Erschienen in: Journal of Eukaryotic Microbiology ; 61 (2014), 1. - S. 95-114

Calcium Regulation in the Protozoan Model, *Paramecium tetraurelia*

Helmut Plattner

Department of Biology, University of Konstanz, P.O. Box 5544, 78457, Konstanz, Germany

Keywords
Ca^{2+}; ciliate; protozoa; signaling; signal transduction.

Correspondence
H. Plattner, Department of Biology, University of Konstanz, P.O. Box 5544, 78457 Konstanz, Germany
Telephone number: (0)7531 882228;
e mail: helmut.plattner@uni konstanz.de

ABSTRACT

Early in eukaryotic evolution, the cell has evolved a considerable inventory of proteins engaged in the regulation of intracellular Ca^{2+} concentrations, not only to avoid toxic effects but beyond that to exploit the signaling capacity of Ca^{2+} by small changes in local concentration. Among protozoa, the ciliate *Paramecium* may now be one of the best analyzed models. Ciliary activity and exo-/endocytosis are governed by Ca^{2+}, the latter by Ca^{2+} mobilization from alveolar sacs and a superimposed store-operated Ca^{2+}-influx. *Paramecium* cells possess plasma membrane- and endoplasmic reticulum-resident Ca^{2+}-ATPases/pumps (PMCA, SERCA), a variety of Ca^{2+} influx channels, including mechanosensitive and voltage-dependent channels in the plasma membrane, furthermore a plethora of Ca^{2+}-release channels (CRC) of the inositol 1,4,5-trisphosphate and ryanodine receptor type in different compartments, notably the contractile vacuole complex and the alveolar sacs, as well as in vesicles participating in vesicular trafficking. Additional types of CRC probably also occur but they have not been identified at a molecular level as yet, as is the equivalent of synaptotagmin as a Ca^{2+} sensor for exocytosis. Among established targets and sensors of Ca^{2+} in *Paramecium* are calmodulin, calcineurin, as well as Ca^{2+}/calmodulin-dependent protein kinases, all with multiple functions. Thus, basic elements of Ca^{2+} signaling are available for *Paramecium*.

WORK published on Ca^{2+} in cells is legion, but mostly it has been performed with mammalian cells. Only rather few studies have dealt with free-living (Plattner et al. 2012; Plattner and Klauke 2001) and with parasitic protozoa (Moreno and Docampo 2003). Recently, there has been increasing interest in Ca^{2+} signaling especially in parasitic protozoa, but this meets many more methodical difficulties than work with free-living species. Another emerging aspect is that Ca^{2+} regulation and signaling must have been developed early in evolution, probably simultaneously with the formation of intracellular compartments and vesicle trafficking (Cai 2008; Plattner and Verkhratsky 2013). This review aims at elaborating features in common with higher eukaryotes and to pinpoint specific features pertinent to ciliates, based mainly on work with *Paramecium*. The data discussed are substantiated by localization studies summarized in Table 1.

CAPABILITIES OF CA^{2+} SIGNALING FROM *PARAMECIUM* TO MAN

Corollaries of the control of intracellular Ca^{2+} and its use for signaling

Already early cells must have struggled with leakage of Ca^{2+} from the sea water, considering the toxicity of intracellular Ca^{2+} even at moderate concentrations. Not only can DNA and protein molecules be precipitated but also phosphate ions which had been established as the pivotal aspect of bioenergetics in all cells (Plattner and Verkhratsky 2013). Therefore, cells had to evolve means to keep the concentration of free (dissolved) intracellular Ca^{2+}, $[Ca^{2+}]_i$, at ~100 nM (Berridge et al. 2003; Clapham 2007), i.e. well below that in the outside medium, $[Ca^{2+}]_o$, which is usually ~1 mM. Due to sequestration into organelles

Table 1. Distribution of selected proteins relevant for Ca^{2+} regulation, signaling, and homeostasis in *Paramecium*

Structure	Molecule	References
Plasma membrane, somatic	PMCA	Wright and van Houten (1990)
	Ca^{2+}/CaM activated Me^{+} channels	Kung et al. (1992)
	Mechanosensitive Ca^{2+} channels	Eckert and Brehm (1979)
Parasomal sacs	*Pt*CRC V 4	Ladenburger and Plattner (2011)
	Calmodulin	Momayezi et al. (1986)
	Calcineurin	Momayezi et al. (2000)
Plasma membrane, ciliary	Voltage dependent Ca^{2+} channels	Machemer and Ogura (1979)
	Guanylate cyclase (G cyclase)	Klumpp et al. (1983)
Inside cilia (membrane)	Calmodulin	Momayezi et al. (1986)
	Centrin	Gonda et al. (2007)
	Guanylate cyclase (Ca^{2+} dependent cGMP formation)	Linder et al. (1999)
	cGMP activated protein kinase (PKG)	Ann and Nelson (1995)
	CDPK	Kim et al. (2002)
	Protein phosphatase 1 (PP1)	Momayezi et al. (1996)
Basal bodies	Calmodulin	Momayezi et al. (1986)
Alveolar sacs	Calsequestrin LP	Plattner et al. (1997b)
	SERCA	Hauser et al. (1998, 2000)
		Plattner et al. (1999)
	*Pt*CRC IV 1 (RyR LP)	Ladenburger et al. 2009)
	*Pt*CRC V 4	Ladenburger and Plattner (2011)
	Guanylate cyclase	Linder et al. (1999)
Endoplasmic reticulum	Calreticulin LP	Plattner et al. (1997b)
	SERCA	Hauser et al. (2000)
	*Pt*CRC I 1 (with $InsP_3$ binding domain)	Ladenburger and Plattner (2011)
	*Pt*CRC IV 1 (RyR LP)	Ladenburger et al. (2006)
Trichocyst docking sites	Calmodulin	Momayezi et al. (1986)
	Calcineurin	Momayezi et al. (2000)
Trichocyst matrix	Ca^{2+} BPs	Klauke et al. (1998)
Infraciliary lattice	Calmodulin	Momayezi et al. (1986)
	Centrin	Beisson et al. (2001)
	CDPK	Kim et al. (2002)
Terminal cisternae	PtCRC VI 3	Ladenburger and Plattner (2011)
Cytostome	Calmodulin	Momayezi et al. (1986)
	*Pt*CRC VI 3	Ladenburger and Plattner (2011)
Food vacuoles and associated vesicles	Calmodulin	Momayezi et al. (1986)
	*Pt*CRC III 4	Ladenburger and Plattner (2011)
Recycling vesicles from food vacuoles	*Pt*CRC III 4	Ladenburger and Plattner (2011)
Undefined cortical vesicles	*Pt*CRC VI 2	Ladenburger and Plattner (2011)
Contractile vacuole complex smooth spongiome	*Pt*CRC II 1 ($InsP_3R$)	Ladenburger et al. (2006)
	*Pt*CRC V 4	Ladenburger and Plattner (2011)
Radial arms (canals)	Calmodulin	Momayezi et al. (1986)
	*Pt*CRC II 1 ($InsP_3R$)	Ladenburger et al. (2006)
	*Pt*CRC V 4	Ladenburger and Plattner (2011)
Contractile vacuole (bladder)	Calmodulin	Momayezi et al. (1986)
	*Pt*CRC II 1 ($InsP_3R$)	Ladenburger et al. (2006)
	*Pt*CRC V 4	Ladenburger and Plattner (2011)
Porus	*Pt*CRC VI 2	Ladenburger and Plattner (2011)
	*Pt*CRC VI 3	Ladenburger and Plattner (2011
Macronucleus, inside	Calcineurin	Momayezi et al. (2000)
Micronucleus (membrane)	*Pt*CRC V 4	Ladenburger and Plattner (2011)
Cleavage furrow (vesicles)	*Pt*CRC V 4	Ladenburger and Plattner (2011)

*Pt*CRC designates Ca^{2+} release channels related to $InsP_3Rs$ and RyRs. CaM calmodulin. Some localizations are based on functional effects and/or results from cell fractionation (e.g. plasma membrane channels, G cyclase, PKG).

and extensive binding of intracellular Ca^{2+} to highly acidic proteins, called high capacity/low affinity Ca^{2+}-binding proteins, total intracellular Ca^{2+} concentration (designated [Ca] free and bound calcium) is ~1 mM in the cytosol and between ~0.1 and ≥ 10 mM in Ca^{2+} stores. All these approximate values have been discussed in detail in the context of the cortical Ca^{2+} stores of *Paramecium*, the alveolar sacs (Hardt and Plattner 2000). Although actual

values may deviate more or less from these pilot values, these corollaries make Ca^{2+} very suitable for signaling for the following reasons. (i) Leakage from outside can be controlled by pumps in the plasma membrane (also an old evolutionary heritage) and in membranes of stores which both keep $[Ca^{2+}]_i$ low (Berridge et al. 2003; Clapham 2007). (ii) Low $[Ca^{2+}]_i$ in the resting cell sets a favorable baseline for Ca^{2+}-based signaling events. (iii) Small increases, mostly by one or two orders of magnitude above $[Ca^{2+}]_i^{rest}$, provide energetically favorable conditions for Ca^{2+} as an intracellular second messenger. (iv) Any increases may also reach mitochondria where Ca^{2+} activates some of the matrix dehydrogenases of the tricarboxylic acid cycle (Tarasov et al. 2012), thus contributing to energy supply for the re-establishment of $[Ca^{2+}]_i$ homeostasis. In summary, evolution has tamed toxic Ca^{2+} and even taken advantage of it for signaling purposes.

Molecular effects of Ca^{2+}

To achieve signal transfer after a stimulus from the outside, the second messenger Ca^{2+} has to exert some effect on one of the many target and effector molecules. Examples are the low capacity/high affinity Ca^{2+}-binding proteins, for instance, calmodulin, synaptotagmin and, with some regard, centrin. These proteins undergo significant conformational changes when they bind Ca^{2+}. For instance, a Ca^{2+}/calmodulin complex may bind to effector molecules (some Ca^{2+}-ATPases/pumps, protein phosphatase 2B calcineurin, etc.) or the respective Ca^{2+}-binding protein is an effector by itself (protein kinases type Ca^{2+}-dependent protein kinases [CDPK], synaptotagmin, centrin). Most of these molecules are known from *Paramecium*. Let us consider some examples in more detail.

(i) Calmodulin is well preserved throughout eukaryotes (Kung et al. 1992) and binds Ca^{2+} in four consecutive EF-hand motifs I IV, i.e. loops of usually ~12 amino acids, in a hierarchal sequence with decreasing Ca^{2+} affinity. Ca^{2+} is weakly bound by coordinative forces, thus allowing easy association and dissociation, i.e. activation and deactivation cycles in rapid sequence. Calmodulin has many sites of action in a cell. For example, maximal activation of the plasma membrane Ca^{2+}-ATPase (PMCA) requires binding of calmodulin to its carboxy-terminal part. Almost all eukaryotes use this pump. Interestingly, the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is devoid of a calmodulin-binding domain (Palmgren and Nissen 2011). Both these molecules transiently form a phospho-intermediate and, therefore, are called P-type Ca^{2+}-ATPases and both occur in *Paramecium* (Wright and van Houten 1990; Hauser et al. 1998).

(ii) In metazoans, synaptotagmin is the Ca^{2+} sensor inducing the fusion of membranes/compartments (Rizo et al. 2006) tethered to each other by SNARE proteins (*s*oluble *N*-ethylmaleimide sensitive factor [NSF] *a*ttachment protein *re*ceptors) in conjunction with monomeric GTP-binding proteins (monomeric G-proteins, small GTPases). Synaptotagmin contains two C2-domains, C2A and C2B, each with a β-barrel motif. From this, a Ca^{2+}-binding loop sticks out which, when activated by Ca^{2+}, can interact with membrane lipids in a way to promote fusion. Isoforms with different Ca^{2+} binding properties occur (Sugita et al. 2002). In gland cells, for instance, different synaptotagmins are distributed over different kinds of vesicles undergoing trafficking (Becherer et al. 2012). A detailed map of intracellular distribution of SNAREs exists for *Paramecium* (Plattner 2010), but any equivalent of synaptotagmin is not known from any protozoan.

(iii) Some cytosolic proteins contain not only EF-hand motifs like calmodulin, but in addition acidic stretches with high Ca^{2+} binding capacity. An example is centrin the molecule enabling some protozoa to rapidly contract, as known from *Paramecium*.

On this background we may now consider in detail the situation in *Paramecium*.

Ca^{2+} extrusion at the cell surface

At the cell membrane, the PMCA-type Ca^{2+}-ATPase (Ca^{2+}-pump) permanently extrudes Ca^{2+} from the cell. As its activity is accelerated by a Ca^{2+}/calmodulin complex (see above) increasing $[Ca^{2+}]_i$ facilitates re-establishment of $[Ca^{2+}]_i^{rest}$. As a P-type ATPase, the PMCA is auto-phosphorylated and auto-dephosphorylated with each activity cycle. In the heart muscle sarcolemma, a Ca^{2+}/Na^+ antiporter (exchanger) supports this ongoing activity, with sodium being equilibrated by a secondary active transport process. Although claimed also for ciliates (Burlando et al. 1999), such activity could not be convincingly ascertained.

Ca^{2+} channels in the cell membrane

The Ca^{2+} influx channels contained in the cell membrane may be activated by different stimuli. Examples are mechanosensitive channels and voltage-dependent Ca^{2+} channels. Such channels are known from protozoa up to man, but their molecular and functional properties may greatly vary. For instance, when a *Paramecium* cell hits its "nose" on an obstacle, this activates mechanosensitive Ca^{2+}-influx channels in the somatic (nonciliary) cell membrane, causing generation of a receptor potential based on K^+-efflux and Ca^{2+}-influx. Here, Ca^{2+} quite unusually serves as a charge carrier. This depolarizing signal activates voltage- (depolarization-) dependent Ca^{2+}-influx channels in the ciliary membranes. Activation means a conformational change in the molecule and concomitantly of its conductivity due to the voltage applied. In consequence, the increase in $[Ca^{2+}]$ inside cilia causes a reversal of the ciliary beat (ciliary reversal). These channels are rapidly inactivated by binding of a Ca^{2+}/calmodulin complex which forms upon Ca^{2+} influx (Brehm and Eckert 1978). Basically, this feedback mechanism is conserved

for L-type Ca^{2+}-influx channels up to neuronal cells in our brain (Levitan 1999; Oliveria et al. 2012) a fundamental mechanism underlying our intellectual capabilities. Later on we will see the involvement of calcineurin in this basic response, which, in man, is also pivotal for brain function and the immune response. Thus, evolution has provided essential tools already at the level of *Paramecium*.

In *Paramecium*, there are also Ca^{2+} influx channels sensitive to hyperpolarization (Preston et al. 1992). Their activation causes accelerated forward swimming, as occurs during mechanical stimulation of a *Paramecium* cell at its rear end. In addition, a *Paramecium* cell contains Na^+ influx and K^+ efflux channels which are activated by Ca^{2+}/calmodulin (Kung et al. 1992).

These examples clearly show some basic aspects of the complexity of Ca^{2+} signaling aiming at a final goal: depending on the stimulus Ca^{2+} enters at different sites of the cell surface, or it is released from different stores, to exert, directly or indirectly, a local effect (e.g. in cilia, exocytosis sites). This anticipates the general rule that every cell, including *Paramecium*, must regulate the Ca^{2+} signal locally at different sites in response to different signals. Figure 1 gives an example what one can see with a fluorochrome during stimulation of trichocyst exocytosis (Klauke and Plattner 1997). Signals are so much restricted that their actual, very local value cannot be demonstrated with a Ca^{2+} fluorochrome. Comparison with the calibrated values in Fig. 1 shows that the actual $[Ca^{2+}]_i$ value at an exocytosis site is estimated between 1 and 10 µM, probably around ~5 µM, i.e. almost one order of magnitude higher than that derived from calibrated fluorochrome signals due to restricted resolution. This estimation is based on the monitoring exocytosis events after injection of Ca^{2+} chelators with different Ca^{2+} affinity (Klauke and Plattner 1997). When many sites are activated, synchronous trichocyst exocytosis may ensue due to signal spillover (Plattner 1987; Plattner et al. 1993). The actual duration of the Ca^{2+} signal during trichocyst exocytosis can be registered only electrophysiologically (Erxleben et al. 1997) by Ca^{2+}/calmodulin-activated currents (Fig. 2). Signals from all activated sites pile up and Ca^{2+} can synchronously activate exocytosis. Also subsequent steps up to exocytosis-coupled endocytosis and retrieval of empty membrane "ghosts" are activated by the signal triggered by exocytosis stimulation (Plattner et al. 1997a), as summarized in Fig. 3.

Why locally defined $[Ca^{2+}]_i$ signals are generally favorable and even required for the cell is discussed below. Beyond this, the wide diversification of plasmalemmal ion channels, including Ca^{2+} influx channels in *Paramecium* (Machemer 1988) suggests two aspects: (i) their emergence early in evolution and (ii) in part convergent and in part divergent evolution of precursor molecules. Examples are conservation of voltage sensitivity of some channels and in part aberrant pharmacology of some other channels (Plattner et al. 2009), respectively.

Organelles serving as Ca^{2+} stores

In mammalian cells, the endoplasmic reticulum (ER) is a Ca^{2+} store of paramount importance (as is the sarcoplasmic reticulum [SR] in muscle cells). This also includes the nuclear membrane compartment. The SR of metazoan muscle cells recalls the alveolar sacs of alveolates and these have been identified as cortical Ca^{2+} stores biochemically (Stelly et al. 1991; Länge et al. 1995) and by electron spectroscopic imaging by Knoll et al. (1993). However, in metazoans endosomes also contain Ca^{2+}, in decreasing

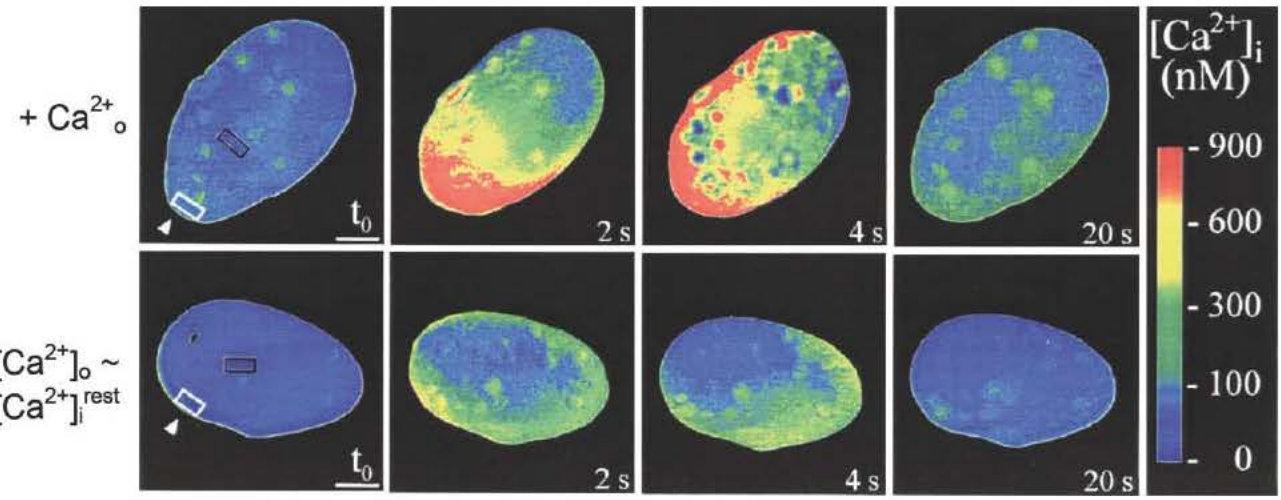

Figure 1 Fluorochrome f/f_0 ratio imaging of Ca^{2+} signaling during stimulation of trichocyst exocytosis in *Paramecium tetraurelia* cells with the RyR agonist caffeine. Strain *trichless* cells were used to avoid dislocation by trichocyst exocytosis, injected with the fluorochrome Fura Red and stimulated at $[Ca^{2+}]_o$ 50 µM (top) or at $[Ca^{2+}]_o$ ~30 nM (bottom). Note that the signal is much higher with $[Ca^{2+}]_o$ 50 µM than at low concentration and signal spreading from the stimulation site (boxed area) already within 2 s. Actual $[Ca^{2+}]_i$ occurring in the small area where focal membrane fusion occurs cannot be resolved for reasons indicated in the text. Scale bar 20 µm. Right column: For $[Ca^{2+}]_i$ scaling capillaries filled with a medium corresponding the intracellular milieu with Fura Red added were analyzed. From Klauke and Plattner (1998).

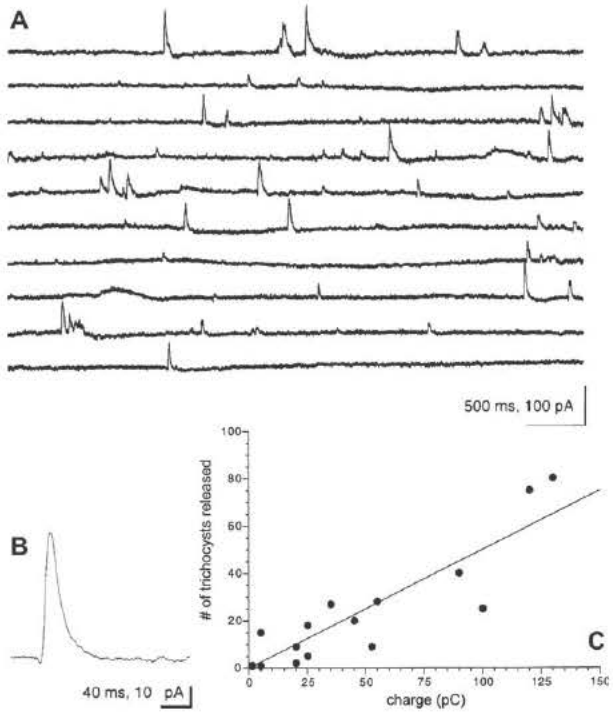

Figure 2 Recording of Ca^{2+} activated currents by whole cell patch electrophysiological analysis with *Paramecium tetraurelia* cells. Cells were patched in a pipette to measure electrical current with or without exocytosis stimulation. Current peaks observed in (**A**) are activated by Ca^{2+}/calmodulin (as shown separately in the original work) and, thus, can indicate subplasmalemmal $[Ca^{2+}]_i$ increases in parallel to spontaneous trichocyst exocytosis. The size of the peaks has been correlated with the actual number of exocytosis events observed (**C**). The minimal peaks in (A) corresponding individual events have been pooled in (**B**). The resulting average half width of the Ca^{2+} signal, 21 ms, characterizes the duration of the Ca^{2+} signal at the cell membrane. From Erxleben et al. (1997).

concentrations from early to late stages (Luzio et al. 2010), as do lysosomes (Christensen et al. 2002). The requirement of Ca^{2+} for phagosome-lysosome fusion is also well established (Jaconi et al. 1990). Note that these organelles represent stores of varying luminal pH. To this, one has to add acidocalcisomes (Docampo et al. 2005).

Although these details have not yet been sufficiently analyzed in any protozoan, crystal vacuoles are known specifically from protozoa including *Tetrahymena* (Coleman et al. 1972) and *Paramecium* (Grover et al. 1997). In general terms, for the different Ca^{2+} stores, the mechanisms of Ca^{2+} sequestration may be different, as are their Ca^{2+}-release channels (CRC). The latter has been verified for *Paramecium* (Ladenburger and Plattner 2011). For mitochondria, see below.

Ca^{2+} uptake mechanisms into stores with deviations from the eukaryotic consensus

As the SERCA-type Ca^{2+} pump is devoid of a calmodulin-binding domain, this molecule is shorter (~100 kDa) than the PMCA (~120 to 130 kDa) (Palmgren and Nissen 2011). In *Paramecium*, the SERCA-type pump occurs in the ER and in the cortical stores, the alveolar sacs (Hauser et al. 1998, 2000; Kissmehl et al. 1998). Figure 4 shows $^{45}Ca^{2+}$ sequestration by isolated alveolar sacs fractions; equally important is the documentation of release by a Ca^{2+} ionophore, to exclude adsorption. That Ca^{2+} uptake is much slower than in mammalian ER/SR has been demonstrated with widely different methods (Mohamed et al. 2003).

Acidocalcisomes, Ca^{2+} storing vesicles with acidic contents (Docampo et al. 2005), have not yet been identified in *Paramecium*, although there is some indirect evidence for their existence (Plattner et al. 2012). The enzyme mainly responsible for sequestering Ca^{2+} into acidocalcisomes of protozoan parasites, such as *Toxoplasma* and *Trypanosoma* species, is a PMCA-type Ca^{2+}-ATPase (Docampo et al. 2005). Vacuolar ATPase and pyrophosphatase (PPase) and Ca^{2+}/X^{+} antiporter systems are also present. Moreover, it has been shown that acidocalcisomes can come into intimate contact with the contractile vacuole in *Dictyostelium* (Marchesini et al. 2002) and in *Trypanosoma* (Rohloff and Docampo 2008). Again such knowledge is not available for *Paramecium* although its contractile vacuole complex is of paramount importance for regulating $[Ca^{2+}]_i$ homeostasis (Stock et al. 2002; Plattner et al. 2012; Schönemann et al. 2013). Here, localization studies (Wright and van Houten 1990) did not aim at showing Ca^{2+}-ATPase in the contractile vacuole complex and cilia were also negative. The latter contrasts with recent work in which the validity of antibodies previously used has been questioned (Yano et al. 2013). Therefore, it may well be possible that the contractile vacuole complex of *Paramecium* would also contain a PMCA-type Ca^{2+}-ATPase, as has been published also for *Dictyostelium* (Marchesini et al. 2002; Moniakis et al. 1999). In summary, one may expect for the contractile vacuole complex of *Paramecium* not only the occurrence of a Ca^{2+}/H^{+} antiporter, but also the occurrence of a PMCA-pump. Nevertheless, in *Paramecium* much of the Ca^{2+} handling is based on a primary active transport of H^{+} by a H^{+}-ATPase (proton pump, V- [vesicular] type ATPase without formation of a phospho-intermediate), as described by Plattner et al. (2012) and below.

Ca^{2+} in bound form in intracellular stores again with deviations among eukaryotes

As [Ca] in stores can amount from sub-millimolar to tens of millimolar (Plattner et al. 2012), Ca^{2+} must be osmotically inactivated by binding to high capacity/low affinity Ca^{2+}-binding proteins. In the mammalian ER, this is mainly calreticulin and in the SR calsequestrin, respectively.

The contents of some mammalian secretory organelles bind Ca^{2+} with high potency. This holds, e.g. for chromaffin granules of the adrenal medulla, where [Ca] may amount to 40 mM. By most authors, these organelles are considered not to be crucial for Ca^{2+} signaling (Becherer et al. 2012). Also the significance of their endowment with $InsP_3Rs$ interacting with chromogranin proteins in the

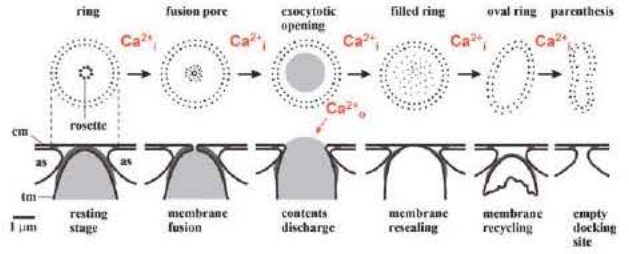

Figure 3 Sequence of events during AED stimulated trichocyst exocytosis and subsequent membrane resealing in *Paramecium tetraurelia*. The top row indicates the freeze fracture appearance (ring shaped circumference and a central rosette indicating exocytosis competence) of exocytosis sites (Plattner et al. 1997a), the bottom row shows the appearance in ultrathin sections. Note progression from ring shape to openings and oval resealing stages during exo endocytosis coupling, as well as decay of rosette particles during formation of the fusion pore. Also note the stimulating effect of the availability of Ca^{2+} in the medium (analyzed separately in experiments with variable $[Ca^{2+}]_o$, Plattner et al. 1997a), not only for progression of all stages but also for the decondensation (explosive stretching) of the trichocyst contents, i.e. the paracrystalline matrix (Bilinski et al. 1981). as alveolar sacs, cm cell membrane, tm trichocyst membrane. From Plattner and Hentschel (2006).

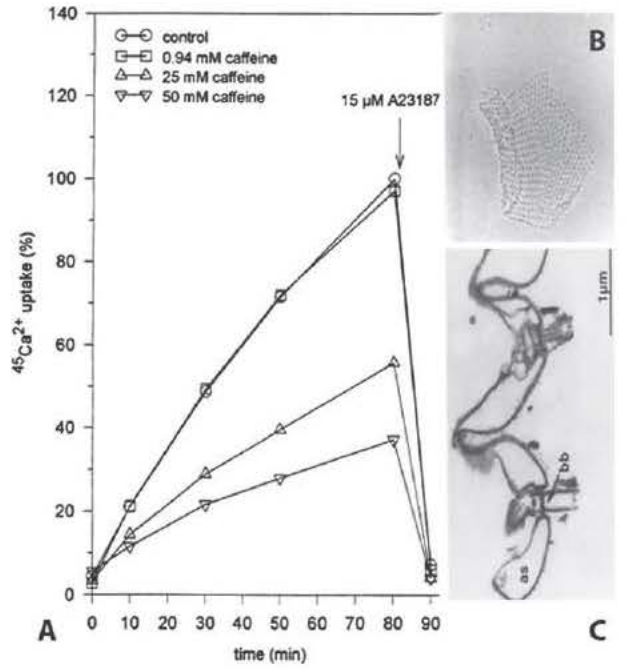

Figure 4 $^{45}Ca^{2+}$ sequestration by alveolar sacs isolated from *Paramecium tetraurelia* cells. The pumping kinetics (**A**) of isolated alveolar sacs (as) seen in the light (**B**) and electron microscope (**C**, with intermittent basal bodies [bb]) are unexpectedly slow. Ca^{2+} uptake is inhibited by caffeine and complete release is achieved by the Ca^{2+} ionophore A23187. (A) is from Länge et al. (1996).

secretory contents (Yoo et al. 2010) has not been scrutinized as yet. The opposite is true for *Paramecium* trichocysts whose mature matrix proteins are devoid of any recognizable Ca^{2+} content and, moreover, rapidly decondense in contact with extracellular Ca^{2+} once an exocytic opening has been formed (Bilinski et al. 1981; Hardt and Plattner 2000; Klauke et al. 1998).

Intracellular Ca^{2+}-release channels and second messenger activation

In higher eukaryotes, the classical activator of Ca^{2+} from the ER is inositol 1,4,5-trisphosphate ($InsP_3$) which, upon stimulation, is formed from phosphatidyl inositol 4,5-bisphosphate ($PInsP_2$) by phospholipase C (PLC) activity (Berridge et al. 2003; Clapham 2007). Only more recently have PLC enzymes with the potential of $InsP_3$ formation been detected in *Toxoplasma gondii* (Fang et al. 2006), in *Paramecium* (Klöppel et al. 2009) and *Tetrahymena* (Leondaritis et al. 2011; Leondaritis and Galanopoulou 2011). Depending on the system, release of Ca^{2+} from the ER can also be initiated by activation of ryanodine receptors. Most likely, the physiological equivalent of the plant toxin ryanodine is cyclic adenosine diphosphoribose (cADPR) produced from nicotine amide dinucleotide phosphate ($NADP^+$) by the enzyme, CD38 (Lee 2012). From the same substrate, the enzyme can also produce nicotinic acid-adenine dinucleotidephosphate (NAADP). NAADP is assumed to activate Two-Pore-Channels (TPC; Galione et al. 2009; Lee 2012). Most recently, there is some discussion about this assignment since Wang et al. (2012) found with mammalian endolysosomes that TPCs are Na^+-channels rather than Ca^{2+} channels and that they are activated by phosphoinositides, rather than by NAADP. However, this in turn has been challenged in a most recent report (Churamani et al. 2013). This controversy now needs clarification.

In higher eukaryotes, $InsP_3Rs$ and RyRs can coexist not only in different organelles of a cell but also within one organelle, such as the ER (McCarron and Olson 2008; Solovyova and Verkhratsky 2003). As will be discussed below, we have $InsP_3Rs$ and RyRs or RyR-like proteins in *Paramecium*. Here, different CRCs can also coexist in the membrane of one specific pool (Ladenburger and Plattner 2011).

Generally, acidic stores are considered the targets of NAADP, from endosomes to lysosomes (Patel and Docampo 2010). Remarkably TPCs are reportedly absent from acidocalcisomes of protozoa (Patel and Muallem 2011). In pilot experiments we saw in *Paramecium* cells, upon microinjection of cADPR or of NAADP, a physiological reaction indicative of a Ca^{2+} increase (Plattner et al. 2012). The respective receptor molecule behind remains unexplored. Similarly, any role of members of the superfamily of Transient-Potential-Receptor-Channels (TRPC) which in higher eukaryotes occur either in the plasmalemma or/and in intracellular store membranes (Patel and Docampo 2009) is unknown in ciliates. Sequences of such channels occur in the *Paramecium* database (Plattner et al. 2012), but any details remain to be elucidated in ciliates. For mitochondria, see below.

Cooperation between Ca^{2+} influx and release from cortical stores

How can an extracellular signal activate an intracellular Ca^{2+} pool? One way is diffusion of a second messenger ($InsP_3$, cADPR, NAADP) capable of activating Ca^{2+} release from specific pools. However, there may occur a tight structural and functional coupling of one of the classical pools, i.e. the ER or the SR, specifically its terminal cisternae, with the cell membrane.

The Ca^{2+} signal generated by the mobilization of ER stores may be enforced by a superimposed Ca^{2+} influx from the extracellular space (store-operated Ca^{2+} influx [entry], SOC[E], also called capacitative Ca^{2+}-influx). By timed stimulation, combined with quenched-flow/fast freezing (Knoll et al. 1991a) and energy-dispersive X-ray (EDX) microanalysis in the electron microscope (EM), this has been shown to occur in *Paramecium* (Hardt and Plattner 1999, 2000); see Fig. 5. Its alveolar sacs are structurally tightly coupled to the cell membrane, thus closely resembling terminal cisternae of the SR. These analyses have been conducted with aminoethyldextrane (AED), a potent secretagogue in *Paramecium*, at higher $[Ca^{2+}]_o$ (Plattner et al. 1984, 1985) or in the presence of $[Ca^{2+}]_o$ ~30 nM, i.e. slightly below $[Ca^{2+}]_i^{rest}$ (Hardt and Plattner 2000), to avoid superposition by Ca^{2+} influx. That the latter occurs has been shown separately, e.g. by rapidly substituting Sr^{2+} for Ca^{2+}. These substitution experiments have definitely shown that Ca^{2+} release from alveolar sacs is immediately superimposed by Ca^{2+} influx (exploiting the widely different energy lines of Sr^{2+} and Ca^{2+} in EDX) (Hardt and Plattner 1999, 2000). Thus, the storage compartment is refilled while being emptied. Rarely such observations, based on other methodologies, have been reported for mammalian cells, e.g. immuno- and skeletal muscle cells (Narayanan et al. 2003). Also fluorochrome analysis at different $[Ca^{2+}]_o$ reveals an endogenous and a superimposed exogenous Ca^{2+} component during trichocyst exocytosis (Klauke and Plattner 1997); see Fig. 1. Finally, genetic elimination of the influx component also allows for Ca^{2+} mobilization from alveolar sacs, as documented also by EDX (Mohamed et al. 2002). Nevertheless, for a rapid exocytotic response and subsequent exo-endocytosis coupling, sufficient Ca^{2+} in the outside medium, $[Ca^{2+}]_o$, is important to drive the SOC mechanism, from exocytotic membrane fusion to contents release and detachment of empty vesicles ("ghosts") (Plattner et al. 1997a). With $[Ca^{2+}]_o < [Ca^{2+}]_i^{rest}$, exocytosis stimulation with AED results only in a small number of exocytosis events. Efficiency increases from $[Ca^{2+}]_o > 0.3$ µM on, i.e. close to the value estimated for $[Ca^{2+}]_i$ during activation.

In skeletal muscle cells, the SR is coupled to the sarcolemma by close interaction of voltage-dependent Ca^{2+} influx channels with the ryanodine receptor-type CRC, i.e. by conformational coupling. Whether the same coupling mechanism applies to the SOC found in *Paramecium* is not known although it is striking how alveolar sacs are intimately attached to the cell membrane, with the CRCs facing the cell membrane (Fig. 3). In heart muscle cells, Ca^{2+} release from the SR is activated differently, namely by a Ca^{2+} influx through plasmalemal voltage-dependent Ca^{2+} channels in the course of depolarization events accompanying each heart beat (Mackrill 1999). Here, a Ca^{2+}-induced Ca^{2+} release (CICR) represents the opposite to the SOC mechanism occurring in skeletal muscle. No CICR is known from ciliates.

Immobile Ca^{2+} buffers cytosolic high capacity/low affinity Ca^{2+}-binding proteins

Such proteins are not Ca^{2+} sensor proteins, i.e. they do not directly contribute to signaling. By rapid binding of Ca^{2+}, they serve as a sink of Ca^{2+} for inactivation. The centrin molecule, including *Paramecium* centrin, has such properties. In *Paramecium,* centrin is concentrated in the infraciliary lattice which contracts in response to increased $[Ca^{2+}]$ (Beisson et al. 2001). Centrin can in part act as a signaling molecule by virtue of its EF-hand motifs but due to numerous acidic regions with considerable Ca^{2+} binding capacity in its linear part, centrin is also a high capacity/low affinity Ca^{2+}-binding protein (Gogendeau et al. 2008; Kim et al. 2002). The activity of the infraciliary lattice centrin as a Ca^{2+} sink has been shown experimentally by knockout experiments (Sehring et al. 2009).

How can a Ca^{2+} signal become locally restricted and why is local $[Ca^{2+}]$ important?

Close to the mouth of a Ca^{2+} influx or a CRC, $[Ca^{2+}]$ will be highest and most channels are rapidly inactivated. The following mechanisms are important. (i) Ca^{2+} becomes diluted with distance by a square function. (ii) It is inactivated by binding to Ca^{2+}-binding proteins depending on their binding and time constant, and (iii) by sequestration into storage organelles and/or extrusion from the cell. (iv) Furthermore, the physiological effects of Ca^{2+} follow a superlinear function of the actual local concentration (Neher 2012). All this entails two major aspects. On the one hand, all these mechanisms serve to restrict the signal and, thus, avoid spreading to irrelevant regions or to reach toxic levels. On the other hand, this requires precise positioning of channels in the cell. If this is provided, the rise of $[Ca^{2+}]$ and the reaction to it can remain limited to a small area. For instance, in adrenal medullary chromaffin cells only those granules that are docked close enough to Ca^{2+} influx channels may be more readily released upon stimulation (Becherer et al. 2003). As far as *Paramecium* is concerned, this cell has an intriguing spatial arrangement of sites of Ca^{2+} activation and cell dynamics. The manifold Ca^{2+} influx and release channels in these cells appear tailor-made for the respective local functions (see below). Otherwise such precision is more broadly known from nerve terminals and neuromuscular junctions.

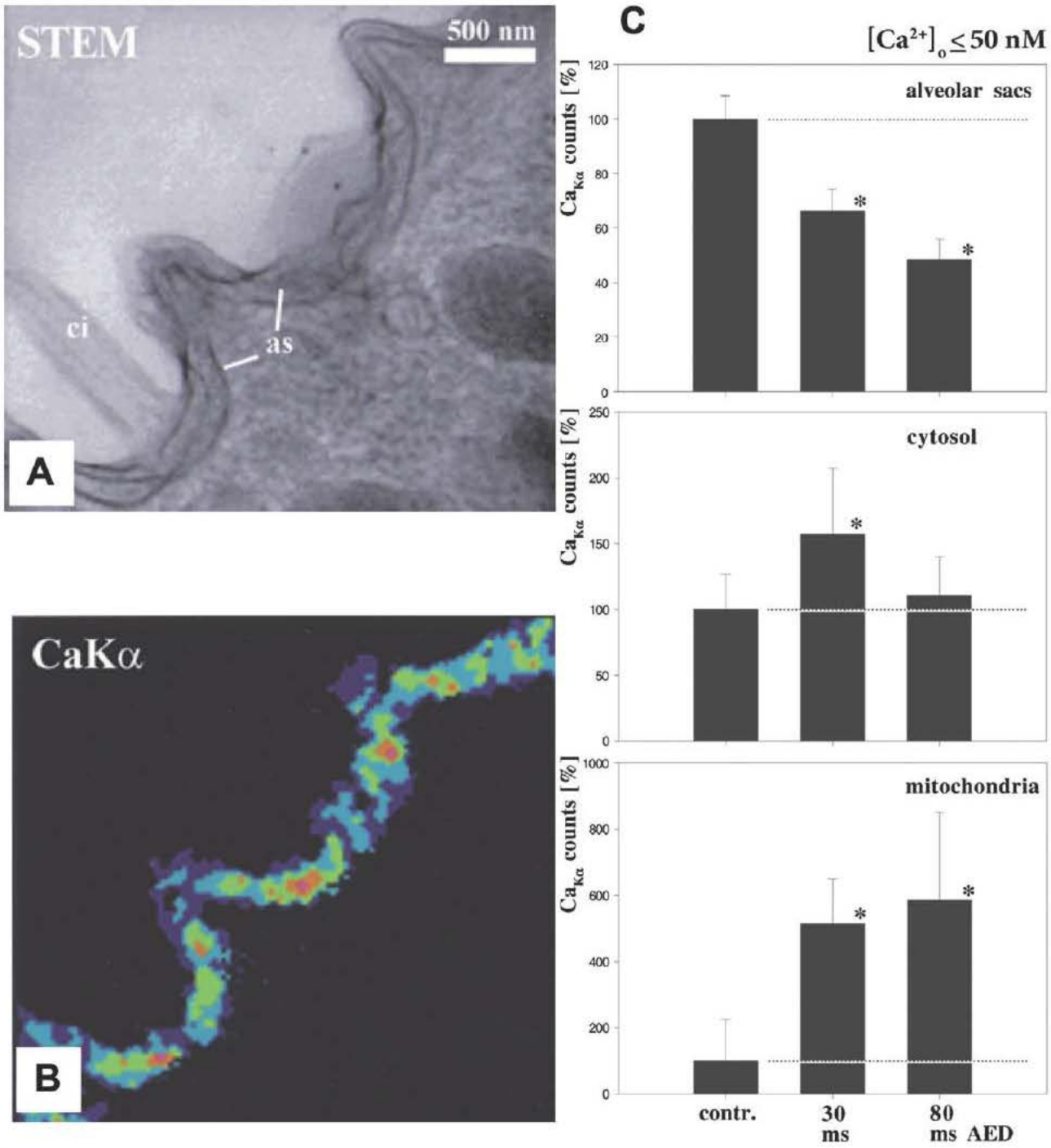

Figure 5 EDX analysis of [Ca] and Ca^{2+} mobilization and redistribution during trichocyst exocytosis in *Paramecium tetraurelia* cells stimulated with AED. (**A**) is a scanning transmission electron micrograph (STEM) obtained from a 0.5 µm thick semithin section, as required for EDX. In (**B**) cal cium is visualized in the same area as in (A) by its specific X ray energy line of 3.691 keV, measured in a window from 3.57 to 3.83 keV, in a non stimulated cell. As the section plane is not always strictly perpendicular to alveolar sacs and also for theoretical reasons (Hardt and Plattner 1999) the false color Ca signal appears broader than the underlying structure. (**C**) presents changes in [Ca] in the compartments indicated, each normal ized to 100% for the unstimulated state (~40 mM in the sacs, Hardt and Plattner 1999), under conditions precluding Ca^{2+} influx from outside ($[Ca^{2+}]_o$ 50 nM, i.e. close to $[Ca^{2+}]_i^{rest}$ [Klauke and Plattner 1997). Data in (C) are collected from a number of measurements from different experiments. Note swift decay in the sacs, short transient increase in the adjacent cytosol and considerable increase in nearby mitochondria; in these areas, basal [Ca] cannot be imaged in (B) before stimulation due to restricted sensitivity of EDX imaging. Bars standard error of the mean; asterisks indicate statistically significant difference from the respective control. From Hardt and Plattner (2000).

FOCUSING STILL MORE ON DETAILS SPECIFIC FOR *PARAMECIUM*

Ca^{2+} leakage and $[Ca^{2+}]_i$ homeostasis

Normally *Paramecium* is cultured in media with $[Ca^{2+}]_o$ 0.1 1 mM. Ca^{2+} fluxes in nonactivated cells have revealed an unexpectedly high leakage rate (Browning and Nelson 1976; Kerboeuf and Cohen 1990) which must be permanently counteracted. This is achieved in several ways. (i) The cell membrane is endowed with a classical PMCA of ~130 kDa (Wright and van Houten 1990) and (ii) the ER as well as the alveolar sacs with a SERCA-type pump (Hauser et al. 1998, 2000). (iii) The contractile vacuole complex proved to dispose of a highly efficient Ca^{2+} extrusion mechanism. Using miniature Ca^{2+}-selective electrodes, Stock et al. (2002) have measured a high $[Ca^{2+}]$ in the fluid of the contractile vacuole complex. As there is alledgedly no primary active $Ca2^{+}$-transporter (yet see above), but only a V-type H^+-ATPase present in the organelle membranes (Fok et al. 1995; Wassmer et al. 2005, 2006), one can assume that a ΔH^+ is exploited by Ca^{2+}/H^+ exchanger (not identified as yet). This assumption is supported by two facts. On the one hand, no acidic pH can be detected with a fluorochrome sensitive to protonation (Wassmer et al. 2009), thus indicating ongoing consumption of the ΔH^+. On the other hand, the H^+-ATPase inhibitor concanamycin B extensively inhibits downregulation of $[Ca^{2+}]_i$ after stimulation of exocytosis (Plattner et al. 2012). By this mechanism, as calculated under defined boundary conditions, a *Paramecium* cell may be able to extrude all of its Ca^{2+} within less than 10 min (Ladenburger et al. 2006). Concomitantly silencing of contractile vacuole complex-resident SNAREs greatly increases the sensitivity to even moderately increased $[Ca^{2+}]_o$ (Schönemann et al. 2013). Similarly, inhibition of the organellar H^+-ATPase compromises cells in their vitality after increasing $[Ca^{2+}]_o$ (Sehring et al. 2009).

A sudden change in $[Ca^{2+}]_o$ causes a rapid transient increase in $[Ca^{2+}]_i$ without any visible functional consequences, i.e. no ciliary reversal or trichocyst exocytosis occurs. This may be accounted for by a group of the CRCs described below, specifically by *Pt*CRC-V-4. These are greatly concentrated in the parasomal sacs, i.e. clathrin-coated pits installed at the basis of cilia (Ladenburger and Plattner 2011). (Consider that several thousand of such sites can be placed on the surface of on *Paramecium* cell near cilia; Erxleben et al. 1997.) We found that these channels contain a short stretch homologous to an $InsP_3$ binding domain (although actual $InsP_3$ binding has not been shown). These CRCs may account for the not well understood high conductivity channels described in *Paramecium* by electrophysiologists (Machemer 1988). It thus looks as if these Ca^{2+}-release channels at the cell boundary would allow for Ca^{2+} "release" from the extremely large extracellular "compartment" into the cytosol. (By comparison, only less than 5 $InsP_3$R-type channel molecules may be localized to the cell membrane of a mammalian gland cell; Dellis et al. 2008.) This type of Ca^{2+}-influx channel may be functionally comparable to a novel type of cation channel with low cation selectivity and high conductivity recently found in hippocampal neurons (Xiong et al. 1997). It may be the alternative to a Ca^{2+}/polyvalent cation receptor, characterized by five transmembrane domains and coupling to trimeric GTP-binding proteins. This is interesting as in *Paramecium* neither such receptors nor such G-proteins have been identified conclusively, although evidence for the possible presence of the latter in *Paramecium* slowly emerges (Lampert et al. 2011) in the literature.

Ca^{2+} stores

Paramecium recalls a muscle cell with regard to its major two Ca^{2+} stores, the ER, and the plasma membrane-associated alveolar sacs. Here, equivalents of both, ER and SR are found, with calreticulin and calsequestrin, respectively. A calreticulin-like protein is localized to the ER and a calsequestrin-like protein to the alveolar sacs (Plattner et al. 1997b). Although this has been established with the best antibodies then available, we could not yet identify these proteins in the *Paramecium* database. From the distribution of CRC in a large set of different vesicle types, we conclude that Ca^{2+} is stored also in the respective organelles all vesicles undergoing trafficking (see below). This is also true for the contractile vacuole complex as it contains calcium (Plattner and Fuchs 1975; Stock et al. 2002) and CRCs (Ladenburger et al. 2006).

Ca^{2+} can also be stored as a polyphosphate, as is typical of acidocalcisomes occurring from protozoa to mammals (Docampo et al. 2005). *Paramecium* contains crystal vacuoles with Ca,Mg-phosphate; according to the X-ray diffraction pattern obtained crystals are identical with struvite. Once more *Paramecium* looks very "human" as such crystals are one form of kidney stones (Grover et al. 1997). Nothing is known about Ca^{2+} uptake into, and mobilization from, these organelles.

$InsP_3$Rs and RyR-related Ca^{2+}-release channels in *Paramecium*

We found 34 such channels in *Paramecium tetraurelia* which can be grouped in six subfamilies (Ladenburger and Plattner 2011). They were identified by domain analysis, specifically by the prediction of an $InsP_3$ binding domain, of a ryanodine/$InsP_3$ receptor homology (RIH) domain and particularly of an appropriate channel domain/pore. This domain has to be found in the carboxy-terminal hydrophobic regions where it contains a selectivity filter between the last two transmembrane regions. All Ca^{2+}-release channels of this type found in *P. tetraurelia*, called *Pt*CRC, have six transmembrane domains (see below). Their selectivity filter is largely conserved as Gly-Ile-Gly-Asp (GIGD), regardless whether an $InsP_3$ binding domain is present or absent. In higher metazoans, this sequence is restricted to RyRs (Boehning 2010) and the $InsP_3$R is characterized by GVGD (V valin). Our analysis shows that GIGD is typical of both CRC types in many lower

eukaryotes (Plattner and Verkhratsky 2013). This and some other features make the *Pt*CRCs prototypes of primeval Ca^{2+}-release channels.

Subfamilies *Pt*CRC-I, *Pt*CRC-II, and *Pt*CRC-III contain an $InsP_3$-binding domain; a shorter form also occurs in some members of the *Pt*CRC-V subfamily. However, only *Pt*CRC-II has been analyzed in more detail. ^{3}H-$InsP_3$ binding has been demonstrated and its activity probably accounts for spontaneous Ca^{2+} puffs emerging from the contractile vacuole complex (Fig. 6) where these channels have been localized at the light microscope and EM level (Ladenburger et al. 2006). In our belief, this *Pt*CRC serves the partial reflux of Ca^{2+} which in its majority is released by expulsion (Stock et al. 2002). Sequestration is probably based on a Ca^{2+}/H^+ antiporter (not yet identified), coupled to the activity of the organellar V-type H^+-ATPase (Fok et al. 1995; Wassmer et al. 2006). The Ca^{2+} reflux seen as local puffs at varying sites of the contractile vacuole complex is thought to serve fine-tuning of cytosolic $[Ca^{2+}]$ (Ladenburger et al. 2006) in analogy with systemic regulation processes in our kidneys. *Pt*CRC-II-type $InsP_3$Rs are constitutively active a fact detected only later on also in DT40 lymphocytes (Cárdenas et al. 2010).

*Pt*CRC-IV-type CRCs, devoid of a $InsP_3$ binding domain, are localized to that part of the alveolar sacs that faces the closely apposed cell membrane, as found by immuno-gold EM localization (Ladenburger et al. 2009; see Fig. 7). Analysis of these CRCs included trichocyst exocytosis stimulation with the secretagogue AED (Plattner et al. 1984, 1985) as well as with the RyR activators caffeine (Klauke and Plattner 1998) and with 4-chloro-m-cresol (Klauke et al. 2000). The basis was Ca^{2+} imaging in response to AED (Klauke and Plattner 1997), to caffeine (Klauke and Plattner 1998; Länge et al. 1996) and to 4-chloro-m-cresol as well as the demonstration of a SOC mechanism (Klauke et al. 2000), complemented by EDX analysis (Hardt and Plattner 2000). We then combined *Pt*CRC-IV silencing with trichocyst exocytosis stimulation and Ca^{2+} imaging (Ladenburger et al. 2009). Both were inhibited. A standard assay to identify a CRC as a RyR, as routinely used with higher eukaryotes, would be Ca^{2+}-dependent ^{3}H-ryanodine binding (Zucchi and Ronca-Testoni 1997). As this depends on several boundary conditions and as there is no defined motif for ryanodine binding, furthermore considering the frequently aberrant pharmacology of ciliates (Plattner et al. 2009), it is not surprising that such binding cannot be registered (Plattner, unpubl. observ.).

To summarize, the following aspects qualify CRCs type IV as RyRs (Ladenburger et al. 2009). (i) As mentioned, they are activated by typical activators of RyR-type CRCs, i.e. caffeine and 4-chloro-m-cresol. (ii) The Ca^{2+} signal recorded with fluorochromes parallels trichocyst release. (iii) The signal is greatly reduced when *Pt*CRC-IV channels are silenced, as is their stimulated trichocyst exocytosis. (Note that in these experiments $[Ca^{2+}]_o$ has been quenched to a calculated value slightly below $[Ca^{2+}]_i^{rest}$ to avoid superposition of Ca^{2+} influx, as described below.) (iv) We have predicted six transmembrane domains for *Pt*CRC-IV (Ladenburger and Plattner 2011), rather than four

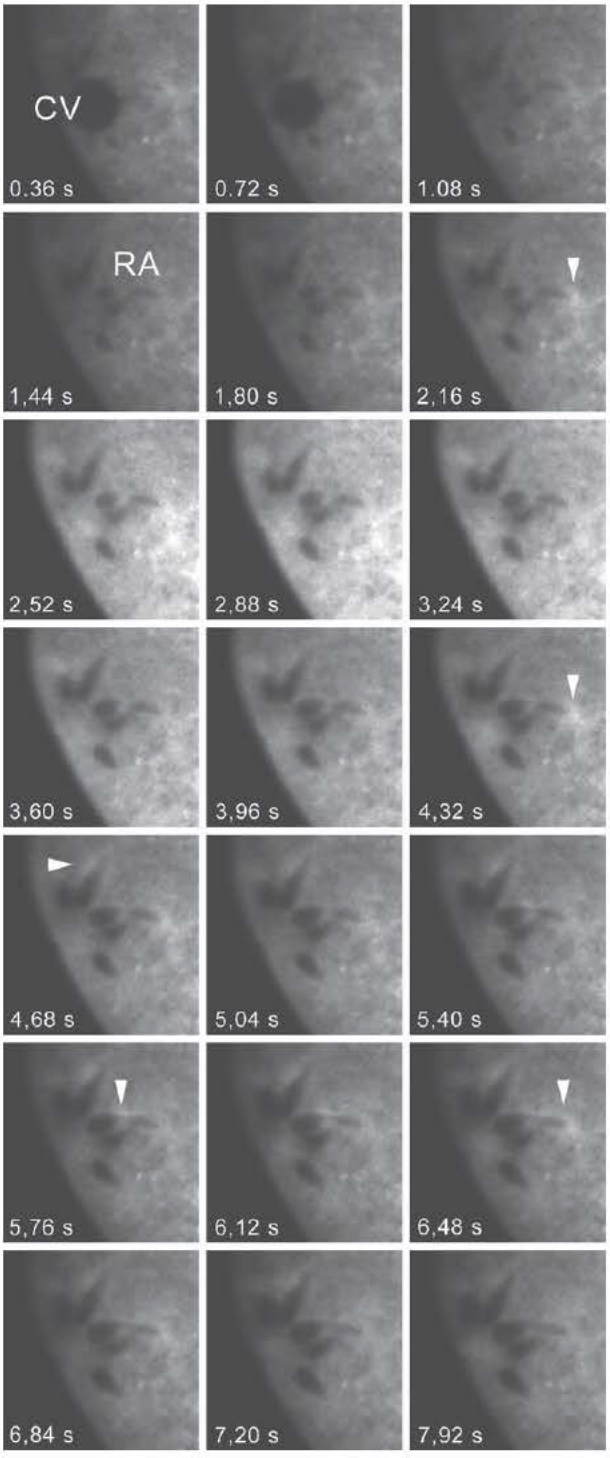

Figure 6 Fluorochrome imaging of spontaneous Ca^{2+} puffs along radial arms of the contractile vacuole complex in a non stimulated *Paramecium tetraurelia* indicates constitutively active *Pt*CRC II 1/$InsP_3$Rs. The cell was injected with the fluorochrome Fluo 4 coupled to dextran. Note at arrowheads transient Ca^{2+} puffs along the radial arms (RA) emanating from the contractile vacuole (CV). Lower left in each frame: actual recording time. From Ladenburger et al. (2006).

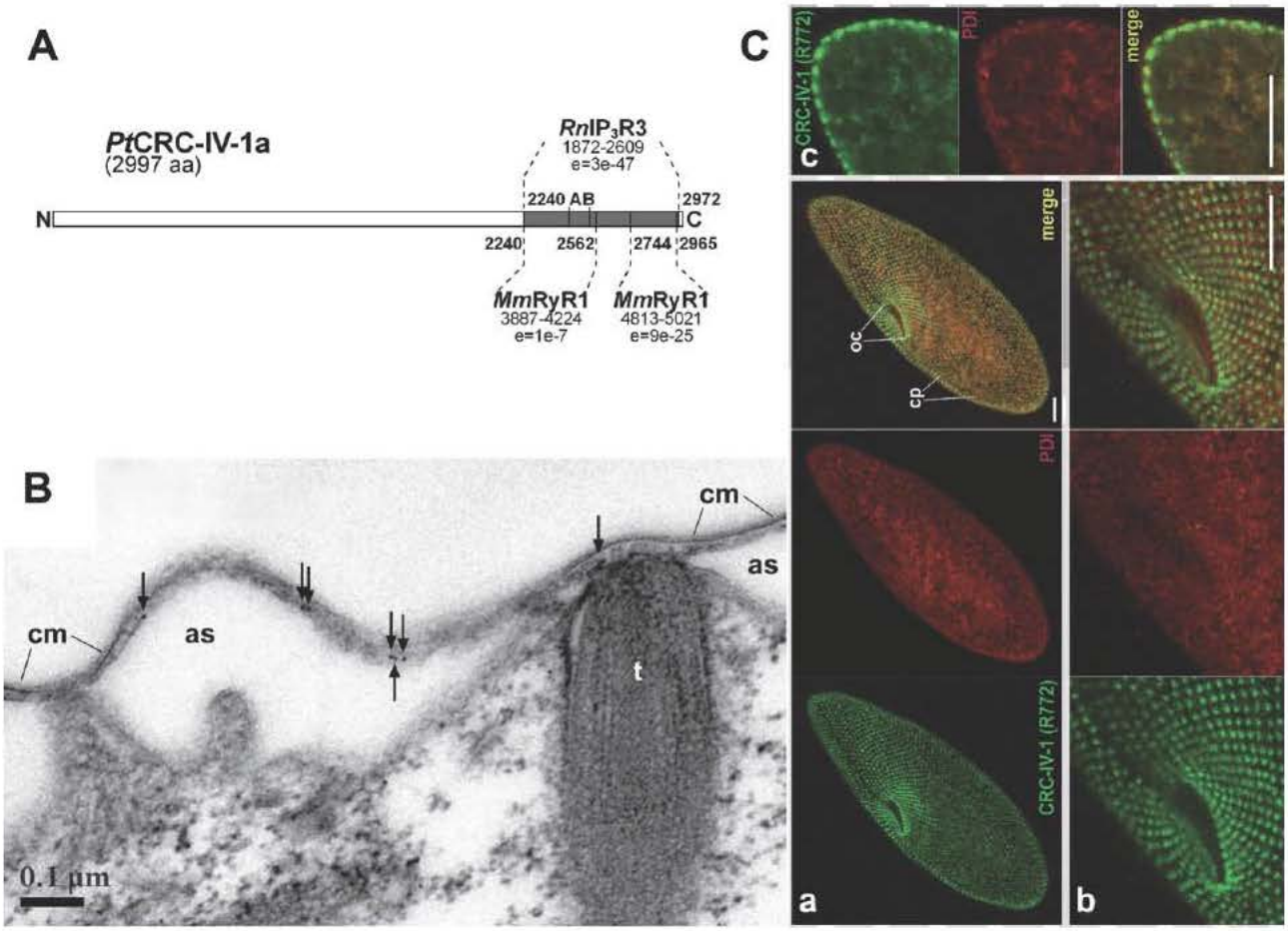

Figure 7 RyR like protein in *Paramecium tetraurelia*, termed *Pt*CRC IV. (**A**) presents the structure and size of subtype 1a (closely resembling 1b), regions of similarity with RyR type 1 from mouse (*Mm*RyR1) and $InsP_3R$ type 3 from rat (*Rn*IP_3R3) as well as the region of *Pt*CRC IV 1a selected for antibody (AB) production. Numbers up to 5,021 indicate amino acid residues of the respective protein included in the comparison of different CRCs; 2,997 indicates the total number of amino acids in *Pt*CRC IV 1a. The *e* values indicate a measure for reliability of identification of specific regions of the *Pt*CRC IV 1a protein in comparison to *Rn*IP_3R or *Mm*IP_3R (highest reliability: *e* $3e^{-47}$, i.e. $3e^{-47}$ error probability). (**B**) is from immuno gold EM localization showing label (arrows) along the outer part of the alveolar sacs (as) membrane below the cell membrane (cm); t marks a trichocyst. (**C**) Immunofluorescence; (CC) documents the localization of *Pt*CRC IV 1 by the antibody (type R722, as specified in [B]) to the ER (characterized by ER specific protein disulfide isomerase [PDI]), (Ca,b) shows the localization to alveolar sacs (devoid of PDI). Symbols: cp cytoproct region, oc oral cavity. Scale bars 10 µm. (A C) are from Ladenburger et al. (2009).

as usually assumed for RyRs (Williams et al. 2001). However, this does not contradict our identification as RyRs because (i) this number has remained controversial until recently (Zalk et al. 2007), (ii) our assumption is based on the most recent comprehensive computational analysis of such domains (Bernsel et al. 2009) and (iii) most recent computational analysis of mammalian RyRs has also predicted six transmembrane domains (Ramachandran et al. 2013). Thus, together with $InsP_3R$-type *Pt*CRCs, we assume six transmembrane domains for all *Pt*CRCs.

Coupling of Ca^{2+} mobilization from alveolar sacs and Ca^{2+} influx

As mentioned, the emptying of the cortical stores (alveolar sacs) during trichocyst exocytosis stimulation is superimposed by an influx of Ca^{2+} from the outside medium, SOC(E). This has been inferred from electrophysiology (Erxleben and Plattner 1994), fluorochrome analyses under conditions specifically appropriate to document SOC (Klauke et al. 2000) and EDX analysis of stimulated wild-type cells during $[Ca^{2+}]_o$ quenching in the medium (Hardt and Plattner 2000); see Fig. 5. Furthermore, EDX analysis of a mutant strain devoid of any Ca^{2+} influx clearly excluded a CICR (Mohamed et al. 2002). In summary, these analyses revealed that Ca^{2+} release from the alveolar sacs contributes, though only a minor fraction, to the total Ca^{2+} signal formed during trichocyst exocytosis, and that it serves as a kind of ignition. The whole arrangement serves precise signaling focussed on exocytosis sites: The CRCs face the cell membrane (Ladenburger et al. 2009), whereas the SERCA faces the cell center (Plattner et al.

1999). In Fig. 8, Ca^{2+} signal spreading in the cell cortex occurring during exocytosis stimulation is compared with the restriction of Ca^{2+} signaling to cilia during depolarization. As signal spill over occurs only centrifugally from the cell soma into cilia, but not centripetally from cilia into the soma (Husser et al. 2004) cilia normally remain functionally clearly decoupled from the soma. As discussed below, this is functionally meaningful. By contrast, neither Ca^{2+} injection (Klauke and Plattner 1997), nor a sudden increase in $[Ca^{2+}]_o$ (Erxleben et al. 1997) entail exocytosis, although both procedures yield an overall Ca^{2+} signal.

With human T-lymphocytes, a SOC(E) mechanism has been described that involves RyRs and similar coupling (Thakur et al. 2012), just as we found with *Paramecium*. Here, alveolar sacs contain *Pt*CRC-IV channels (Fig. 7) and these are relevant for this signaling pathway (Ladenburger et al. 2009). Disappointingly other components contributing to this signaling mechanism, such as Stim and Orai (Cahalan 2009; Wu et al. 2007), could not be detected in the *Paramecium* database.

Mitochondria as Ca^{2+} stores

Mitochondria can rapidly take up Ca^{2+} by a uniporter (Hajnóczky and Csordás 2010) which has been characterized on a molecular scale in mammalian cells only quite recently (Raffaello et al. 2012). Release of Ca^{2+} is mediated by a Ca^{2+}/Na^+ exchanger (Palty and Sekler 2012). Although a homolog of the Ca^{2+} uniporter has been detected in the *Tetrahymena* genome (Bick et al. 2012), no molecular details are known for the Ca^{2+}/Na^+ antiporter in ciliates. (Note that occurrence of partial sequences of such antiporters in the *Paramecium* database would not allow any stringent identification as such molecules may be operating at different sites of the cell.) However, in EDX analyses following timed quenched-flow/cryofixation during different times of AED-stimulation of trichocyst exocytosis (Fig. 5C), we see a swift increase in [Ca] in mitochondria and a decay within fractions of a second (Hardt and Plattner 2000). By contrast, parallel imaging of $[Ca^{2+}]$ by rhodamine fluorescence shows a much slower decay with a $t_{1/2}$ of ~3 min (Plattner et al. 2012). The EDX data fit well the rapid activity of a uniporter and a slower release mechanism. The Ca^{2+} retained in mitochondria in a soluble form (seen by rhodamine) may stimulate some dehydrogenases of the tricarboxylic acid cycle (Tarasov et al. 2012) also in *Paramecium* (see "Energetic aspects").

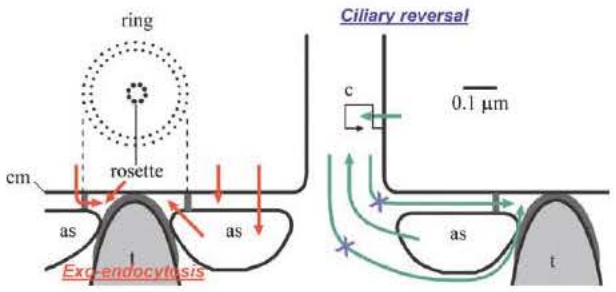

Figure 8 Ca^{2+} fluxes occurring in *Paramecium tetraurelia* cells during stimulated trichocyst (t) exocytosis (red arrows) and during ciliary reversal induction (green arrows), as derived from various work cited in the text. Left part: upon exocytosis stimulation, Ca^{2+} is released from alveolar sacs (as) in a first step, followed by influx of Ca^{2+} from the outside medium (SOC). The type of influx channels, their precise localization with regard to the exocytosis site (characterized by freeze fracture particle assemblies, ring and rosette) in the cell membrane (cm) and how they are linked to alveolar sacs is not known. Right part: reversal of the beat direction of cilia (c) is initiated by an influx of Ca^{2+} via voltage dependent channels in the ciliary membrane; the hooky arrow (black) symbolizes the negative feedback, as the increased organellar $[Ca^{2+}]$ inhibits further Ca^{2+} influx. Green arrows emerging from the cilium are crossed to indicate that no signal spill over to exocytosis sites occurs, whereas spill over in centrifugal direc tion does occur when Ca^{2+} is mobilized from alveolar sacs, e.g. during exocytosis stimulation. For further explanations, see text.

Behavioral responses paralleled by Ca^{2+} signaling

As already outlined above, *Paramecium* reverses the direction of its active ciliary stroke in response to a Ca^{2+} influx from the medium into cilia. In nature this is induced by activation of anteriorly enriched mechanosensitive Ca^{2+} influx channels (Eckert and Brehm 1979). This causes cell membrane depolarization which in turn activates voltage-gated Ca^{2+} channels. Remarkably, these two channel types are located in different domains, i.e. the somatic (nonciliary) and the ciliary membrane, respectively (Machemer and Ogura 1979). (Molecular mechanisms under consideration are discussed below.) Under physiological conditions, there is no spill over of Ca^{2+} into the soma (Husser et al. 2004), except when cells are overstimulated (Plattner et al. 2006). By contrast, massive trichocyst exocytosis stimulation causes the Ca^{2+} signal to spill into the cilia (Husser et al. 2004), thus causing ciliary reversal (Knoll et al. 1992; Plattner et al. 1984, 1985). This involves formation of cyclic GMP (Knoll et al. 1992; Yang et al. 1997) by a Ca^{2+}/calmodulin-activated guanylate cyclase (Schultz and Klumpp 1993) and cGMP exerts an effect via a protein kinase G, PKG (Ann and Nelson 1995). This effect remains to be determined in detail. Both, the guanylate cyclase and the PKG are localized to cilia (Ann and Nelson 1995; Linder et al. 1999). In vivo, the combination of both mechanisms, trichocyst release and ciliary reversal, can be advantageous to escape the attack of predatory ciliates (Knoll et al. 1991b) a defense mechanism detected by Harumoto and Miyake (1991).

Another phenomenon is the periodically alternating backward and forward swimming (each for ~8 s) in response to exogenously added GTP, but not other nucleotides (Clark et al. 1993). This is paralleled by periodic changes in $[Ca^{2+}]_i$ signals (Sehring and Plattner 2004). Based on inhibitor studies, the source of Ca^{2+} underlying this signal was attributed to alveolar sacs (Wassenberg et al. 1997). Considering that the pharmacology used is aberrant in *Paramecium* (Plattner et al. 2009) one may envisage some other stores which are important particularly for the first peak seen with a fluorochrome after GTP stimulation (Sehring and Plattner 2004).

Which one of the many organelles endowed with CRCs (see below) this would be has not been determined as yet. The physiological meaning of this reaction is hypothetic, e.g. it may be a response to increased population density (Sehring and Plattner 2004). The type of a likely purinergic receptor remains to be analyzed. No such receptors are known for GTP (Coddou et al. 2011) and only few protozoa are known to contain purinergic receptors (Burnstock and Verkhratsky 2009).

Ca^{2+} sensors and effectors in *Paramecium*

Does calmodulin play a role in Ca^{2+} signal transduction in *Paramecium*? Calmodulin is localized to cilia and to the docking sites of trichocysts (Momayezi et al. 1986). Defective calmodulin impedes the assembly of functional trichocyst exocytosis sites (Kerboeuf et al. 1993). However, very specific anticalmodulin drugs (inhibitors) which are also active in *Paramecium* do not inhibit trichocyst exocytosis (Erxleben and Plattner 1994). All this is compatible with the relevance of calmodulin for the assembly of fusion sites in mammalian cells (Junge et al. 2004), although a direct activity during fusion is unlikely.

In cilia calmodulin-binding proteins are in part associated with the axoneme and in part with the membrane (Evans and Nelson 1989). A role of the hierarchial occupancy of the Ca^{2+}-binding loops, I IV, by Ca^{2+} or alternatively by Mg^{2+} in the near carboxy-terminal loops has been discussed in the context of ciliary reversal (Machemer and Teunis 1996). In fact, such antagonism affects binding to target molecules (Jama et al. 2011). Nevertheless, in cilia this mechanism remains speculative, as there are still other candidates for Ca^{2+} signal transfer (Bonini et al. 1991). One important candidate is ciliary centrin as it regulates voltage-dependent Ca^{2+} channels (Gonda et al. 2007); here, the occupancy of the carboxy-terminal EF-hand loop of centrin, rather than of calmodulin, proved important.

Although any direct role for calmodulin in signal transfer, if any, appears rather restricted, calmodulin can modulate signal transfer in many regards. Let us look for an indirect activity calmodulin may exert in *Paramecium* via calcineurin.

Multiple functions may be assigned to some protein phosphatases including calcineurin. Its catalytic subunit A contains a binding site for subunit B and for calmodulin, as well as an autoinhibitory domain (Klee et al. 1998; Rusnak and Mertz 2000). Subunit B binds Ca^{2+} and the binding site for subunit B at subunit A is highly conserved also in *Paramecium* (Fraga et al. 2010). Calcineurin can be designated a multipurpose phosphatase. For instance, the Ca^{2+}/calmodulin complex inactivating L-type Ca^{2+}-influx channels in hippocampal pyramidal neurons is actually inactivated via dephosphorylation by calcineurin (Oliveria et al. 2012). Similarly, in *Paramecium*, silencing of some of the calcineurin paralogs affects stimulated backward swimming (Fraga et al. 2010). Although the underlying mechanisms has not been elucidated as yet one should recall that ciliary Ca^{2+}-influx channels in *Paramecium* are also inhibited by a Ca^{2+}/calmodulin complex (see above). The same is true of inhibition of trichocyst exocytosis by microinjected antibodies against mammalian calcineurin (recognizing homologs in *Paramecium*) (Momayezi et al. 1987). Similar effects have been achieved with a variety of mammalian secretory systems; as discussed by Fraga et al. (2010), this may be caused by multiple effects of calcineurin. For instance, CRCs of the RyR-type in different cell types are sensitive to calcineurin, or calcineurin-directed drugs (Brillantes et al. 1994; Wagenknecht and Radermacher 1997; Xiao et al. 1997). Whether this is also true of *Pt*CRC-IV channels associated with the alveolar sacs in *Paramecium* (Ladenburger et al. 2009) remains to be elucidated.

The best, long known function of calcineurin is the activation of transcription factors (Shenolikar 1994), e.g. during activation of T-lymphocytes. May a similar pathway of gene activation also take place in ciliates? In ciliates, a massive Ca^{2+} increase during stimulated exocytosis may give a signal to the nucleus. Though in *Tetrahymena* little is known about Ca^{2+} signaling per se, the situation may be quite similar to that we described in *Paramecium*. Under such conditions, e.g. massive mucocyst exocytosis stimulation, the transcription of many genes is upregulated (Haddad and Turkewitz 1997). More recently this has also been found in *Paramecium* (Arnaiz and Sperling 2011).

Another long known aspect is the dephosphorylation of dynamin for driving clathrin-coated vesicle endocytosis in mammalian cells (Marks and McMahon 1998). In agreement with the frequently reported fact that protein phosphatases are localized close to their site of action (Inagaki et al. 1994) we found calcineurin considerably enriched on parasomal sacs of *Paramecium* (Momayezi et al. 2000). In *Tetrahymena*, these sites have been shown to contain a dynamin-like protein (Elde et al. 2005).

CDPKs contain a calmodulin-like domain integrated in the kinase molecule (Sussmann et al. 1996). They are specific inventions of protists and higher plants. In *Paramecium* CDPKs phosphorylate several ciliary Ca^{2+}-binding proteins as well as centrin of the infraciliary lattice (Kim et al. 2002). The latter may be relevant for cell surface structuring and the centrin-enriched lattice provides a sink (immobile buffer) for Ca^{2+} after massive exocytosis stimulation (Sehring et al. 2009). As according to database mining the number of protein kinases, including Ca^{2+}-sensitive ones, is unexpectedly large (Bemm et al. 2009) one has to expect for protein kinases many more functions pertinent to Ca^{2+} signaling.

Although the basic mechanism of synaptotagmin as a Ca^{2+} sensor for membrane fusion is common to all higher eukaryotes, a synaptotagmin equivalent has not been identified unambiguously in protozoa as yet. In *Paramecium*, the database contains similar proteins including one type with eight (rather than two in orthodox synaptotagmin) C2-domains which otherwise is most similar to synaptotagmin (Plattner et al. 2012). The wide variety of CRCs we find in *Paramecium* may provide Ca^{2+} for locally different sensors with different Ca^{2+} sensitivity, as known from synaptotagmin in mammalian cells (Sugita et al. 2002).

Energetic aspects

Exocytosis stimulation entails a significant decrease in total [ATP] over ~5 s and a slower increase within ~30 s (Vilmart-Seuwen et al. 1986; Müller et al. 2002). One may argue that the cell invests ATP to get rid of an excess of Ca^{2+}, but this aspect may be controlled in several ways. One aspect is the occurrence of dephosphorylation of a 63 kDa phosphoprotein, pp63, within 80 ms, i.e. the time required for synchronous exocytosis (Zieseniss and Plattner 1985; Höhne-Zell et al. 1992). This pp63 has been identified as phosphoglucomutase (Hauser et al. 1997), with two isoforms occurring in *Paramecium*. It is a substrate of calcineurin (Kissmehl et al. 1996, 1997a) and re-phosphorylation is executed by a casein kinase which quite unusually is inhibited by Ca^{2+} (Kissmehl et al. 1997b; Vetter et al. 2003). Thus, dephosphorylation is very fast, although rephosphorylation is retarded in the course of Ca^{2+} signaling. We have only indirect evidence that this interplay may keep phosphoglucomutase in a dephosphorylated active state, as hypothesized by Plattner and Kissmehl (2005), thus allowing it to feed glucose substrate into the glycolytic pathway for further use in mitochondria. From all we know about mitochondrial Ca^{2+} in *Paramecium* and the general effect of a $[Ca^{2+}]$ increase in mitochondria on energetics (see above) we have to expect an increase in ATP production also in these organelles. Thus, events in the cytosolic compartment might be coupled with the effect of mitochondrial $[Ca^{2+}]$ increase during exocytosis stimulation (see above).

EVOLUTIONARY ASPECTS, CONCLUSIONS AND OPEN QUESTIONS

Evolutionary aspects

Many details of cellular Ca^{2+} dynamics are currently known from *Paramecium*, probably more than from most other protozoa. The question arises when in evolution such a complex signaling system may have been installed. Alveolates are estimated to have emerged about 2×10^9 yr ago (Hedges et al. 2004), ciliates about 850×10^6 (Douzery et al. 2004), Apicomplexa about 550×10^6 (Douzery et al. 2004) and their current parasitic close relatives about 13×10^6 yr ago (Ricklefs and Outlaw 2010). We may reasonably assume that the requirement of Ca^{2+}, alone for the needs of complex intracellular trafficking pathways, had to be matched already at the beginning of the evolution of Alveolates (Plattner and Verkhratsky 2013). In the other extreme, Apicomplexa have changed their Ca^{2+} signaling system with several respects, as it is not possible, for instance, to identify any $InsP_3Rs$ or RyR-like proteins (Plattner et al. 2012).

From the multitude of rather similar, but in detail divergent CRCs in *Paramecium*, one may conclude that an ancient precursor of current-time CRCs, types $InsP_3R$ and RyR, has existed already in early times of eukaryotic evolution. There may have taken place some convergent evolution in detail to match the requirements of Ca^{2+} conductance for membrane interactions and other phenomena requiring regulation (Plattner and Verkhratsky 2013). Mechanosensitive Ca^{2+} channels may also be an ancient inheritage, not only to guide swimming cells by ciliary reversal (see above) to areas devoid of obstacles, but also to regulate internal tensions occurring, for instance, in the contractile vacuole complex and in food vacuoles (phagolysosomes). We speculate along these lines because we find Stomatin in such membrane areas and because this scaffolding protein is frequently associated with mechanosensitive channels in metazoans (Reuter et al. 2013).

Altogether it appears that in parallel to the complicated vesicle trafficking machinery also a complicated Ca^{2+} signaling machinery, with all molecular details (or most of them), will have evolved in ciliates.

CONCLUSIONS

In *Paramecium*, the inventory of Ca^{2+} regulating and Ca^{2+} regulated molecules are present in full. A plethora of Ca^{2+}-channels has been found. Those in the cell membrane have been extensively studied over decades by electrophysiologists, those inside the cell only quite recently. The spectrum of Ca^{2+}-channels encompasses mechanosensitive and voltage-dependent channels, $InsP_3Rs$ and RyR-like CRC proteins. Also characteristic Ca^{2+}-ATPases/pumps, type PMCA and SERCA are present. This inventory is complemented by Ca^{2+} sensor proteins, such as calmodulin and by CDPK and phosphatases, with the exception of synaptotagmin. Although better known from plants and recently also from protozoan parasites, including Apicomplexa, kinases type CDPK most probably serve many functions also in *Paramecium*. See Fig. 9 for a summary of basic mechanisms of Ca^{2+} signaling and homeostasis, and Table 1 for the localization of relevant components.

Despite some progress in work concerning Ca^{2+} in *Paramecium* many questions remain to be solved. The following are some examples:

- How is the signal perceived and transduced when exocytosis is stimulated by AED?
- Is there a classical G-protein-coupled 7-transmembrane protein receptor with coupling to PLC?
- Which is the identity of the Ca^{2+}-sensor for membrane fusion, in the absence of synaptotagmin?
- How are alveolar sacs coupled to the cell membrane, considering the absence of Orai and Stim proteins in the *Paramecium* database?
- Although in *Paramecium* a calreticulin-like protein could be localized in the ER and a calsequestrin-like protein in the alveolar sacs using monospecific antibodies, their molecular characterization remains to be established.
- Are all of those *Pt*CRCs which, according to their sequence similarities, are closely related to $InsP_3Rs$ and RyRs functionally active? And how are they locally activated?

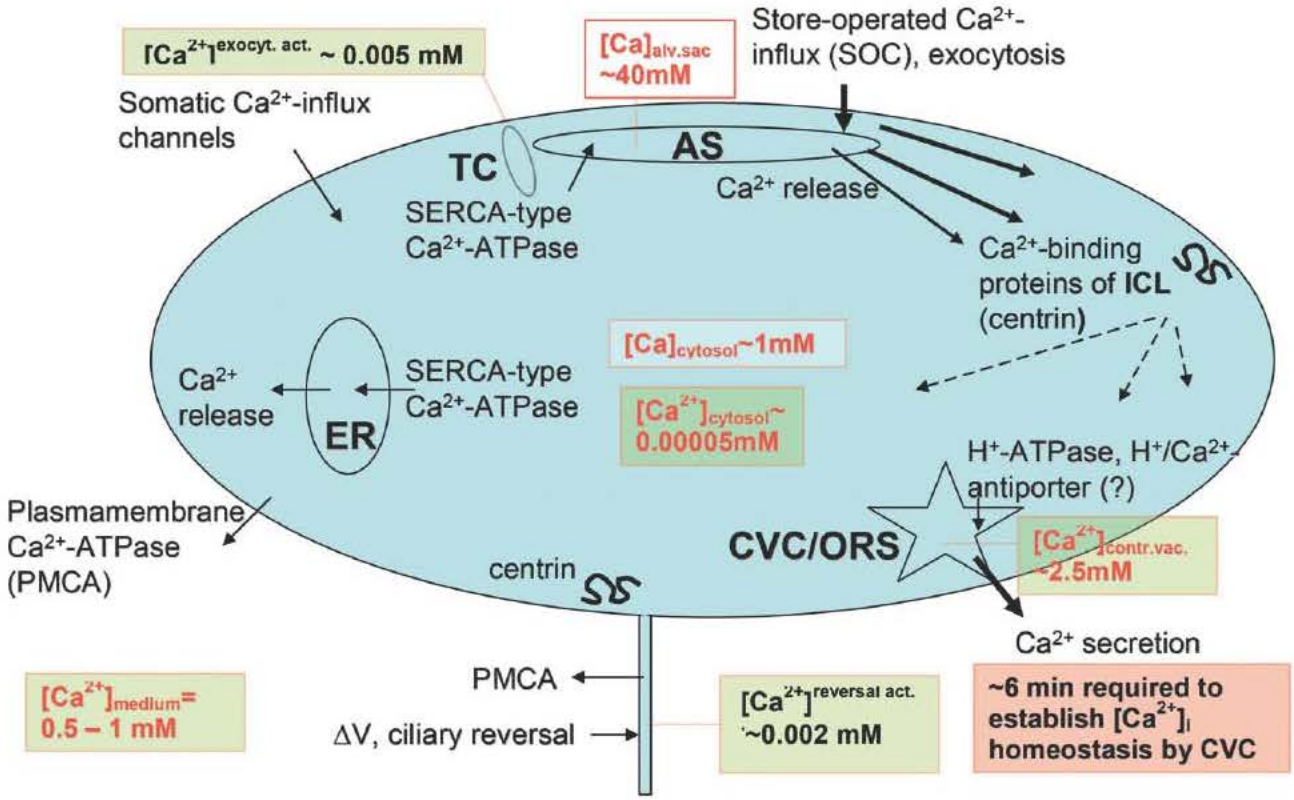

Figure 9 Survey of Ca^{2+} storage, mobilization, and handling, as established for *Paramecium* cells. The structures considered are alveolar sacs (AS), the ER and cilia (with depolarization dependent [ΔV] Ca^{2+} influx channels), the infraciliary lattice (ICL) as an immobile Ca^{2+} buffer system as well as the contractile vacuole complex (CVC ORS, osmoregulatory system). Boxes indicate total [Ca] and dissolved [Ca^{2+}], respectively, in dif ferent areas of the cell. Pumps/transporters available for the downregulation of Ca^{2+} encompass PMCA, SERCA and, indirectly, the H^+ ATPase in conjunction with a hypothetical H^+/Ca^{2+} (or similar) antiporter for secondary active Ca^{2+} transport into the contractile vacuole complex, followed by secretion during contractile vacuole systole. Differences in actual Ca^{2+} concentrations between compartments are significant, thus allowing directed Ca^{2+} fluxes. In alveolar sacs, [Ca] of ~40 mM (Hardt and Plattner 2000) can be rapidly mobilized to release Ca^{2+}, but lumenal [Ca^{2+}] is not known. Any increased cortical Ca^{2+} is rapidly bound to centrin of the infraciliary lattice (Sehring et al. 2009); from there Ca^{2+} dissipates into the cytosol for extrusion mainly via the contractile vacuole system (shown by the retardation of $[Ca^{2+}]_i$ downregulation by inhibiting H^+ ATPase activity Plattner et al. 2012). Thus, after exocytosis stimulation, $[Ca^{2+}]_i$ homeostasis can be re established within ~6 min (Ladenburger et al. 2006). [Ca^{2+}] at trichocyst (TC) exocytosis sites during stimulation has been estimated by Klauke and Plattner (1997), that in cilia during ciliary reversal has been derived from electrophysiology (Oertel et al. 1977), as summarized by Plattner et al. (2006). Note that centrin, associated with basal bodies, can rapidly immobilize any Ca^{2+} dissipating from the ciliary basis. For further explanations, see text.

- Any other Ca^{2+} channels addressed here remain to be identified at the molecular level.
- Does the ciliary membrane contain a PMCA?
- Does the contractile vacuole complex contain a Ca^{2+}-ATPase and a Ca^{2+}/H^+ antiporter?
- What precisely is the function of calmodulin at secretory organelle docking sites and during ciliary reversal?
- Which are the specific functions, out of probably many, of calcineurin?
- Which specific functions exert CDPKs in Ca^{2+} signaling?

There remains enough work to be performed in the decades to come, provided funding of basic research will still be available for aspects like these. Surely, it may be an argument that work with a harmless ciliate, which can be easily cultured and which offers considerable advantage by its regular design, may also give a handle to similar problems with parasitic protozoa which are much less easy to work with. I mention this because politicians and universities may decide more and more that work without immediate practical use may not be supported further on.

ACKNOWLEDGMENTS

The author's work cited herein has been supported by the German Research Council (Deutsche Forschungsgemeinschaft).

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