Chapter 2
Principles of Intracellular Signaling in Ciliated Protozoa—A Brief Outline

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Abstract Ciliates have available most of the intracellular signaling mechanisms known from metazoans. Long-range signals are represented by firmly installed microtubules serving as gliding rails aiming at specific targets. Many components are distinctly arranged to guarantee locally restricted effects. Short-range signals include Ca^{2+}, provided from different sources, and proteins for membrane recognition and fusion, such as SNAREs, GTPases and high affinity Ca^{2+}-binding proteins (still to be defined). A battery of ion conductances serves for electric coupling from the outside medium to specific responses, notably ciliary activity, which also underlies gravitaxis responses. Eventually cyclic nucleotides are involved, e.g. in ciliary signaling. Furthermore, an elaborate system of protein kinases and phosphatases exerts signaling mechanisms in widely different processes.

2.1 Introduction—Basic Aspects of Signaling in Ciliates

As for every eukaryotic cell one may ask also for ciliates which cellular processes require signaling, how signaling is executed and over which distances, whether principles are shared with metazoans and plants, whether mechanisms are maintained during evolution, abolished or newly invented. Together with *Dictyostelium*, the ciliates *Paramecium* and *Tetrahymena* represent the protozoa which, at this time, are best analyzed with regard to signaling. It is useful to differentiate between long- and short-range signaling, e.g. by microtubules or electrical signals and by molecular interactions or spatially restricted Ca^{2+} signals (Plattner and Klauke 2001), respectively.

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2.1.1 Basic Phenomena Applicable to Ciliates

Signaling pertinent to ciliary activity in ciliated protozoa is as elaborate, or even more than in metazoan (Machemer 1988a) as these cells are highly mobile and capable of reacting to various environmental stimuli (Machemer 1988b; Bell et al. 2007). To achieve this, mechanical, electrical, biochemical and molecular signals, i.e. long range and short-range signals, can be combined in some variation to the basic theme.

Ciliates have at their disposal a highly sophisticated vesicle trafficking system, as illustrated (http://www5.pbrc.hawaii.edu/allen/) and summarized (Allen and Fok 2000) for *Paramecium* and *Tetrahymena* (Frankel 2000). The routes have to be addressed here. (i) Endoplasmic reticulum (ER) → Golgi apparatus → lysosomes + dense core-secretory organelles (trichocysts in *Paramecium* and mucocysts in *Tetrahymena*). (ii) Constitutive exocytosis of surface coat materials (Flötenmeyer et al. 1999) and dense core-secretory organelle exocytosis (Plattner et al. 1985; Plattner and Kissmehl 2003a). (iii) Phagocytosis, from cytostome → phagosome → endosomal and lysosomal input → phagolysosome (called "food [digesting] vacuole") → discharge of spent vacuoles at the cytoproct. (iv) Endocytosis via early endosomes → links to phagosomes + lysosomes. (v) Vesicle recycling from the cytoproct to the nascent phagosome. (vi) In addition, the contractile vacuole complex impresses not only by its dynamic activity (Allen and Naitoh 2002) in the context of ongoing osmoregulation (Allen et al. 2009), but it also represents a site endowed with the machinery typical of vesicle trafficking (Plattner 2015b) although vesicle trafficking within the organelle is less obvious. Steps (iii) to (v) have been documented in detail for *Paramecium* (Allen and Fok 2000) as well as for *Tetrahymena* (Frankel 2000). Beyond short-range signaling, steps (i), (iii) and (v) include long-range signaling. All these pathways serve for proper delivery and positioning of signaling elements so that they can execute their signaling function at distinct sites of the cell.

2.1.2 Molecular Key Players

Recent availability of a macronuclear genome database for the most frequently used species, *P. tetraurelia* and *T. thermophila*, has enabled the identification, localization and assessment of the functional relevance of key players. In *Paramecium* such work has included mainly SNARE (*soluble N*-ethylmaleimide sensitive factor [NSF] *attachment protein receptors*) proteins, actin and H^+-ATPase, as summarized previously (Plattner 2010) as well as Ca^{2+}-release channels (CRC) of the type inositol 1,4,5-trisphosphate receptors ($InsP_3R$) and ryanodine receptor-like proteins (RyR-LP) (Ladenburger and Plattner 2011; Ladenburger et al. 2006, 2009; Plattner 2015a), as summarized recently (Plattner and Verkhratsky 2015). This is complemented by monomeric GTP (guanosine trisphosphate) binding proteins
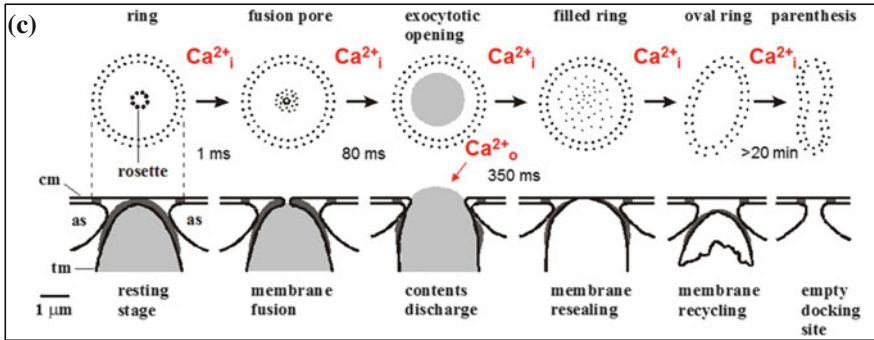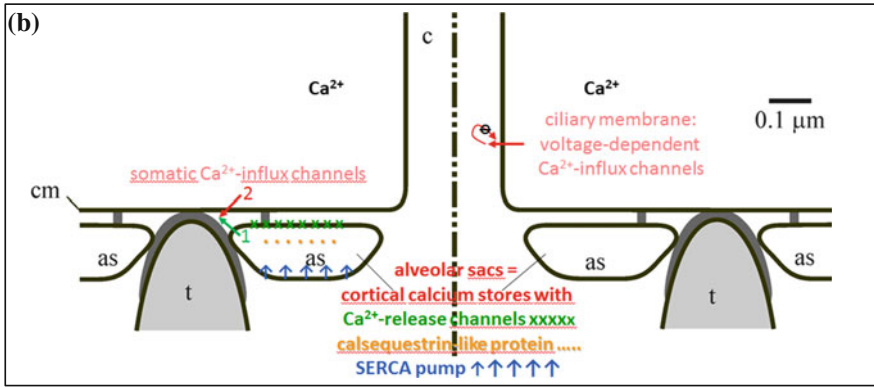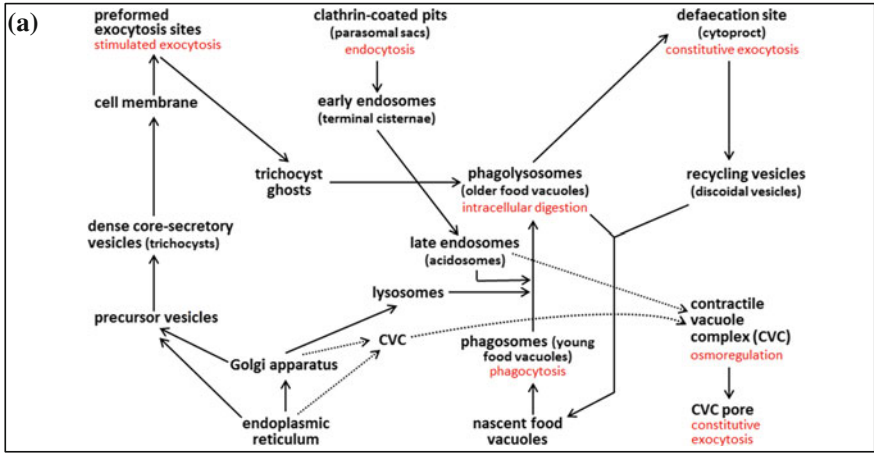
(G-proteins), the GTPases, not only in higher eukaryotes (Zhen and Stenmark 2015) but also in ciliates (Bright et al. 2010). Isoforms, i.e. paralogs or ohnologs (in case of diversification following whole genome duplications, particularly in *P. tetraurelia*), can be assigned to different steps and routes of vesicle trafficking and, thus, mirror the high complexity of the ciliate cell.

2.1.3 Long- and Short-Range Signals

The distinction between short-range and long-range signals has been extensively elaborated elsewhere (Plattner 2016a). A typical long-range signal is the docking of trichocysts (Aufderheide 1978) along microtubules which emanate from ciliary basal bodies and, thus, serve as transport rails (Plattner et al. 1982). This has to be complemented by short-range signals. For instance SNAREs and G-proteins are important for vesicle docking and finally membrane fusion. Local Ca^{2+} increase is another signal which has to arise from a nearby source since Ca^{2+} signals decay rapidly (Neher 1998). This also guarantees selective activation of distinct sites and also avoids cytotoxicity (Plattner and Verkhratsky 2015). Local restriction of Ca^{2+} signaling is most obvious, for instance, by the assignment of different CRCs types to different trafficking organelles, from the cell surface to deep inside, in *Paramecium* (Plattner 2015a). Moreover, ciliates fascinate particularly by their highly regular design that predetermines their vesicle trafficking routes and signaling sites based on epigenetic phenomena (Frankel 2000; Beisson 2008). Accordingly, cilia and secretory organelles are arranged in a strikingly regular surface pattern.

2.2 Overview of Trafficking Regulation Along Different Signaling Pathways

Basic trafficking pathways in ciliates are outlined in Fig. 2.1a. Box 1 outlines different kinds of cytoplasmic signaling operating in ciliates. Despite the old evolutionary age of ciliates, signaling mechanisms are quite similar to those in animals and—with exceptions—in plants. Similarities encompass the role of monomeric GTP-binding proteins (G-proteins acting as GTPases) (Bright et al. 2010), H^+-ATPase, SNARE proteins and their chaperone, NSF, as well as the regulation of membrane fusion by a local Ca^{2+} signal (Plattner 2010). The importance of luminal acidification of trafficking vesicles is derived from the observation that a transmembrane signal generated by the conformational change of H^+-ATPase intramembranous V0 part causes binding of GTPase modulators (Hurtado-Lorenzo et al. 2006), thus facilitating docking and membrane fusion. Specificity of vesicle interaction is finally mediated by SNAREs (Plattner 2010) and GTPases (Bright et al. 2010). Sequences encoding GTPases and GTPase modulators, such as GAP

(a) Diagram of membrane trafficking pathways including preformed exocytosis sites (stimulated exocytosis), clathrin-coated pits (parasomal sacs) (endocytosis), defaecation site (cytoproct) (constitutive exocytosis), cell membrane, early endosomes (terminal cisternae), trichocyst ghosts, phagolysosomes (older food vacuoles) (intracellular digestion), recycling vesicles (discoidal vesicles), dense core-secretory vesicles (trichocysts), late endosomes (acidosomes), lysosomes, contractile vacuole complex (CVC) (osmoregulation), precursor vesicles, CVC, phagosomes (young food vacuoles) (phagocytosis), Golgi apparatus, endoplasmic reticulum, nascent food vacuoles, CVC pore (constitutive exocytosis).

(b) c; Ca²⁺; Ca²⁺; ciliary membrane: voltage-dependent Ca²⁺-influx channels; 0.1 μm; somatic Ca²⁺-influx channels; cm; as; t; alveolar sacs = cortical calcium stores with Ca²⁺-release channels xxxxx; calsequestrin-like protein .....; SERCA pump ↑↑↑↑↑.

(c) ring; fusion pore; exocytotic opening; filled ring; oval ring; parenthesis; Ca^{2+}_i; Ca^{2+}_i; Ca^{2+}_i; Ca^{2+}_i; Ca^{2+}_i; rosette; 1 ms; 80 ms; Ca^{2+}_o; 350 ms; >20 min; cm; as; as; tm; 1 μm; resting stage; membrane fusion; contents discharge; membrane resealing; membrane recycling; empty docking site.

◄ **Fig. 2.1** Signaling pathways in the *Paramecium* cell. **a** Vesicle trafficking pathways encompass different main streams, such as the exocytotic, the endocytotic, the phagocytotic pathway and less overt trafficking in the contractile vacuole complex. *Dotted arrows* are less well established, particularly membrane input into this organelle via acidosomes, as derived from various recent papers about other protists. Also for proteins passing or bypassing the Golgi apparatus has not yet been sufficiently specified in detail. **b** Cortial organelles, such as cilia and exocytosis sites are regulated separately. Depolarization induces ciliary beat reversal by Ca^{2+} influx via ciliary voltage-dependent Ca^{2+} channels, abolished via negative feedback (Θ) by intraciliary $[Ca^{2+}]$ increase. CRCs in alveolar sacs, type RyR-LPs, are facing the plasmamembrane, opposite to the SERCA pump. Alveolar sacs contain a calsequestrin-like high capacity/low affinity CaBP. Trichocyst exocytosis is governed by a SOCE mechanism (store-operated Ca^{2+}-entry), i.e. Ca^{2+} release from alveolar sacs in a first step, followed by Ca^{2+} influx via somatic (non-ciliary) channels in a tightly coupled second step. **c** Summary of events during trichocyst exocytosis. *Top* Freeze-fracture images of fusion/resealing stages and their estimated duration, derived from synchronous stimulation/quenched-flow/rapid freezing analysis. Note decay of rosette particle aggregates and rapid formation of a fusion pore which expands and, thus, allows Ca^{2+} access to the secretory contents which triggers their explosive discharge by densondensation (stretching). *Below* Parallel situations seen on ultrathin sections. **a** Data pertinent to trichocyst processing are based on previous reviews (Plattner et al. 1993; Plattner 2014), those for endo-/phagocytotic trafficking are mainly derived from Allen and Fok (2000) and **c** trafficking in context of the contractile vacuole complex is based on recent reviews (Plattner 2015b, 2016a) **b** is modified from Plattner (2014), **c** is modified from Plattner et al. (1993, 1997)

(guanine nucleotide activation protein) and GEF (guanine nucleotide exchange factor), also occur in the *P. tetraurelia* database (Plattner and Kissmehl 2003b).

The Ca^{2+} signal is generated by intracellular CRCs of which different types are assigned to different organelles (Ladenburger and Plattner 2011; Plattner and Verkhratsky 2013). The Ca^{2+}-sensor causing fusion, as known from higher eukaryotes, is a low capacity/high affinity Ca^{2+}-binding protein (CaBP) which usually contains two high affinity Ca^{2+}-binding C2 domains (β-barrels with a Ca^{2+}-binding loop), such as synaptotagmin (Rizo et al. 2006; Südhof 2014). Although such CaBPs have not yet been specified in ciliates, equivalents of synaptotagmin occur in the *P. tetraurelia* database (Farrell et al. 2012). Extended synaptotagmins (e-syntag) with more than two C2 domains are known from some mammalian cells (Min et al. 2007), but they also occur in the *Paramecium* database (H. Plattner and R. Kissmehl, unpublished observations). Calmodulin (CaM) is another low capacity/high affinity CaBP, with four EF-hand type loops, each with high affinity Ca^{2+}-binding capacity. CaM operates at many sites also in ciliates. In the CaM molecule, the extensive conformational change upon hierarchical Ca^{2+} binding in the EF-hand loops I to IV represents the transduction of a chemical to a molecular-mechanical signal (Park et al. 2008). Thus, CaM can regulate a variety of surface influx channels (Saimi and Kung 2002), phagocytosis (Gonda et al. 2000) and probably endocytosis, also in ciliates.

Box 1 also shows that Ca^{2+} for activation may eventually also come from the outside medium for some specific effects, e.g. for activating some nucleotide cyclases, kinases and phosphatases, in the context of ciliary activity. This includes the signaling function of cyclic nucleotides, such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) and activation of the

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Box 1. Kinds of cytoplasmic signals operating in ciliates – a survey

Electrical signals: depolarization, hyperpolarization → change of ciliary beat

Acidification of organelle lumen

      H^+-ATPase/pump → binding of GTPases + their modulators → membrane docking
           and fusion by interference of SNARE proteins (SNARE = soluble N-
           ethylmaleimide sensitive factor [NSF] attachment protein receptors) and low
           capacity/high affinity Ca^{2+}-binding proteins (CaBPs) with C2-domains

Ca^{2+}, from outside or from intracellular stores (Ca^{2+}_o, Ca^{2+}_i)

      → activation of some nucleotide cyclases, some protein kinases and phosphatases, of
           CaBPs and of membrane fusion and fission (synaptotagmin with two
           C2 domains, extended synaptotagmin with more C2 domains): to be settled for
           ciliates

Nucleotides

      cyclic adenosine monophosphate (cAMP) → protein kinase A (PKA)
      cyclic guanosine monophosphate (cAMP) → protein kinase G (PKG)
           → protein phosphorylation, also in ciliates
      cyclic adenosinediphosphoribose (cADPR) from NAD (nicotinamide
           adeninedinucleotide) → activation of some (ryanodine receptor-type) Ca^{2+}-
           release channels (CRCs) in mammalian cells: activators effective in ciliates,
           but not yet assigned to specific channels
      nicotinic acid adenine dinucleotidephosphate (NAADP), formed from NADP,
           → activation of two-pore channels, in acidic compartments: also in ciliates?

Protein phosphorylation

      PKA, PKG: as defined above
      Ca^{2+}-dependent protein kinases (CDPKs) with integrated calmodulin- (CaM)-like
           motifs

Protein dephosphorylation by protein phosphatases (PPs) type PP1, PP2A, PP2B

      PP2B (calcineurin)
         with subunit (SU) A (catalytic SU, containing SU-B- and CaM-binding domain)
         and SU-B (regulatory SU, with Ca^{2+}-binding domain) also in ciliates

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respective protein kinases, protein kinase A (PKA) and protein kinase G (PKG) (Bonini and Nelson 1990); for review see Plattner (2016a). Also some metabolic Ca^{2+} channel activators, such as *c*yclic *a*denosine*dip*hospho*ri*bose (cADPR) and *n*icotinic *a*cid *a*denine *d*inucleotide*p*hosphate (NAADP) are derived

from nucleotides, i.e. nicotinamide adeninedinucleotide (NAD) and nicotinic acid adenine dinucleotidephosphate (NAADP), respectively, as known from vertebrates (Lee 2012). For cADPR and NAADP effects there is only circumstantial evidence in *Paramecium* (Plattner et al. 2012).

A total of 2600 kinases has been found in the *P. tetraurelia* genome (Bemm et al. 2009), thus contributing by 7 % to the macronuclear genome. In *T. thermophila* the proportion is 3.8 % (Tian et al. 2014). Both values stress their importance for signal transduction. The difference between the two genera may originate from whole genome duplication in *Paramecium*. A considerable difference between protein kinases in animal cells and in ciliates is the absence in the latter of a "CaM kinase", i.e. a kinase activated by a complex of calmodulin (CaM) and Ca^{2+}. Whereas such CaM-kinases in metazoans contribute to the regulation of neuronal activity, they are replaced in ciliates by "Ca^{2+}-*dependent protein kinases*" (CDPKs). These contain CaM-like sequences integrated in the kinase molecule (Kim et al. 1998).

Box 1 also indicates the occurrence in ciliates of protein phosphatases (PPs), e.g. PP1, PP2A and PP2B. PP2B, which is identical with calcineurin, encompasses two subunits, catalytical subunit A and regulatory subunit B, from ciliates (Fraga et al. 2010) to man where it regulates immune-response and long term potentiation, i.e. learning. In ciliates, multiple roles can be expected for calcineurin, including exo-/endocytosis regulation (Momayezi et al. 1987; Fraga et al. 2010).

2.3 Subcompartmentalization of Signaling Including Signaling in Cilia

Signals can be rather precisely restricted to subcompartments, e.g. cilia (Box 2), for which Box 3 shows details. Mechanical stimulation of a ciliated protozoan cell causes depolarization or hyperpolarization, depending on whether stimulation occurs at the anterior or posterior part of the cell (Eckert and Brehm 1979; Machemer 1988a, b). This is enabled by a graded differential distribution of specific ion channels over the somatic (non-ciliary) cell membrane. The respective receptor potential formed by different ion conductances activates different mechanisms in cilia. For instance, depolarization activates voltage-dependent Ca^{2+}-channels selectively occurring in cilia (Machemer and Ogura 1979) and, thus, a Ca^{2+}-carried action potential. (This signaling occurs no more in metazoans beyond Ctenophores.) Increased intraciliary Ca^{2+} shuts off this Ca^{2+} influx (Brehm and Eckert 1978). Hyperpolarization accelerates forward swimming (Preston et al. 1992).

During de- and hyperpolarization, different cyclic nucleotides are formed, activating PKG and PKA, respectively (Box 3). Ciliary activation mechanisms are independent of Ca^{2+}-activated processes during exocytosis, except when massive exocytosis stimulation entails an exuberant Ca^{2+} signal (Husser et al. 2004). In

Box 2. Examples of subcompartmentalization of signals in ciliates

External mechanical stimulation

$\rightarrow$ Ca^{2+} signaling in cortical domains, e.g. for receptor potential formation

anterior stimulation $\rightarrow$ depolarization/action potential $\rightarrow$ ciliary reversal

posterior stimulation $\rightarrow$ hyperpolarization $\rightarrow$ accelerated forward

swimming

for details of ciliary activity, see Box 3

Activation of non-ciliary (somatic) membrane phenomena for exocytosis: see Box 5

Constitutive local intracellular Ca^{2+} signaling via organelle-specific Ca^{2+}-release channels

(CRCs) in all trafficking vesicles

Organelle specific protein phosphorylation processes for other activation mechanisms: see

Box 8

Box 3. Ciliary beat activity in ciliates

(A) Anterior mechanical stimulus (hitting an obstacle)

somatic cell membrane $\rightarrow$ receptor potential: depolarization by activation of anterior

mechanosenstive Ca^{2+}-channels, repolarization by K^+-efflux

Effect of depolarization on cilia: action potential by

activation of voltage-dependent (ΔV) Ca^{2+} influx channels in ciliary membrane

$\rightarrow$ guanylate cyclase activation $\rightarrow$ cGMP formation $\rightarrow$ PKG activation

$\rightarrow$ phosphorylation of target proteins in ciliary axoneme;

in parallel: activation of axonemal calmodulin (CaM) $\rightarrow$ different effects

No Ca^{2+} spillover into cell soma

Inactivation of ciliary reversal by closing ciliary ΔV-channels by a Ca^{2+}/CaM complex

and binding of excessive Ca^{2+} to immobile buffer (CaM, centrin)

(B) Posterior mechanical stimulus

Hyperpolarization by K^+ efflux (somatic cell membrane)

$\rightarrow$ hyperpolarization-activated Ca^{2+}-channels (somatic cell membrane),

adenylate cyclase activation $\rightarrow$ cAMP formation $\rightarrow$ PKA activation $\rightarrow$

phosphorylation of target proteins in ciliary axoneme

(C) Gravikinesis/gravitaxis

Positive gravitaxis: "statocyst"-mediated intracellular signal perception (*Loxodes*)

Negative gravitaxis: very much predominant form of gravitaxis (*Paramecium*, *Tetrahymena*)

activation by hyperpolarization via posteriorly enriched K^+-channels; postulated link

to cortical F-actin $\rightarrow$ upward movement

summary, a mechanical signal is transformed into a long-range electrical signal generated at the somatic cell membrane that is transduced into cilia where it causes short-range Ca^{2+} signaling and a mechanical response in ciliary activity.

Mechanisms described for basic ciliary activity (Fig. 2.1b) also apply to chemotaxis and to gravitaxis/gravikinesis (Box 3). Chemotaxis requires the activation of distinct ion conductances to achieve specific behavioral responses (Saimi and Kung 2002; Bell et al. 2007; Yano et al. 2015). Positive gravitaxis is rare in ciliates where negative gravitaxis, causing upward swimming in the gravity field, by far prevails. For this, *Paramecium* is the best analyzed example (Machemer et al. 1998; Hemmersbach and Braun 2006; Machemer 2014). Accordingly cAMP and PKA are assumed to be involved in negative gravitaxis (Hemmersbach et al. 2002). Investigators assume that, for sensing gravity, channels have to be linked to filamentous actin (F-actin) (Machemer 2014). In fact, actin has been localized to the cell cortex (Sehring et al. 2007) and, even more precisely, to the narrow space between cell membrane and alveolar sacs (Kissmehl et al. 2004).

2.4 Organelle Trafficking Signals

2.4.1 Molecular Background

Long-range signals, such as firmly installed microtubules, can guide vesicles to their target sites (Box 4). This is true of trichocysts (Aufderheide 1978; Plattner et al. 1982) and organelles of the phagocytotic cycle (Allen and Fok 2000). Short-range signals involved are GTPases, SNAREs, H^+-ATPase, as outlined in Sect. 2.1, together with actin. For GTPases (Bright et al. 2010) and the other key players, organelle specific isoforms are available (Plattner 2010). The multimeric H^+-ATPase molecule is composed of an intramembranous V0 basepiece and a catalytic head, V1, which may dis- and re-assemble by interaction with an elongate, variable a-SU (Sun-Wada and Wada 2015). Considering the key role of H^+-ATPase (Sect. 2.2), the unsurpassed number of 17 a-subunits in *Paramecium* may mediate adjustment to local requirements (Wassmer et al. 2005, 2006, 2009). Among SNAREs, longin-type sequences in *Paramecium's* "synaptobrevins" may contribute to organelle specificity, in addition to the usual domain sequences (Schilde et al. 2006, 2010). In *P. tetraurelia*, plasmalemmal Syntaxin 1 (*Pt*Syx1) is engaged in trichocyst exocytosis (Kissmehl et al. 2007). For more details, see Plattner (2010, 2016a).

Vesicles undergoing trafficking are endowed with CRCs identical with, or related to InsP$_3$Rs and RyRs (Ladenburger and Plattner 2011; Plattner and Verkhratsky 2013); see Box 5. An exception are trichocysts which seem to be devoid of luminal Ca^{2+}, in contrast to what is known from some other dense core-secretory vesicles, endosomes and phagocytotic organelles of higher eukaryotes (Hay 2007). The presence of the key players mentioned above, including

Box 4. Organellar trafficking signals

Long-range signals

      microtubular "rails" as firmly established guidelines (emanating from oral cavity [for

            phagocytosis] and from ciliary basal bodies [for trichocyst docking],

            respectively)

Short-range signals/molecular recognition sites

      cooperative role of H^+-ATPase molecules (acidification of organelle lumen) $\rightarrow$

            binding of organelle-specific small GTPases (+ modifying proteins, e.g. GAP

            = guanosine nucleotide activation protein, GEF = guanosine nucleotide

            exchange factor, as known from higher eukaryotes)

      vesicle docking: SNAREs + GTPases

            $\rightarrow$ docking at target organelle/membrane: organelle-specific GTPase-binding

            proteins yet to be identified

      local Ca^{2+} signal and Ca^{2+} sensor activation $\rightarrow$ membrane fusion

Vesicle budding: coatamer proteins (COPs), clathrin, adaptor proteins

Golgi apparatus: ill-defined molecular cues and signals in ciliates awaiting srutiny

CRCs, in the endo-/phagocytotic cycle of *Paramecium* may reflect the intensity and multitude of vesicle trafficking known from ultrastructural studies (Allen and Fok 2000) [In *Paramecium*, not all of these vesicles are acidic (Wassmer et al. 2009), and not all lysosomal enzymes have an acidic pH-optimum (Fok and Paeste 1982; Fok 1983)]. Appropriate CRCs may drive membrane interactions in concert with, or independently from other key players. The importance of local availability and regulation of Ca^{2+} during membrane docking and fusion is discussed in the accompanying paper (Plattner 2016b). The numerous members of the six CRC subfamilies found in *Paramecium* may fine tune Ca^{2+} signals and membrane interactions depending on local requirements.

2.4.2 *Dense Core-Secretory Vesicle Exocytosis*

Ca^{2+} regulation of trichocyst exocytosis involves three steps (Box 5, Fig. 2.1b, c): (i) Ca^{2+} release from alveolar sacs via RyR-like proteins and (ii) immediately superimposed Ca^{2+} influx from the outside medium (Klauke and Plattner 1997; Ladenburger and Plattner 2011; Plattner 2014). Both mechanisms acting in concert are called store-operated Ca^{2+} entry, SOCE—a mechanism maintained up to mammals. A large excess of Ca^{2+}, much more than seen by fluorochromes, has to flood trichocyst exocytosis sites to become activated, just as in some

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Box 5. Why a multiplicity of Ca^{2+}-release channels?

Paramecium contains 34 genes for Ca^{2+}-release channels (CRCs, 6 subfamilies), to be assigned to the superfamily of $InsP_3R$/Ryanodine receptor (RyR) type CRCs, distributed over different trafficking organelles for local signaling

Example A - RyR-like channels: alveolar sacs (cortical Ca^{2+} stores)

activation by RyR activators caffeine, 4-chloro-m-cresol or by polyamines (AED)

$\rightarrow Ca^{2+}$ release from alveolar sacs by RyR-LP of CRC-IV subfamily,

superimposed by Ca^{2+}-entry from the outside medium (store-operated Ca^{2+}-entry, SOCE) $\rightarrow$ trichocyst exocytosis based on

(i) membrane fusion (depending on $[Ca^{2+}]_i$ increase by SOCE) and $\rightarrow$

(ii) Ca^{2+}_o access to trichocyst contents for inducing decondensation (vigorous ejection)

membrane resealing and ghost detachment (exocytosis-coupled endocytosis), also driven by $[Ca^{2+}]_i$ increase

Example B - $InsP_3Rs$: occurring in the contractile vacuole complex; serving

(i)   for fine-tuning of $[Ca^{2+}]$ in the cytosol and

(ii)   for internal membrane restructuring (hypothetic) during contraction cycles

$InsP_3R$/RyR- type channels also include mixed types, in compartments undergoing trafficking

Additional Ca^{2+}-release channels in ciliates

two-pore channels NAADP-activated, in acidic stores? Occurrence likely

TRP-type and mechanosensitive channels: also not yet specified in ciliates

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neuroendocrine cells (for details, see Plattner 2016a). (iii) Discharge of contents follows formation of an exocytotic opening and requires the entry of Ca^{2+} from the outside and binding to some secretory components, thus causing decondensation by conformational change (Plattner et al. 1997; Klauke et al. 1998; Plattner 2014). This in turn depends on proper processing of secretory protein precursors (Pouphile et al. 1986; Bowman et al. 2005).

2.4.3   The Phagocytotic Cycle

This aspect is reviewed here in more detail, as it demonstrates the complex sequence of interacting signaling molecules although these are only partially known.

The phagocytotic cycle in *Paramecium* requires multiple signaling (Allen and Fok 2000), including firmly established microtubules as long-range signals and variable stage-dependent short-range signals. In detail the sequence is as follows. (i) At the cytopharynx, at the bottom of the cytostome, vesicles recycling from advanced stages of food vacuoles, together with vesicles from the cytoproct, deliver membrane material for a bulging nascent food vacuole. Thus, a phagosome is formed at converging microtubular rails, the "postoral fibers". (ii) After detachment, acidosomes (late endosomes) fuse with the phagosome, thus endowing it with H^+-ATPase molecules for luminal acidification. (iii) This is followed by fusion with lysosomes, thus forming phagolysosomes. (iv) Lysosomal enzymes are retrieved later on during cyclosis, (v) as are parts of the membrane for delivery to the cytopharynx. (vi) The contents of spent food vacuoles are released by exocytosis at the cytoproct and membranes are recycled as indicated for step (i) (Allen and Fok 2000).

In *Paramecium tetraurelia*, key players for signaling in the different stages (Box 6) encompass exchanging sets of SNAREs (Schilde et al. 2006, 2010; Kissmehl et al. 2007), subunits (SU) of H^+-ATPase (Wassmer et al. 2005, 2006), and actin, as outlined in a separate chapter (Plattner 2016b). In *Tetrahymena*, different types of GTPases are exchanged during cyclosis (Bright et al. 2010). In *Paramecium*, the exchange of numerous actin isoforms, types 1, 3, 6, 8, 11–14 and 16 as well as their patchy or unilateral arrangement in some stages is a most striking phenomenon (Sehring et al. 2007). This may serve propulsion of the organelle and/or regulation of accessibility to fusion and/or budding of vesicles. All this documents a series of interacting long- and short-range signaling during cyclosis.

Box 6. The phagocytotic cycle in ciliates

At cytopharynx

    cell membrane enlargement by fusion of recycling vesicles → association with actin

        → nascent food vacuole pinches off to form a phagosome

During cyclosis

    → fusion with acidosomes (late endosomes) providing H^+-ATPase → luminal

        acidification → fusion with lysosomes to form a phagolysosome (mature food

        vacuole)

    endowment with varying SNAREs, small GTPases, and

        actin coats (for details see text), whereas Ca^{2+}-release channels are throughout

        of type CRC-III (InsP$_3$R-type)

    selective membrane input and retrieval, contents digestion

    pH gradually increasing to ~7

At cytoproct

    contents discharge by exocytosis and formation of recycling ("discoidal") vesicles

2.4.4 The Contractile Vacuole Complex

Surprisingly, the contractile vacuole complex contains all components relevant for vesicle trafficking, except actin, in even higher variability and with strict localization to specific substructures, such as the vacuole, the pore and the meshwork of the smooth spongiome (Box 7). The organelle has a very complex design (Allen and Naitoh 2002). It not only can expel fluid with an excess of Ca^{2+} and other ions (Stock et al. 2002), but it also shows some reflux of Ca^{2+} into the cytosol via constitutively active $InsP_3Rs$ (Ladenburger et al. 2006). This may serve not only for fine tuning of cytosolic Ca^{2+} but also to drive the extensive membrane fusion and fission processes within the organelle during systole/diastole cycles (Plattner 2015b).

Box 7. Signaling in the contractile vacuole complex

Signals assumed for self-assembly of new anlagen: centrin, CaM, γ-tubulin, NIMA kinase,
      as discussed elsewhere (Plattner 2015b)

Signals assumed for organelle growth: delivery of vesicle with specific v-/R- and t-/Q
      SNAREs

Local tubularization (spongiome) and reversible planar-tubular transitions: F-BAR proteins
      (hypothetic), as discussed elsewhere (Plattner 2015b)

Acidification by V-type H^+-ATPase
      ΔH^+ exploited for Ca^{2+} sequestration (hypothetic $X^+/H^+/Ca^{2+}$ exchanger, in addition to
      Ca^{2+}-ATPase (see Plattner 2016a) and expulsion of H_2O, Ca^{2+} and other ions by
      periodic exocytotic release at the pore
      CRCs of type $InsP_3R$ for constitutive partial Ca^{2+} reflux into cytosol: for $[Ca^{2+}]_i$ fine
      tuning and probably for spongiome restructuring

Pore for periodic contents release by exocytosis: with specific SNAREs and CRCs at the pore
      periodic signal for vacuole contents release: mechanosensitive channels (suggested by
      occurrence of stomatin [Reuter et al. 2013] and in agreement with other systems
      [Plattner 2015b]), in conjunction with pore-specific SNAREs and CRCs

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2.4.5 Additional Signals

Little is known about other types of Ca^{2+} release channels in ciliates, such as two pore-channels (TPC) and transient receptor potential-channels (TRPC) and their activators (Box 5). Particularly metabolic CRC activators (Lee 2012), such as cADPR, NAADP, remain to be assigned to different channels and organelles in

ciliates. Such channels have to be expected also in ciliates, based on microinjection studies (Plattner et al. 2012).

Vesicle budding at the Golgi apparatus and other organelles as well as at the plasmamembrane requires a set of additional proteins, such as coatamer proteins (COPs) and clathrin, together with their adaptor proteins known from higher eukaryotes up to mammals (Rothman 2014). In ciliate cells, coatamer coats are suggested to occur by electron microscopy in the cis- and trans-side of the Golgi apparatus (Allen and Fok 1993; Garreau De Loubresse 1993) and clathrin coats in addition by molecular biology according to Elde et al. (2005) who also reported the expression of adaptor proteins, AP-1, AP-2, AP-3 and AP-4 in *T. thermophila*. While none of them appear important for lysosome biogenesis (Briguglio et al. 2013), AP-2 is important for endocytosis via coated pits (Elde et al. 2005). Sequences encoding all these adaptor proteins have also been found in the *P. tetraurelia* database, in addition to the ARF/SAR-type G-protein known as a target of the drug, brefeldin A (Plattner and Kissmehl 2003b). The same is true of clathrin heavy chains and of COPs.

In summary, for vesicle trafficking ciliates have at their disposal most of the signaling components known from multicellular organisms. Note, however, that InsP$_3$R/RyR-like molecules are absent from higher plants (Plattner and Verkhratsky 2015), whereas they occur in some green algae (Wheeler and Brownlee 2008). Globally a ciliate's signaling machinery closely resembles that of metazoans.

2.5 Protein Phosphorylation for Activation and Deactivation of Signaling Processes

2.5.1 Phosphorylation Processes

As mentioned in Sect. 2.3, signaling in cilia includes PKA and PKG activity for enhanced forward and backward swimming, respectively (Kim et al. 1998; Kutomi et al. 2012). Activating cyclic nucleotides are generated within one ciliary stroke (Yang et al. 1997). Together with CDPKs they belong to the superfamily of Seryl/Threonyl kinases (Box 8). Phosphoproteins are substrates of the different phosphatases. Among them, PP1 dephosphorylates a ciliary phosphoprotein formed during ciliary reversal in *Paramecium* (Klumpp et al. 1990). PP2B/calcineurin probably has a broad spectrum of activity, depending on its A-subunit, whereas the two genes for the B-SU in *Paramecium* result in an identical translation product, with a well conserved binding domain in the A-SU (Fraga et al. 2010).

As indicated in Box 8 and discussed in more detail somewhere else (Plattner 2016a), the occurrence of Tyrosyl phosphorylation may be largely restricted in ciliates to cell cycle and mitosis regulation. Work with mammalian cells exposed to

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Box 8. Protein phosphorylation for signaling and activation processes in ciliates

(A) Seryl/Threonyl phosphorylation

      Protein kinases (PK)

            protein kinase A, PKA (cAMP-activated)

            protein kinase G, PKG (cGMP-activated)

            CDPK ($\underline{C}a^{2+}$-$\underline{d}$ependent $\underline{p}$rotein $\underline{k}$inase, with a CaM-like domain) substituting

            for CaM-kinase (activated by a separate Ca^{2+}/CaM-complex) in animal cells

      Protein phosphatases (PP): PP1, PP2A, PP2B (Ca^{2+}/CaM-dependent PP = calcineurin)

(B) Dedicated Tyrosyl phosphorylation

Some predicted for ciliates from proteomic analysis (still to be confirmed)

      mainly concerning cell cycle and mitosis regulation (MAPKs = $\underline{m}$itogen-$\underline{a}$ctivated

            $\underline{p}$rotein $\underline{k}$inases)

      new aspects emerging from phosphoproteomic analysis

Euplotes gamones indicates signaling via a mitogen-activated protein kinase (MAPK) cascade with Tyrosine phosphorylation (Vallesi et al. 2010; Cervia et al. 2013). See also chapter by Luporini.

2.5.2 Signal Downregulation

Also ciliates possess different ways to downregulate signals (Box 9). Cyclic nucleotides are deactivated by diesterases and phosphoproteins are dephosphory-lated by protein phosphatases. For instance, the association of calcineurin with parasomal sacs (Momayezi et al. 2000), the clathrin-coated pits in ciliates, is compatible with dynamin dephosphorylation known from mammalian coated pits.

   Ca^{2+} signals are downregulated by different mechanisms with different kinetics (Box 9). The most rapid is binding to centrin (Sehring et al. 2009) a CaBP with high capacity/low affinity (in addition to low capacity/high affinity) binding sites local-ized in the cell cortex of *Paramecium* (see Plattner 2016a). This is orders of mag-nitude more rapid than downregulation by Ca^{2+}-ATPases/pumps (Plattner 2016a) of which type Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) (Hauser et al. 1998) or plasmamembrane Ca^{2+}-ATPase (PMCA) (Elwess and Van Houten 1997) have been analyzed in *Paramecium*. PMCA also occurs in cilia of *Tetrahymena* (Dentler 1988) and *Paramecium* (Yano et al. 2013). These two are P-type ATPases because they autocatalytically form a phospho-intermediate which then dephosphorylates itself. Ca^{2+} exchangers, though not yet identified, show up in ciliate databases; they are driven by a H^+-gradient formed by a H^+-ATPase (V-type, in vesicles) operating without a phospho-intermediate formation. Although such

> **Box 9. Shut-down of signalling in ciliates**
>
> Inactivation of Ca^{2+}
>
>   binding to high capacity/low affinity Ca^{2+}-binding proteins, e.g. centrin
>
>   reduction by pumps and transporters
>
> Ca^{2+} extrusion and sequestration by Ca^{2+}-ATPases/pumps
>
>   PMCA (<u>p</u>lasma<u>m</u>embrane <u>C</u>a^{2+}-<u>A</u>TPase)
>
>   SERCA (<u>s</u>arcoplasmic/<u>e</u>ndoplasmic <u>r</u>eticulum <u>C</u>a^{2+}-<u>A</u>TPase)
>
>   hypothetical: X^+/Ca^{2+} exchangers, e.g. H^+/Ca^{2+} antiporter (postulated
>
>   specifically for contractile vacuole complex)
>
> Inactivation of cyclic nucleotides by diesterases
>
> Reversion of phosphorylation state
>
>   protein phosphatase PP1, possibly also PP2C, for deactivation of ciliary reversal
>
>   PP2B/calcineurin: pleiotropic effects to be expected, e.g. dynamin activation for
>
>   vesicle budding and regulation of Ca^{2+} stores, e.g. by effects on CRCs

exchangers urgently call for scrutiny in ciliates it appears that they are much more efficient in signal downregulation than the pumps (Ladenburger et al. 2006; Plattner 2016a).

2.6   Signaling by Surface Receptors

These aspects are summarized in Box 10. The occurrence of trimeric GTP-binding proteins (G-proteins) is likely (De Ondarza et al. 2003; Lampert et al. 2011), but not firmly established in protozoa in general (Krishnan et al. 2015) and in ciliates in particular since important details have not been examined yet, as discussed in more detail elsewhere (Plattner 2016a). The same is true of G-protein-coupled receptors (GPCRs). All this also applies to the secretagogue, aminoethyldextran, which, in *Paramecium,* is most efficient in activating highly synchronous exocytosis (Plattner et al. 1985; Plattner and Kissmehl 2003; Knoll et al. 1991) by a SOCE mechanism for trichocyst exocytosis (Hardt and Plattner 2000; Plattner 2014). For hints to MAPK activity and Tyrosyl phosphorylation, see Sect. 2.5.

Purinergic receptors can be assumed to occur in *Paramecium* as these cells, upon exposure to ≥10 µM GTP, perform periodic back- and forward swimming accompanied by depolarization (Clark et al. 1993) and Ca^{2+} waves oscillating with the same periodicity (Sehring and Plattner 2004). This is unusual insofar as purinergic receptors normally respond to ATP or, less common, to UTP. We assume a function in keeping cells from dispersal to low density which is known to inhibit cell division and maintenance of the population.

Box 10. Surface receptor signalling in ciliates

Trimeric GTP-binding proteins (G-proteins) and G-protein-coupled receptors (GPCRs)

existence in ciliates under considerable debate, fragmentary information

Mitogen-activated protein kinase (MAP kinase; MAPK): related activities are currently

assumed also for ciliates

Effects of exogenous GTP ($\geq$10 μM): Ca^{2+} oscillations in parallel to de-/repolarizations

the first $[Ca^{2+}]_i$ peak (larger than subsequent periodic peaks) requires Ca^{2+}_o

$\rightarrow$ further (smaller) cyclic activity peaks in ~8 s oscillations supported by Ca^{2+} from

internal stores (type of store for internal Ca^{2+} mobilization during GTP

activation: unknown) $\rightarrow$ ongoing periodic activation $\rightarrow$ desensitization

and signal downregulation (mechanism unknown) $\rightarrow$ decaying Ca^{2+} signal

Unknown: purinergic receptors, Ca^{2+}/polyvalent cation-sensing receptor (a GPCR?)

Chemotaxis chemoreceptors

Operating via specific ion conductances (see text)

2.7 Conclusions

Intracellular signaling by pheromones (gamones) in ciliates (Luporini et al. 2014) is summarized separately in this volume. Epigenetic signaling is also covered separately in this volume by Nowacki; for surveys, also see Chalker et al. (2013) and Simon and Plattner (2014). Most of the other signaling mechanisms described here seem to be evolutionarily old and maintained from protozoa on, particularly ciliates, up to top-ranking metazoans. The impressive complexity of ciliate cells and their elaborate trafficking system may have required a complex signaling system—an old heritage from early eukaryotic ancestors (Dacks and Field 2007; Plattner and Verkhratsky 2015).

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