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Signals Regulating Vesicle Trafficking in *Paramecium* Cells

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Abstract Most data available from *Paramecium*, fewer from *Tetrahymena*, disclose essentially the same principles of signaling as in metazoans up to man. Microtubules serve for long-range signaling, whereas SNARE proteins, H^+-ATPase, GTPases and actin provide short-range molecular signals, with Ca^{2+} as a most efficient, locally and spatially restricted signal particularly for membrane fusion. This is enabled by the strategic positioning of Ca^{2+}-release channels, type $InsP_3$ receptors and ryanodine-receptor-like proteins, also in ciliates. In *Paramecium*, the most evident trafficking routes encompass exo-/endocytosis, endo-/phagocytosis and the contractile vacuole complex. The high specificity, precision and efficiency of vesicle trafficking regulation in ciliates is facilitated by their highly regular, epigenetically controlled cell structure, with firmly installed microtubular rails.

6.1 Basic Aspects of Vesicle Trafficking and Signaling Pathways in Ciliates

Ciliates are well designed cells, with a cell cortex composed of unit fields called kinetids, about 1 × 2 μm in size. Each kinetid possesses a cilium (or two) in its center and intermittently positioned dense core-secretory organelles. These are trichocysts in *Paramecium* and mucocysts in *Tetrahymena*—the most intensely studies genera. This epigenetically controled surface pattern (Beisson and Sonneborn 1965; Wloga and Frankel 2012) entails important consequences for signaling, e.g. by the universal second messenger, Ca^{2+}, as it allows for alternative activation of cilia and of vesicle exocytosis, respectively, due to strict local and temporal signal confinement (Klauke and Plattner 1997; Husser et al. 2004). Exocytosis is an example of short-range signaling enabled by a pre-assembled signaling machinery and a local Ca^{2+} impulse.

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Microtubules emanating from ciliary basal bodies act as long-range signals for the transport of trichocysts to docking sites (Aufderheide 1977; Glas-Albrecht et al. 1991). Close to basal bodies, parasomal sacs serve for formation of clathrin-coated endosomes (Elde et al. 2005), alternating with constitutive exocytosis of surface coat materials (Flötenmeyer et al. 1999). Other landmarks in the cell cortex are the cytostome from where phagosomes pinch off, fed by acidosomes (late endosomes) and recycling vesicles originating from the cytoproct—the site of spent food vacuole release (Allen and Fok 2000). Again, microtubules form a long-range signaling system in this "ventral" part of the cell by guiding vesicles to the nascent food vacuole. The outlets of the two contractile vacuole complexes are positioned "dorsally", also under epigenetic control.

Three vesicle trafficking pathways are recognizable: (i) from endoplasmic reticulum via Golgi apparatus to the cell surface for the exocytotic route, paralleled by a reverse endocytotic pathway via endosomes for membrane recycling and/or degradation in lysosomes; (ii) the phagocytotic pathway, coupled to endocytosis and recycling, for digestion of food particles; finally, (iii) the contractile vacuole complex for periodic expulsion of an excess of water and ions (Allen and Naitoh 2002). This counteracts the rapid permeation of water and Ca^{2+} and—beyond pulsation activity—also involves less overt membrane dynamics and signaling (Plattner 2015c).

Before genomic databases became available, Allen and Fok (2000) have pioneered the field of vesicle trafficking in *Paramecium* by using organelle-specific monoclonal antibodies for immunolocalization studies (http://www5.pbrc.hawaii.edu/allen/). Subsequently molecular biology allowed for more stringent molecular identification, localization and gene silencing of key players in signal-based vesicle trafficking. Short-range signals include SNARE proteins, the SNARE-specific chaperone NSF, H^+-ATPase/pump, actin, Ca^{2+}-release channels (CRC), as analyzed in *Paramecium* (reviewed by Plattner 2010a), and small GTPases analyzed in *Tetrahymena* (Bright et al. 2010). SNAREs stand for "*s*oluble *N*-ethylmaleimide sensitive factor (NSF) *a*ttachment protein *re*ceptors", NSF being a SNARE-specific chaperone (Rothman 2014; Südhof 2014). SNAREs, together with Rab-type monomeric GTP-binding proteins/GTPases and their organelle-specific binding proteins (Grosshans et al. 2006) and high sensitivity Ca^{2+}-binding proteins are important for short-range signaling enabling membrane interaction and fusion (Rothman 2014; Südhof 2014).

Note that genes and proteins of *Paramecium tetraurelia*, are designated with the prefix "Pt", e.g. PtSyb for synaptobrevin. As there are subfamilies of variable size, a designation may be PtSyb5-1. The prefix Pt is important since synaptobrevins are difficult to correlate with their equivalents up to mammals with regard to localization and function. Although this is more easy, e.g. with syntaxins, we maintained this nomenclatorial principle throughout. For CRCs we used a comparable designation, for instance, PtCRC-IV-1 for a subfamily member. Considering the number of paralogs (also called ohnologs when arisen by whole genome duplications) in a protein/gene family of *P. tetraurelia* it was not possible to analyse them all and their localization and function may be similar or even identical. Therefore, in this

review, the types of subfamily members are usually not specified. More details can be retrieved from the original publications and reviews cited, e.g. Plattner (2010a, b) for SNAREs and Plattner and Verkhratsky (2013) for CRCs.

Figure 6.1a illustrates the general principle of signalling for vesicle trafficking, Fig. 6.1b the requirement of a strictly confined Ca^{2+} signal for membrane fusion, Fig. 6.1c the molecular interactions during intracellular vesicle trafficking, as exemplified by food vacuole (phagolysosome) formation, and Fig. 6.1d the particular situation of an exocytosis site in ciliates.

6.2 Long-Range Trafficking Signals

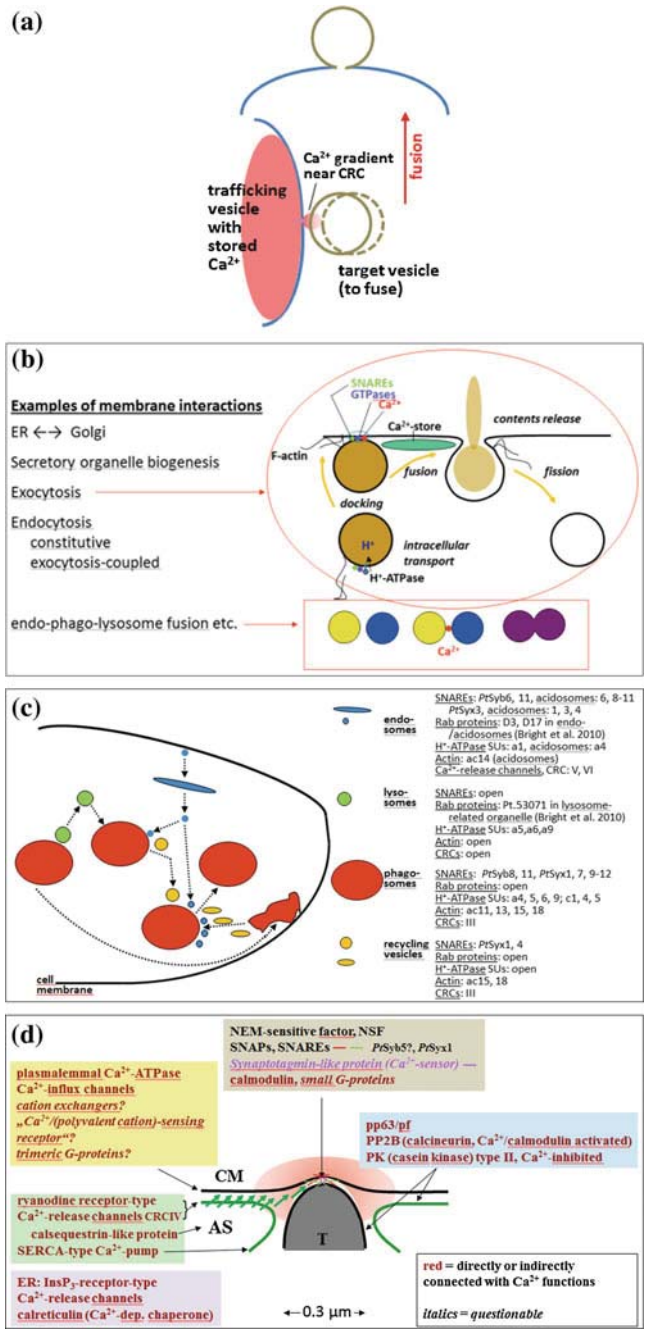
Since biogenesis of the surface membrane complex requires proper positioning of components, such as cilia, parasomal sacs and trichocysts, one may ask for cause and consequence. On the one hand one may ask for the causation of this regular arrangement and, on the other hand, for its consequences for signalling processes. Reversing this arrangement by spontaneous misplacement in *Euplotes* (Frankel 1973) or experimentally by microsurgery in *Paramecium* (Beisson and Sonneborn 1965) maintains and transfers such local disorientation, thus indicating epigenetic regulation of surface pattern formation and resulting signalling (Plattner 2015a).

Analysis of surface pattern formation has revealed several crucial components of ciliary basal bodies (Beisson and Jerka-Dziadosz 1999). Saltatory docking of trichocysts (Aufderheide 1977) along microtubules originating at ciliary basal bodies (Plattner et al. 1982; Glas-Albrecht et al. 1991), quite unusually, occurs in plus-to-minus direction of microtubules—a phenomenon maintained in evolution, e.g. in mammalian epithelia and T-lymphocytes (Griffiths et al. 2010). Some possible mechanisms relevant for the distinct positioning of the contractile vacuole complexes, also microtubule-based, have been discussed recently (Plattner 2013, 2015c).

Important parts of the phagocytotic pathways and delivery of recycling vesicles in ciliates are also guided by microtubules. Crucial work from Allen and Fok (2000) revealed that recycling vesicles travel from the cytoproct to the cytostome for reuse, as shown with *Paramecium multimicronucleatum* (Fok and Allen 1993) and *Tetrahymena thermophila* (summarized by Wloga and Frankel 2012).

In conclusion, pre-established microtubular arrays, together with appropriate motor proteins, allow for long-range signaling and, thus, render vesicle trafficking very efficient. Since many types of vesicles bounce around in the cytoplasm one has to ask what molecular signal makes a vesicle jump onto the bandwagon suitable for delivery to its final address. In the end this is determined by vesicle-resident proteins, e.g. an “early endosome antigen” (EEA)—a short-range signal, as described below.

Prerequisite to the strict signalling phenomena in the cell cortex is the targeted delivery of vesicles and their membrane components to proper sites. The selective expression pattern during reciliation following deciliation, e.g. in *T. thermophila*


(a)
trafficking vesicle with stored Ca2+
Ca2+ gradient near CRC
fusion
target vesicle (to fuse)
(b)
Examples of membrane interactions
ER ←→ Golgi
Secretory organelle biogenesis
Exocytosis
Endocytosis
constitutive
exocytosis-coupled
endo-phago-lysosome fusion etc.
SNAREs
GTPases
Ca2+
Ca2+-store
contents release
F-actin
fusion
fission
docking
H+
intracellular transport
H+-ATPase
Ca2+
(c)
endo-somes
SNAREs: PtSyb6, 11, acidosomes: 6, 8-11 PtSyx3, acidosomes: 1, 3, 4
Rab proteins: D3, D17 in endo-/acidosomes (Bright et al. 2010)
H+-ATPase SUs: a1, acidosomes: a4
Actin: ac14 (acidosomes)
Ca2+-release channels, CRC: V, VI
lyso-somes
SNAREs: open
Rab proteins: Pt.53071 in lysosome-related organelle (Bright et al. 2010)
H+-ATPase SUs: a5,a6,a9
Actin: open
CRCs: open
phago-somes
SNAREs: PtSyb8, 11, PtSyx1, 7, 9-12
Rab proteins: open
H+-ATPase SUs: a4, 5, 6, 9; c1, 4, 5
Actin: ac11, 13, 15, 18
CRCs: III
recycling vesicles
SNAREs: PtSyx1, 4
Rab proteins: open
H+-ATPase SUs: open
Actin: ac15, 18
CRCs: III
cell membrane
(d)
NEM-sensitive factor, NSF
SNAPs, SNAREs — PtSyb5?, PtSyx1
Synaptotagmin-like protein (Ca2+-sensor) —
calmodulin, small G-proteins
plasmalemmal Ca2+-ATPase
Ca2+-influx channels
cation exchangers?
„Ca2+/(polyvalent cation)-sensing receptor"?
trimeric G-proteins?
pp63/pf
PP2B (calcineurin, Ca2+/calmodulin activated)
PK (casein kinase) type II, Ca2+-inhibited
CM
ryanodine receptor-type Ca2+-release channels CRC IV
calsequestrin-like protein
AS
SERCA-type Ca2+-pump
T
ER: InsP3-receptor-type Ca2+-release channels
calreticulin (Ca2+-dep. chaperone)
0.3 µm
red = directly or indirectly connected with Ca2+ functions
italics = questionable

◀ **Fig. 6.1** **a** Principles of vesicle/membrane interaction, with the involvement of SNAREs, GTPases, H^+ ATPase, actin and Ca^{2+}. Acidification of the vesicle lumen by the organellar H^+ ATPase signals binding of additional components, leading to vesicle docking at a target membrane and fusion upon a Ca^{2+} signal. This principle applies not only to exocytosis (*top*) but also to intracellular membrane interactions (*bottom*). **b** Illustrates the local restriction of Ca^{2+} signals for membrane fusion, e.g. by release via local CRCs. **c** Vesicle trafficking and molecules involved in the endo /phagocytotic process. At the right, key players relevant for the respective steps are listed. Note, for instance, the exchange of actin, CRC and SNARE isoforms. **d** Components assembled at and around a trichocyst docking site include not only proteins relevant for docking and fusion, but also for Ca^{2+} storage in, and release from alveolar sacs (AS), as well as Ca^{2+} pumps, Ca^{2+} binding proteins and proteins relevant for energetic aspects, such as pp63/pf, PP2B and a casein kinase, shown at the *right/center*. *Green arrows* indicate Ca^{2+} flux upon stimulation. Some components, listed with a question mark, require identification in ciliates. AS = alveolar sac, CM = cell membrane, T = trichocyst. For further details, see text and Plattner (2016) (Color figure online)

(Miao et al. 2009), can provide insight into the relevance of individual components. Frequently this is pinpointed by following up in ciliates the relevance of mutations underlying mammalian ciliary pathology, as summarized previously (Simon and Plattner 2014). It should be noted that vesicles are not delivered into cilia in any system analysed. Unexpectedly we observed a ring of PtSyb10 around the ciliary basis (Schilde et al. 2010). Since synaptobrevins are normally harbored in vesicles, rather than in target membranes (see below) this might indicate retention due to excessive vesicle delivery, whereas this normally seems to be counterbalanced by membrane retrieval.

Another aspect is that Ca^{2+} can separately regulate widely different processes, such as trichocyst exocytosis and ciliary activity. Why is a cell then not confused when stimulated? Normally there is no spill-over of Ca^{2+} from cilia to exocytosis sites which, therefore, remain inactive (Husser et al. 2004). However, the opposite process does take place when a predator attacks a *Paramecium* cell. In consequence, explosive local trichocyst release keeps the predator at a distance and ciliary reversal allows the potential prey to rapidly escape (Knoll et al. 1991a). Both processes depend on Ca^{2+} and spillover of Ca^{2+} into cilia (Husser et al. 2004) allows, in this case, to bypass the physiological activation of ciliary reversal. Normally this is induced by cell membrane depolarization and Ca^{2+} influx selectively into cilia via voltage dependent channels [For review see Plattner and Klauke (2001) and Plattner (2014)]. Beyond this exceptional situation of a long-range Ca^{2+} signal there are several mechanisms to confine a Ca^{2+} signal very strictly (Sect. 6.3).

6.3 Short-Range Trafficking Signals

6.3.1 General Aspects

Evidently integral membrane proteins can serve as local landmarks for specific interaction with a partner membrane (Fig. 6.1a). This applies to SNAREs, anchored

by their carboxy-terminal region, as well as to C2-type high sensitivity Ca^{2+}-sensor proteins, type synaptotagmin, inserted by their amino-terminal region. Both are crucial for membrane fusion (In *Paramecium* we have found only synaptotagmin-like proteins, still to be characterized; see Sect. 6.3.3). For a summary of key players in membrane recognition and fusion, see Plattner (2010a). Also some soluble proteins, type Rab, have to be locally available. As mentioned, they bind to organelle-specific integral membrane proteins (essentially unknown in ciliates). All these molecules have to match with their counterparts in the target membrane.

Ca^{2+} for signaling originates either from release from a nearby store, or interacting vesicles contain Ca^{2+} themselves and are able to release Ca^{2+} via CRCs integrated in their membrane (Plattner 2015b); see Fig. 6.1b. Cytosolic Ca^{2+} concentration has to rise very locally and for a very restricted time, for different reasons (Plattner and Verkhratsky 2015). First, Ca^{2+} at too high concentrations is toxic; second, Ca^{2+} diffuses rapidly; third its concentration is rapidly downregulated by binding to Ca^{2+}-binding proteins, and over longer times by sequestration and extrusion from the cell; fourth, its effect depends of concentration in a supralinear function (Neher 1998a). All this calls for microdomain regulation in ciliates (Klauke and Plattner 1997; Plattner and Klauke 2001), just as in neurons (Neher 1998b). Local Ca^{2+} signals for membrane fusion are so short-lived that their actual size and duration can only be extrapolated from intracellularly applied Ca^{2+} chelators with different binding and time constant (Neher 1998a, b). In *Paramecium*, Ca^{2+} for trichocyst exocytosis comes from alveolar sacs (Sect. 6.3.3), the well established cortical stores (Stelly et al. 1991; Hardt et al. 2000; Plattner and Klauke 2001). Local values of ~ 5 μM are required for exocytosis (Klauke and Plattner 1997). Considering that, in mammalian cells, most trafficking organelles contain Ca^{2+} themselves (Hay 2007) and the occurrence of CRCs in such organelles (except trichocysts) in *Paramecium* (Ladenburger and Plattner 2011), one can reasonably assume that Ca^{2+} signaling in microdomains occurs throughout vesicle trafficking pathways also in ciliates. For any further discussion, see Plattner (2014).

Some essential aspects of signaling are outlined in Fig. 6.1a. A vesicle contains SNARE proteins (called v- or R-SNAREs due to an R [Arg] residue in the center of the SNARE domain). For docking, a target membrane has to contain a matching set of t-/Q-SNARES (Q = Glu). SNAP-25 is an additional SNARE that, because of its wide distribution throughout the *Paramecium* cell is hardly appropriate to further specify short-range signaling. As mentioned, additional specificity is provided by the reversible binding of monomeric GTP-binding proteins (GTPases type Rab for vesicle trafficking), as analysed in *Tetrahymena* (Bright et al. 2010). These in turn bind to organelle-specific residents proteins (hardly identified in ciliates). A total of 44 SNAREs, v-/R- (Schilde et al. 2006, 2010) and t-/Q-SNARES (Kissmehl et al. 2007) have been assigned in *Paramecium* to specific organelles, some containing more than one type, including some rather similar ohnologs (Plattner 2010b) due to

recent whole genome duplications. 57 Rabs are reported from *Tetrahymena* (Bright et al. 2010).

The activity of Rabs is governed by activating and inhibitory proteins (hardly known from ciliates), respectively. In higher eukaryotes their attachment to the respective target membrane depends on the luminal pH value (Hurtado et al. 2006). Concomitantly, vesicles undergoing trafficking contain a H^+-ATPase (Mellman 1992). The conformational change of the H^+-ATPase/pump resulting from the actual luminal acidification enables the binding of Rab regulators and Rab proteins, probably also in ciliates. Here, we also find this multimeric H^+-ATPase in the organelles undergoing trafficking (Wassmer 2005, 2006, 2009). Particularly remarkable is the unprecedented number of a-subunit isoforms which allows for the attachment of the catalytic V1 part to the membrane-integrated H^+-conducting base piece, V0; both, V0 and V1 are a complex of variable monomers. The high number of theoretical combinations with the a-subunit may allow for a range of subunit combinations for local requirements. Similarly organelle-specific CRCs may account for specific local requirements. Work with higher eukaryotes suggests that targeting of some of the components under consideration for short-range signalling is mutually interdependent, as reviewed previously (Plattner 2010a).

6.3.2 Signaling During the Endo-Phagolysosomal Cycle

Figure 6.1c outlines vesicle trafficking and signalling molecules involved in food vacuole (phagolysosome) formation. Already before the molecular era, Allen and Fok (2000) have elucidated the principal pathways of phagocytosis in *Paramecium multimicronucleatum*.

Formation of food vacuoles starts at the cytostome. Here, the cell membrane seems to bulge in; in reality this part of the cell membrane—in the absence of alveolar sacs, ciliary basal bodies and of trichocysts—is extended by recycling vesicles. These are delivered from spent food vacuoles after contents discharge at the cytoproct and by other recycling vesicles originating from progressed stages of food vacuoles. The nascent vacuole becomes a phagosome which is a non-acid organelle devoid of H^+-ATPase. Only after pinching off, late endosomes, called acidosomes because of their endowment with H^+-ATPase, fuse with the phagosome (Allen et al. 1993). On their way through the cell (cyclosis) food vacuoles fuse with lysosomes and, thus, become phagolysosomes which digest food particles. At a later stage, lysosomal components are retrieved and the spent food vacuole releases indigestible materials at the cytoproct (Allen and Fok 2000). When stained with permeable acidity markers, which are retained in protonated form and change color depending on luminal pH, a color change indicates the sequence: neutral → acid → neutral (Wassmer et al. 2009).

The sequence of events described is supported by long-range and short-range, molecular signaling. Here, like in other acidic compartments, such as endosomes, occurrence of an H^+-ATPase was demonstrated (Fok et al. 2002; Wassmer et al. 2009). As mentioned, changing acidification also implies change of Rab protein binding (Hurtado-Lorenzo et al. 2006) at the organelle surface. Changing binding partners, from endosome to phagolysosome, are indicated in Fig. 6.1c. Whereas this background information mainly comes from *Paramecium* (*P. multimicronucleatum* for classical cell biology, *P. tetraurelia* for molecular biology), data concerning changing association of Rab proteins throughout the digesting cycle come from *T. thermophila*, with a side glance on *P. tetraurelia* (Bright et al. 2010).

Figure 6.1c summarizes what is known about the changing isoforms of SNAREs, H^+-ATPase subunits, actin and CRCs along the endo-phagolysosomal pathway, as determined in our laboratory, supplemented by data on changing Rab proteins collected by the Turkewitz laboratory (Bright et al. 2010). This comprehensive work reveals extensive specification of Rabs in compartments designated as follows: endocytotic vesicles, posterior potential recycling vesicles, lyso/phagosomes, all phagosomes, selected phagosomes and cytoproct-associated phagosomes with 4, 4, 1, 1, 6, and 3 Rab types, respectively, complemented by five Rabs associated with the oral apparatus (including the cytosome) and three associated with the cytoproct. Considering that, in *P. tetraurelia*, usually several similar ohnologs occur, the number of Rabs may be several times higher, although probably with redundant localizations and functions.

6.3.3 Dense Core-Secretory Vesicle Biogenesis, Docking and Signaling for Exocytosis

In ciliates, the endoplasmic reticulum is very elaborate, whereas the Golgi apparatus is inconspicuous; in *Paramecium* the Golgi apparatus is split in several hundred Golgi fields (dictyosomes) with very few membrane stacks (Allen 1988). Biogenesis of both, mucocysts and trichocyst, follows this classical assembly line. Precursor vesicles fuse and secretory proteins are trimmed (Briguglio et al. 2013; Gautier et al. 1994). When not posttranslationally cleaved, assembly to crystalline contents and transfer to the cell membrane are inhibited with both, mucocysts (Briguglio et al. 2013) and trichocysts (Pouphile et al. 1986; Gautier et al. 1994). Therefore, there must be a luminal signal, linked to a surface signal, for delivery along microtubules to the cell membrane.

Upon arrival at the cell membrane trichocysts induce restructuring of the docking site by formation of a “fusion rosette” (Beisson et al. 1976). The identity of the proteins forming a rosette is not clear (Plattner 2010a) although we know that its assembly requires the activity of the SNARE chaperone, NSF (Froissard et al.

2002). One of the problems is that one rosette particle, to account for its diameter, would have to contain ~70 syntaxins (Plattner 2010b). Nevertheless, the t-/Q-PtSyx1 can be safely assumed to occur at exocytosis sites although, by immunolocalization, it is scattered over the entire cell membrane (Kissmehl et al. 2007). No V0 parts of the H^+-ATPase are seen at exocytosis sites (Wassmer et al. 2005), thus excluding a membrane fusion model involving these molecules (Plattner and Kissmehl 2003). The only v-/R-SNARE detected on the trichocyst membrane is PtSyb5 (Schilde et al. 2010). In *T. thermophila*, the Rab protein TtRabD41 has been detected at exocytosis sites (Bright et al. 2010).

A Ca^{2+}-sensor, type synaptotagmin, still requires identification and localization in ciliates. Such molecule is mandatory for rapid exocytosis—considering that all trichocysts can be rapidly released within 80 ms upon stimulation (Knoll et al. 1991b, Plattner et al. 1993)—the highest rate ever found with dense core-secretory organelles (Plattner and Kissmehl 2003). Candidate Ca^{2+}-sensors found in the *Paramecium* genomic database have eight C2 domains (Kissmehl and Plattner unpubl. observ.), rather than the usual two, for high sensitivity Ca^{2+} binding (Südhof 2014). Such extended synaptotagmins (e-syts) also occur in other systems, up to mammalians, and they allow membrane-to-membrane links over a broader interspace (Pérez-Lara and Jahn 2015). This would fit the trichocyst-cell membrane interspace at docking sites. Another cursory note based on database mining indicates in *P. tetraurelia* occurrence of a homolog of DOC2.1—a two C2 domain-bearing Ca^{2+}-binding protein in apicomplexan parasites (Farrell et al. 2012).

Figure 6.1d illustrates which additional proteins are assembled at, and around a trichocyst docking site. Calmodulin is required for the assembly of SNAREs (Kerboeuf et al. 1993). Specific isoforms of actin (Sehring et al. 2007a, b), together with a 63 kDa phosphoprotein (pp63/parafusin, pf), phosphatase 2B (calcineurin), and a Ca^{2+}-inhibited protein kinase are distributed around trichocyst tips and the nearby subplasmalemmal space. They are considered important for energetic aspects of signaling during stimulated trichocyst exocytosis (Plattner and Kissmehl 2003). A plasmalemmal Ca^{2+}-ATPase/pump serves for keeping subplasmalemmal Ca^{2+} concentration low, whereas a SERCA-type Ca^{2+}-ATPase/pump refills the stores (see Plattner 2014). Upon stimulation, release of Ca^{2+} from alveolar sacs, the cortical Ca^{2+}-stores (Stelly et al. 1991; Hardt and Plattner 2000), is mediated by ryanodine receptor-like protein (RyR-LP) channels (Ladenburger et al. 2009; Plattner 2015b). This microanatomical arrangement forces Ca^{2+} flux over trichocyst tips, thus mediating rapid response, as required for predator defence (Harumoto and Miyake 1991).

The CRCs, as well as the luminal high capacity/low affinity Ca^{2+}-binding proteins are different in endoplasmic reticulum and in alveolar sacs, respectively. Other molecules of potential interest, such as a surface Ca^{2+}/polyvalent cation-sensing receptor and trimeric G-proteins are under debate. Such details concerning signaling in ciliates are discussed in a more extensive review (Plattner 2016).

Once an exocytotic pore is formed, Ca^{2+} can rapidly flow into the trichocyst lumen where it triggers the explosive decondensation (stretching) and release of the

crystalline contents (Bilinski et al. 1981) by binding to specific secretory proteins (Klauke et al. 1998).

The Ca^{2+} signal flushing over exocytosis sites suffices to drive also exocytosis-coupled endocytosis of trichocyst "ghosts" and their internalization (Plattner et al. 1997), thus making these sites again available for docking of new trichocysts. After stimulation, in the cytosol, Ca^{2+} is most rapidly downregulated by binding to the Ca^{2+}-binding protein, centrin (Sehring et al. 2009), whereas the pumps are primarily devoted to overall household regulation (Plattner 2016).

6.3.4 Contractile Vacuole Complex

This complex organelle is most dynamic, as it contracts and expands in ≤10 s intervals. From the contractile vacuole, radial canals emanate which are associated with a flexible three-dimensional network, the spongiome (Allen and Naitoh 2002). Its part proximal to the canals is smooth and periodically collapses after contraction (systole) and, thus, may represent a membrane reservoir for the expansion of the canals and of the vacuole during diastole. Canals are disconnected from the vacuole before every systole (Tominaga et al. 1998). In the electron microscope the peripheral part of the spongiome appears decorated by the head parts (V1) of the H^+-ATPase (Fok et al. 2002; Wassmer et al. 2005) and, therefore, is called the decorated spongiome.

SNAREs (PtSyx2, 14, 15, and PtSyb2, 6, 9) and Ca^{2+}-release channels (type PtCRC-II, i.e. inositol 1,4,5-trisphosphate receptors [$InsP_3Rs$]) occur in different parts of the organelle in *P. tetraurelia*, together of PtSyx2 and 15 as well as PtSyb2 and 9 at the vacuole outlet (the pore). Mechanosensitive Ca^{2+} channels occur in the database and are also localized to the contractile vacuole complex in other species. All this suggests the following signaling scenario (Plattner 2013, 2015c). (i) A proton gradient enables osmotic filling with water and ions from the cytosol (Allen and Naitoh 2002), including an excess of Ca^{2+} (Stock et al. 2002). (ii) Increasing turgor causes reversible rearrangement of membranes in the smooth spongiome with the help of SNAREs (Plattner 2013). (iii) Partial constitutive reflux of Ca^{2+} from the organelle through the CRC-II/$InsP_3$R-type channels into the nearby cytosol (Ladenburger et al. 2006) can drive membrane rearrangements in the smooth spongiome and vesicle fusion for ongoing protein replacement. (iv) Mechanosensitive channels may also mediate fusion of the vacuole with the cell membrane, thus opening the pore. (v) In *T. thermophila*, three Rab proteins have been detected, TtRabD2, 10 and 14 (Bright et al. 2010).

Before cytokinesis, de novo formation of additional contractile vacuole complexes is observed. Localization studies with ciliates and with other protozoa suggest signaling by proteins many of which are known to govern cytokinesis (Plattner 2015c).

6.4 Conclusions and Open Questions

Despite considerable progress, there are important gaps in our knowledge about vesicle trafficking and underlying signaling mechanisms in ciliates although they now belong to the best analysed protozoa. In *Paramecium*, the abundance of ohnologs serves for a kind of gene amplification, but some may undergo neo-functionalization. It would be particularly rewarding to analyze the functional relevance of the unprecedented number of H^+-ATPase a-subunits. May they serve for fine tuning of local signalling? Also important is the establishment of the identity of Rab target proteins. We also still have to learn a lot about the Golgi apparatus in ciliates.

Beyond these and some other gaps, many details of general validity about vesicle trafficking and underlying signaling mechanisms have been established specifically in ciliates, mainly with *Paramecium* and *Tetrahymena*. With P. *tetraurelia* our primary goal was to sound out, on a broad scale, the inventory available, while punctual scrutiny of some more important aspects was a secondary goal. Work may now be extended to detailed analysis of ciliate-specific aspects, as has been done for some important aspects, mainly with *T. thermophila*. Both systems together have taught us that signaling mechanisms underlying vesicle trafficking and beyond have been invented already at this level of evolution. The redundancy of structural elements, particularly in the cell cortex, and amplification effects of synchronized processes, such as synchronous exocytosis induction and synchronous reciliation after deciliation, can greatly facilitate the analysis of signalling processes.

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