer et al. 1999). Similarly, acid phosphatase was localized to *T. thermophila*

mucocysts (Tiedtke and Görtz 1983), but later on this was recognized as a redistribution artifact (Tiedtke et al. 1993). The same is true of actin in trichocysts of the dinoflagellate, *Prorocentrum micans* (Livolant and Karsenti 1982) and actin in *P. tetraurelia* trichocysts (H. Plattner, unpublished observ.). In conclusion, muco- and trichocysts are very liable to avidly adsorb antigens when they get access due to the preparation protocol used. Let us consider functionally more important proteins with regard to real or artificial redistribution.

Calmodulin and chromogranin A in trichocysts?

An unexpected finding was the association of calmodulin with the trichocyst (body) matrix in *Paramecium* (Rauh and Nelson 1981). However, the inhibitory effect of calmodulin inhibitors on trichocyst release may be attributed to an effect at the membrane level, rather than to inhibition of matrix expansion (Garofalo et al. 1983). Using different affinity and immuno-labeling procedures, calmodulin in the matrix has been identified as a redistribution artifact generated by the unintentional permeabilization of the trichocyst membrane during preparation (Kersken et al. 1984; Momayezi et al. 1986). Nevertheless, our papers have then been cited as evidence in favor of the original claim when the authors themselves had disproved their former data based on microsequencing of gel bands (Tindall et al. 1989).

Chromogranin A is a component of chromaffin granule contents (Winkler et al. 1986) where it functions as a Ca^{2+}-binding protein (Reiffen and Gratzl 1986). Using antibodies against chromogranin A, reactive bands have been detected in SDS-PAGE gels from *Paramecium* trichocysts (Peterson et al. 1987a). A scaffolding function able to organize trichocyst structure has been attributed to the molecule. However, again with partial microsequencing, no evidence for the presence of chromogranin A in trichocysts has been found (Tindall et al. 1989).

Are trichocysts acidic compartments?

Similarly, the assumption that trichocysts are acid compartments (Busch and Satir 1989) was based on involuntary membrane damage. Acidity was demonstrated using membrane permeable weak bases as probes that, upon protonation, are retained in acidic organelles. In contrast to that previous study, to visualize acridine orange trapping, we applied image intensification to avoid membrane leakiness; no indication of acidity has been detected under these conditions (Lumpert et al. 1992; Wassmer et al. 2009). The same was observed with trichocyst precursor stages at the EM level, using 3-(2,4-dinitroanilino)3′-amino-N-methyldipropylamine (DAMP). (DAMP has been used as a hapten to produce antibodies for visualization by a gold conjugate; Garreau De Loubresse et al. 1994). Considering that tmp's are negatively charged (Tindall et al. 1989) because of an excess of acidic amino acid residues (Gautier et al. 1996; Steers et al. 1969), this may explain the artificial binding of the permeable positively charged probes when getting access through leaky trichocyst membranes.

As mentioned (section "Trichocyst membrane"), trichocyst membranes integrate a H^+-ATPase/GFP fusion protein (Wassmer et al. 2006) and, thus, should be able to import protons. In conjunction with the lack of Ca^{2+} in mature trichocysts, this raises the postulate of a Ca^{2+}/H^+ exchanger system operating across the trichocyst membrane (Garofalo and Satir (1984). This or a similar but indirect transport mechanism would be appropriate to keep the lumen of mature, dischargeable trichocysts free of Ca^{2+}, before an exocytotic opening is formed. Acidification cannot be seen (Wassmer et al. 2009), as to be expected, when any ΔH^+ would be consumed for any exchanger or indirect transport activity.

EXOCYTOTIC MEMBRANE FUSION, SECRETORY CONTENTS DISCHARGE, AND EXOCYTOSIS-COUPLED ENDOCYTOSIS

Synchronous exocytosis stimulation by AED, a very efficient secretagogue in *Paramecium* (section "Exocytotic membrane fusion"; Plattner et al. 1984, 1985a,b), combined with timed quenched-flow and EM analysis revealed that exocytotic membrane fusion and contents release were accomplished within 80 ms. (This encompasses all events in all cells of the population analyzed in an experiment.) Time-resolved EM analysis is discussed in Plattner (2014a); for a not time-resolved light microscopy documentation, see Fig. 3. This 80 ms period is followed by ~270 ms of membrane resealing, indicating exocytosis-coupled endocytosis (Knoll et al. 1991a). Since one trichocyst release event lasts only < 1 ms (Matt et al. 1978), the time intervals required for one single event, exocytosis and endocytosis combined, would logically be much shorter than measured in the entire population of trichocysts and cells. Not only AED (Plattner et al. 1984, 1985a,b), but also the ryanodine receptor agonists, caffeine (Klauke and Plattner 1998) and 4-chloro-m-cresol (Klauke et al. 2000) activate trichocyst exocytosis, though much less synchronously (Plattner and Klauke 2001). All these effects are paralleled by a transient cytosolic Ca^{2+} signal (Plattner 2015a). This rapid response is enabled by primary release of Ca^{2+} from alveolar sacs via ryanodine receptor-like Ca^{2+}-release channels (Ladenburger et al. 2009) and secondary Ca^{2+} influx from outside, both being tightly coupled as a store-operated Ca^{2+}-entry (SOCE) mechanism (Klauke et al. 2000; Mohamed et al. 2002; Plattner 2014a; Plattner and Klauke 2001). These requirements for trichocyst exocytosis have essentially been confirmed with *P. caudatum* (Iwadate and Kikuyama 2001).

To achieve efficient Ca^{2+} signaling, tight apposition of alveolar sacs to the cell membrane and to each of the docked trichocysts is prerequisite (Plattner 2015b). To achieve exocytosis competence, several molecular determinants have to be coassembled. Based on EM localization and gene silencing studies, we consider the SNARE proteins synaptobrevin type *Pt*Syb5 (Schilde et al. 2010) and syntaxin type *Pt*Syx1 (Kissmehl et al. 2007) relevant for

trichocyst exocytosis. Accordingly, silencing of the SNARE-specific chaperone, NSF (NEM-sensitive factor), inhibits the assembly of functional docking sites (Froissard et al. 2002). Their maturation is paralleled by the assembly of "rosettes", i.e. membrane protein aggregates in the center of a "ring" structure seen in freeze-fractures (Fig. 9).

Which could be the Ca^{2+} sensor cooperating with SNAREs? The EF-hand type Ca^{2+}-sensor, calmodulin, though found at trichocyst docking sites (Momayezi et al. 1986), appears rather responsible for the assembly of the docking sites (Kerboeuf et al. 1993), just as in higher eukaryotes (Quetglas et al. 2002; Wang et al. 2014). As a signal transducer for exocytosis, its time constant is too slow. We have found in the *Paramecium* database an equivalent of the usual fast sensor, synaptotagmin, but this contains eight, rather than the usual two C2-type Ca^{2+}-binding domains (R. Kissmehl and H. Plattner, unpublished observ.). (Note that C2 domains are β-sheets with a loose Ca^{2+}-binding loop.) This corresponds to e-syts (extended synaptotagmins), found up to some mammalian systems (Min et al. 2007). Alternatively, a sequence with two C2 domains, an ortholog of DOC2 in the related parasitic Apicomplexa, has been identified in the *P. tetraurelia* database (Farrell et al. 2012). Therefore, the fast C2 domain-containing Ca^{2+}-sensor to be expected at exocytosis sites (Plattner 2014a) should now be amenable to detailed characterization.

Annexins, though only insufficiently characterized in ciliates, are another component attached to the trichocyst tip in a Ca^{2+}-dependent manner (Knochel et al. 1996). Altogether not only trichocyst docking, but also their detachment appears to be Ca^{2+}-dependent, as the organelles can be detached in the intact cell, among other means, by a Ca^{2+} ionophore (Pape and Plattner 1990) or by EDTA (Glas-Albrecht and Plattner 1990). Similarly, trichocyst-free cortex fragments can be obtained by isolation in a buffer containing EDTA (Stelly et al. 1991).

During AED-induced synchronous exocytosis, vigorous discharge of trichocyst contents is synchronized with membrane fusion without any detectable delay. As outlined in section "Ca^{2+}-binding proteins", contents ejection depends on the access of Ca^{2+} from the outside medium to the trichocyst matrix. Also, the ensuing process of membrane resealing is very rapid, so that exo-encocytosis coupling in *Paramecium* altogether requires only 350 ms (Knoll et al. 1991a) in the whole cell population analyzed by quenched-flow. This may be considered a "kiss-and-run" mechanism although this term is usually attributed to systems with fluid contents, such as neurons (Kononenko and Haucke 2015); in contrast, dense core-vesicle systems normally operate much more sluggishly. In sum, every one of the individual steps of the exo-endocytosis process in *Paramecium* is accelerated by increasing $[Ca^{2+}]_o$ (Plattner et al. 1997b). Spontaneous exocytosis of trichocysts also occurs, though at low frequency (Erxleben et al. 1997; Plattner et al. 1985a,b). This may also be functionally important (see section "Effects of trichocyst contents on bacteria"), and not only massive exocytosis during a predator attack (section "Defensive function of trichocyst ejection").

The first demonstration of membrane fusion and trichocyst contents release as two distinct, separate steps was achieved by Bilinski et al. (1981a). Later on, this was supported by analysis of *P. caudatum* strain *tnd1* (Klauke et al. 1998) which is unable to release its trichocysts (Watanabe and Haga 1996) as described in the section "Ca^{2+}-binding proteins". We have observed that the styrene lipid stain, FM1-43 (Ryan et al. 1993) induces membrane fusion just like a secretagogue (Fig. 10), probably by perturbing membrane lipids. However, at low $[Ca^{2+}]_o$, trichocyst contents are not discharged, exocytosis sites are slowly resealed and trichocysts with their FM1-43 labeled membrane and all their contents are detached (Klauke and Plattner 2000). This phenomenon has been called "frustrated exocytosis". Such resealed and FM1-43-stained trichocysts can be internalized and undergo a new secretory cycle (Fig. 10); this includes redocking with $t_{1/2}$ 35 min and release upon stimulation under standard $[Ca^{2+}]_o$ conditions. This is in contrast to trichocyst "ghosts" formation after regular exocytosis. The links between trichocyst contents and membrane, as described in the section "Trichocyst membrane", may make the difference in signaling the full/empty state. Empty trichocyst membranes ("ghosts") are removed from the cell surface with $t_{1/2}$ 3 9 min, depending on the strain (Plattner et al. 1985a,b, 1993). Ghosts vesiculate and vesicles are fed into the phago-/lysosomal system (Allen and Fok 1984; Ramoino et al. 1997).

TRICHOCYST FUNCTION

Defensive function of trichocyst ejection and of ciliary reversal

The first unambiguous demonstration of a defensive function of trichocysts against predator attacks has been achieved by cocultivation of *P. tetraurelia*, *P. caudatum*, and *P. jenningsi* with the carnivorous ciliate, *D. margaritifer* (Harumoto and Miyake 1991). Paramecia without extrudable trichocysts survived at much lower rate than wildtype cells. Based on a preceding short report by Miyake et al. (1989), Knoll et al. (1991b) have extended such analyses to LM observations. Contact with the predator provoked the local release of a bunch of trichocysts that was restricted to the site of contact with the predator (Fig. 5a d). This was paralleled by ciliary reversal occurring in parallel to $[Ca^{2+}]_i$ transients accompanying stimulated trichocyst exocytosis, as we could document by fluorochrome analysis a spill-over of Ca^{2+} into cilia (Husser et al. 2004). Thus, rapidly occurring ciliary reversal must be considered an auxiliary component of the escape mechanism during a predator attack (Knoll et al. 1991b). As also has been discussed in the section "Glycoproteins and secretory lectins", the secretory lectins may play an additional important role in the overall defense mechanism, in addition to some other functions.

No toxicysts were released by the attacker. Both phenomena, local trichocyst release and ciliary reversal have been considered by us as part of the defensive mechanism (see below). Altogether, a defensive effect is well established, in contrast to some other potential functions of trichocysts (Haacke-Bell et al. 1990). Notably, trichocysts exert no toxic or damaging effects on the attacking cell.

As mentioned, with the best instrumentation then available we had estimated the time required for decondensation of a trichocyst as < 1 ms (Matt et al. 1978; Plattner et al. 1993). A rough calculation reveals that trichocyst stretching is much faster than the swimming speed of the cell. Assuming a matrix length of 3 μm and eightfold stretching within < 1 ms, this would be a speed of > 24 μm × ms^{-1}. In contrast, the swimming speed is ~300 μm × s^{-1}, i.e. 0.3 μm × ms^{-1}. In summary, the difference of > 80fold can provide a safe escape from the spot of attack, although additional factors, such as ciliary reversal and effects of secretory lectins, also have to be taken into account (see below).

Trichocyst expulsion protects paramecia against several more predatory species, such as the ciliate *Climacostomum* (Sugibayashi and Harumoto 2000) and *Monodinium* (Miyake and Harumoto 1996), and even against some metazoans, including rotifers and arthropods (Buonanno et al. 2013). No protective effect is seen against the carnivorous ciliate species, *Didinium nasutum* (Wessenberg and Antipa 1970). These cells first release pexicysts for attachment and then toxicysts. The difference in the response may reside in membrane surface components. In contrast to the species which are successfully warded off, successful attackers may not contain the surface molecules which can trigger trichocyst exocytosis immediately upon contact. Considering the high efficiency of the polyaminated carbohydrate, AED, as a secretagogue (Fig. 5e,f; Plattner et al. 1984, 1985a,b), the natural trigger molecules may be positively charged surface components contained in the glycocalyx of the aggressor. This hypothesis is amenable to experimental test.

Is ciliary reversal a second component of the defense mechanism? Experiments with *P. tetraurelia pawn* mutants, which are able to release trichocysts but unable to perform ciliary reversal upon depolarization (Kung and Naitoh 1973), resulted in the same protective function as with wildtype cells (Harumoto 1994; Sugibayashi and Harumoto 2000). This led the authors to conclude that ciliary reversal would not play any protective role, in contrast to the report by Knoll et al. (1991b). This discrepancy can be resolved as follows. Closer light microscopic inspection, combined with recording of intraciliary Ca^{2+} signals (Fig. 12), clearly documented a spillover of Ca^{2+} during exocytosis stimulation into cilia and a clear-cut ciliary reversal reaction in the mutant cells (Husser et al. 2004), i.e. in situations where any role of ciliary reversal had been disputed.

A third defensive mechanism could potentially be contributed by the lectins contained in the tip of *Paramecium*'s trichocysts (Fig. 11; section "Glycoproteins and

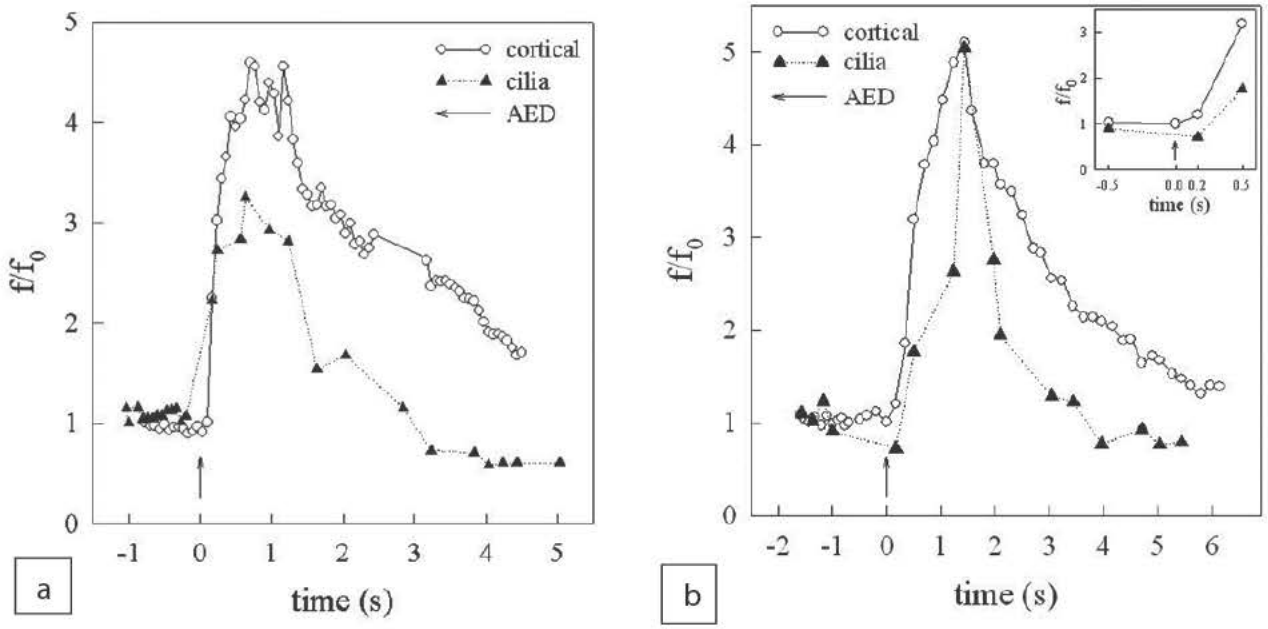

Figure 12 The Ca^{2+} signal recorded in the cell cortex and in the ciliary layer with the fluorochrome Fluo 3 f/f_0 ratio imaging method during trichocyst exocytosis stimulation by AED. Ca^{2+} spreads not only in the cell cortex, but also reaches cilia (recorded in a ~5 μm broad extracellular layer adjacent to the cell surface) in *P. tetraurelia* (**a**) wildtype and (**b**) *pawn* (*d4 500r*) mutant cells. Note that *d4 500r* cells react not only by trichocyst exocytosis, but also by ciliary reversal upon AED stimulation (in contrast to depolarization), so that ciliary reversal can contribute to the escape mechanism, as described in the text. From Husser et al. (2004).

secretory lectins"). Exocytosis of *Dileptus*' toxicysts may be inhibited by crosslinking of components of *Dileptus*' cell surface, achieved by the secretory lectins. (Lectins generally represent bi- to multivalent ligands, Gupta et al. 2012). This can be seen in analogy to the inhibitory effect of polyclonal antibodies (prepared against the surface of slightly fixed *Paramecium* cells) on trichocyst exocytosis (Momayezi et al. 1987). In fact, no toxicyst release by *Dileptus* has been seen during such a "dangerous encounter" of the two ciliates. In addition, lectins might adsorb and, thus, inactivate toxins. Moreover, considering the liability of the trichocyst matrix to bind a variety of proteins (section "Additional trichocyst components"), this may reflect some additional effects of secretory lectins, e.g. a detoxification effect. At this time, these aspects are speculative and require scrutiny.

In summary, the defensive mechanism of trichocyst release by the *Paramecium* cell can be based on several functions. First, locally and explosively released trichocysts act as spacer bars. Second, switching to the "reverse gear" by ciliary reversal can facilitate escape. Third, the exocytosis of toxicyst by the aggressor can possibly be inhibited by crosslinking of surface components by *Paramecium*'s secretory lectins. The existence in the wild of *P. tetraurelia* strains unable of releasing trichocysts (Nyberg 1978, 1980) may reflect the absence of predators feeding on paramecia.

Effects of trichocyst contents on bacteria

As outlined in the section "Glycoproteins and secretory lectins", *Paramecium*'s trichocysts also contain soluble glycoproteins (Allen et al. 1988; Glas-Albrecht et al. 1990; Lüthe et al. 1986), in addition to lectins recognizing galactose and mannose residues (Haacke-Bell and Plattner 1987). Both types of proteins are released during exocytosis. Either one may cause, for instance, aggregation of bacteria (Fig. 11), in analogy to alginate (Weitere et al. 2005) or other biofilm producing compounds (Dopheide et al. 2011). Furthermore, *Paramecium* cells, in small volumes, tend to aggregate under certain conditions (Saunders 1925). Widely different ciliate species can contribute to biofilm formation in nature (Dopheide et al. 2009). As a hypothesis to be tested (glyco)proteins and the lectins released may keep cells from dispersal, as is the case with trichocyst contents of the dinoflagellate, *Ostreopsis* (Honsell et al. 2013). Aggregation of bacteria could increase their availability as food resource and, thus, indirectly improve their ingestion. Considering avid binding of some exogenous proteins by trichocyst proteins (Kersken et al. 1984; Momayezi et al. 1986), as outlined in section "Additional trichocyst components", binding of toxins released by bacteria or other organisms is another hypothetical option (Honsell et al. 2013; Matz and Kjelleberg 2005). Along these lines, there may be a role for spontaneous, constitutive exocytosis of trichocysts (Erxleben et al. 1997; Plattner et al. 1985a,b).

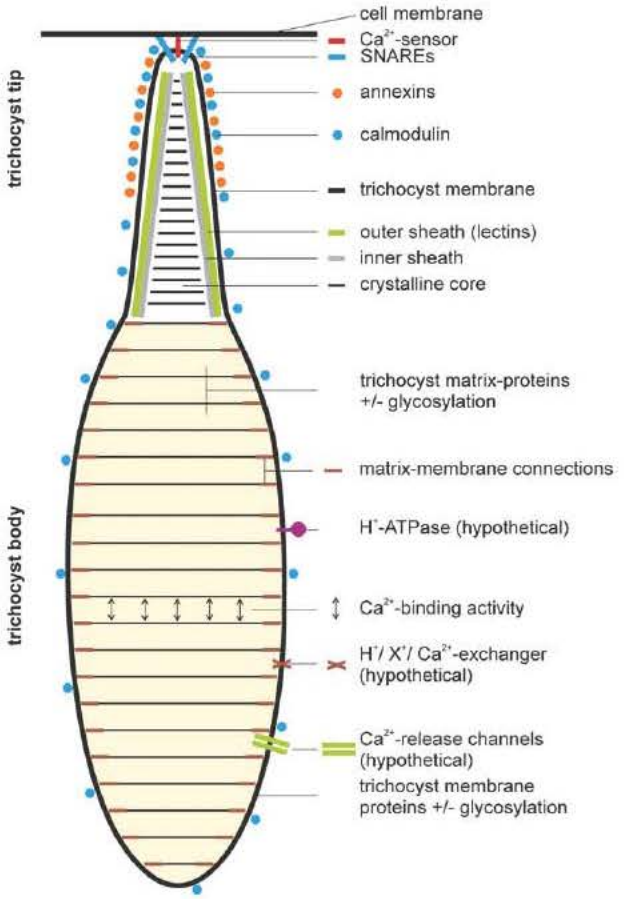

Figure 13 Scheme of a *Paramecium* trichocyst docked at the cell membrane. Note "tips" and "body" region. The docking site proper contains SNARE proteins and an unidentified Ca^{2+} sensor protein, both required for stimulated exocytosis via membrane fusion. Calmodulin not only occurs at the docking site proper (whose assembly it enables), but it also surrounds the trichocyst tip, similarly to annexins. The upper domain of the tip is also enforced by microtubule like structures (not shown). Below the tip membrane, fluffy material ("outer sheath") has been identified as secretory lectins, followed by a dense "inner sheath" and a crystalline core whose periodic banding does not change during trichocyst expulsion. The body part is characterized by its periodic banding that expands severalfold during exocytosis, when the body undergoes decondensation (about sevenfold stretching). Some of the proteins found in the trichocyst body are glycosylated (as are some of the membrane proteins) and some can bind Ca^{2+}. The trichocyst matrix is connected to the membrane by proteins detectable from the Golgi apparatus on. The occurrence of the following three components is postulated from functional analyses, including gene silencing experiments, described in the text: H^+ ATPase, $H^+/X^+/Ca^{2+}$ exchangers and Ca^{2+} release channels. Molecular data are superimposed to a scheme published by Bannister (1972).

CONCLUSIONS

Figure 13 is a summary of the design of a *Paramecium* trichocyst, based on structural description by Bannister (1972), with superimposed molecular and functional data as described throughout this review. Trichocyst release by

stimulated exocytosis is the fastest and most synchronous dense core-secretory vesicle system known (Plattner and Kissmehl 2003; Plattner et al. 1993). In the future, this system may also allow one to assess the feedback signal governing enhanced transcriptional activity observed after massive stimulated trichocyst release (Arnaiz et al. 2010). Over the years, synchrony of the process, enabled the analysis of basic aspects of secretory activity (Plattner 2014a,b). Emerging molecular biology, with the availability of a *Paramecium* database, enabled the identification of important key players and to provide a more solid basis to the manifold details collected about signaling during trichocyst exocytosis. Thus, some key players pertinent to Ca^{2+} signaling could be identified. The rapid release of trichocyst contents depends on the following characteristics. First, on the defined, regular localization of their release sites preassembled under epigenetic control, as discussed in more detail recently (Plattner 2015a,b) and, second, on the rapid decondensation of the trichocyst contents. The way how Ca^{2+} can initiate the explosive ejection of contents, once it enters the trichocyst lumen in accord with membrane fusion, remains only partially understood. This activity is most probably due to rapid cooperative conformational change of tmps some of which can bind Ca^{2+}. Together with rapid signal transduction, this appears to be the main basis of defense against predators such as *Dileptus*. This may be supported by two additional mechanisms: One is ciliary reversal superimposed to trichocyst exocytosis due to Ca^{2+} spillover into cilia. Another one, though hypothetic, is an inhibitory effect of secretory lectins, released from the trichocyst tip, on toxicyst expulsion by the predator cell. In summary, trichocysts remain enigmatic and fascinating defensive projectiles but many details still have to be clarified by further research.

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