Calcium signaling in closely related protozoan groups (Alveolata): Non-parasitic ciliates (*Paramecium*, *Tetrahymena*) vs. parasitic Apicomplexa (*Plasmodium*, *Toxoplasma*)

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ABSTRACT

The importance of Ca^{2+} -signaling for many subcellular processes is well established in higher eukaryotes, whereas information about protozoa is restricted. Recent genome analyses have stimulated such work also with Alveolates, such as ciliates (*Paramecium*, *Tetrahymena*) and their pathogenic close relatives, the Apicomplexa (*Plasmodium*, *Toxoplasma*). Here we compare Ca^{2+} signaling in the two closely related groups. Acidic Ca^{2+} stores have been characterized in detail in Apicomplexa, but hardly in ciliates. Two-pore channels engaged in Ca^{2+} -release from acidic stores in higher eukaryotes have not been stingly characterized in either group. Both groups are endowed with plasma membrane- and endoplasmic reticulum-type Ca^{2+} -ATPases (PMCA, SERCA), respectively. Only recently was it possible to identify in *Paramecium* a number of homologs of ryanodine and inositol 1,3,4-trisphosphate receptors (RyR, IP_3R) and to localize them to widely different organelles participating in vesicle trafficking. For Apicomplexa, physiological experiments suggest the presence of related channels although their identity remains elusive. In *Paramecium*, IP_3Rs are constitutively active in the contractile vacuole complex; RyR-related channels in alveolar sacs are activated during exocytosis stimulation, whereas in the parasites the homologous structure (inner membrane complex) may no longer function as a Ca^{2+} store. Scrutinized comparison of the two closely related protozoan phyla may stimulate further work and elucidate adaptation to parasitic life. See also "Conclusions" section.

Keywords:
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1. Introduction

Early in evolution, eukaryotes have established small changes in intracellular free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, as a second messenger system, in many cases connected to a variety of external stimuli. Such mechanisms and corollaries of Ca^{2+} signaling have been extensively summarized for multicellular organisms [1–4]. Basic aspects are as follows. Ca^{2+} can come from outside the cell by influx and/or from internal stores, and both components may cooperate. Ca^{2+} signals can trigger widely different effects, from cell contraction, secretion and gene activation to cell death etc. Thus, to be specific, precise spatial and temporal regulation of $[\text{Ca}^{2+}]_i$

in microdomains is required [5–7]. This is achieved by precisely positioning Ca^{2+} influx channels and/or Ca^{2+} release channels at strategic sites and rapid downregulation of the signal. Local regulation is even more important as, on the one hand, the effect achieved shows an extremely superlinear dependency on local $[\text{Ca}^{2+}]_i$ [5], while on the other hand rapid inactivation of the signal by diffusion, binding to cytosolic Ca^{2+} -binding proteins (CaBPs, immobile Ca^{2+} buffers), sequestration into stores, and extrusion from the cell rapidly counteract the stimulatory effect of a $[\text{Ca}^{2+}]_i$ increase after activation [8]. Strict regulation also serves to impede toxic effects of too high concentrations of Ca^{2+} [9].

Only some of these aspects have been analyzed in some detail in protozoa, as summarized for the groups to be compared here, ciliates [10] and Apicomplexa [11,12]. Comparison of current knowledge on Ca^{2+} signaling in ciliates (predominantly *Paramecium*) with older reviews [13–15] shows that some implications could be more clearly specified, while others could not be verified. Beyond that, since about 1995, introduction of imaging

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methods and of molecular biology has allowed unforeseeable progress.

Among Ca^{2+} -influx channels, from protozoa on, some are activated by depolarization (voltage-dependent [gated] Ca^{2+} channels), while others are activated mechanically or by specific ligands or other compounds [16]. Among Ca^{2+} stores, some are located in the cell cortex and structurally coupled to influx channels. For instance, in higher eukaryotes, stores may be activated by conformational coupling (sarcoplasmic reticulum [SR] of skeletal muscle) or by Ca^{2+} influx [Ca^{2+} induced Ca^{2+} release, CICR, in SR of heart muscle] [17]. Alternatively Ca^{2+} influx can be activated by emptying of cortical stores (store-operated Ca^{2+} influx, SOC) in response to a stimulus (Section 3.2). For the latter, two proteins are known to interact in mammalian cells [18]: Stim acts as a transmembrane reporter of luminal [Ca^{2+}] in cortical ER which signals store emptying to the cell membrane where Orai mediates Ca^{2+} influx (Section 3.2).

In contrast, many stores are distributed deeper inside the cell. They encompass a large part of the ER, mitochondria, and some widely different vesicle subpopulations involved in trafficking – from endosomes to lysosomes and secretory vesicles. This variety, including some more or less acidic compartments, can be complemented – from protozoa to man – by dedicated acidic Ca^{2+} stores, such as the acidocalcisomes [19–22]. In higher eukaryotes, activation of non-acidocalcisomal Ca^{2+} stores is achieved by second messengers, such as inositol-1,4,5-trisphosphate (InsP_3 , derived from phosphatidylinositol-4,5-bisphosphate, PlnSP_2) which binds to the InsP_3 receptor, IP_3R [23], or by cyclic adenosinediphosphoribose (cADPR which activates the ryanodine receptor, RyR [24]). Nicotinic acid-adenine-dinucleotide phosphate (NAADP^+) binds to two-pore channels, TPC, in the membranes of some acidic compartments – members of the endo-lysosomal trafficking system – which it thus activates [25–27]. Both, cADPR and NAADP^+ are derived from NAD and NADP, respectively [28].

A variety of drugs has been used to characterize the different types of Ca^{2+} release channels in mammalian cells, while their physiological activators have been detected only gradually, some remaining under discussion up to recently. However, specificity and efficacy of channel-modulating drugs, such as ryanodine and caffeine (1,3,7-trimethylxanthine) for RyRs , may vary with concentration in the case of ryanodine [29], between cell types and particularly at different levels of evolution. The effects can vary widely from protozoa to metazoa, or – more specifically – mammalian cells [30]. Intracellular Ca^{2+} release channels, therefore, have been more difficult to approach than channels in the cell membrane which are also easily amenable to electrophysiological analysis.

Only quite recently several developments have facilitated further analysis. This has involved rapid progress in elaborating genomic databases (DB) for many species, and increasing availability of domain DBs. This is of particular importance considering the requirement of defined domain structure particularly also for proteins dedicated to the handling of Ca^{2+} . Data obtained by molecular biology can then be correlated with antibody (AB) and green fluorescent protein- (GFP-) tagging in vivo as well as with functional analyses (gene silencing) and recording of [Ca^{2+}]_i signals generated under defined conditions.

Ca^{2+} transients are rapidly counterbalanced by binding of Ca^{2+} to cytosolic CaBPs [5]. These encompass some with EF-hand Ca^{2+} -binding loops, such as calmodulin (CaM) and centrin, while others possess C2 domains (β -sheets with a Ca^{2+} -binding loop). Among them are the Ca^{2+} -sensor proteins (Section 5) for membrane-membrane interaction (synaptotagmin and related proteins), copines, phospholipase C (PLC) and the regulatory subunit (SU) of the Ca^{2+} /CaM-activated protein phosphatase type 2B (PP2B=calcineurin, CaN). Such high affinity/low capacity CaBPs proteins cooperate, or compete, with Ca^{2+} effectors in the cytosol or

Fig. 1. Outline of the approximate evolutionary relationship between members of the phylum Alveolata. Evolutionary distances are not drawn to scale. After Aravind et al. [38].

on membranes. In contrast, many stores contain low affinity/high capacity CaBPs, such as calreticulin (ER) and calsequestrin (SR). The abundance of acidic aminoacids in these proteins enables rapid binding of large quantities of Ca^{2+} in the store lumen from where it can easily be released.

Finally, in higher eukaryotes the plasma membrane can extrude Ca^{2+} by a plasmalemmal Ca^{2+} -ATPase/pump (PMCA) or by cation exchangers, e.g. a $\text{Na}^+/\text{Ca}^{2+}$ antiporter. Mitochondria possess a Ca^{2+} uniporter for rapid uptake and a $\text{Na}^+/\text{Ca}^{2+}$ antiporter for rapid release. Internal stores, such as ER and SR, possess a sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) which – in contrast to PMCA – lacks the CaM-binding domain. Both belong to the P-type of ATPases because they form a phospho-intermediate during each pumping cycle [31,32]. In contrast, acidic stores can exploit the chemiosmotic H^+ -gradient (ΔH^+), generated by a H^+ -ATPase, using a $\text{H}^+/\text{Ca}^{2+}$ exchanger. The vesicular Ca^{2+} -pumps (V-type Ca^{2+} -ATPases) form no phospho-intermediate. Among protozoa, some of these molecules and, in addition, some other pumps are in use, such as H^+ -pyrophosphatases (H^+ -PPases), as well as alternative cation antiporter systems.

All this makes the basic process of Ca^{2+} signaling very complex, but this complexity also enables a variety of local responses in a cell with a considerable degree of fine-tuning. For basic references, see Berridge et al. [2], Clapham [3], Dodd et al. [4], and Palmgren and Nissen [32] and the following text. In protozoa, knowledge even of some of the basic principles has been rather fragmentary until quite recently – and in part it still is.

2. Calcium signaling in Alveolates

2.1. Why compare ciliates and apicomplexan parasites?

As mentioned, all Alveolates contain flat cortical sacs, designated “alveolar sacs” in Ciliophora/ciliates (R.D. Allen at <http://www5.pbrc.hawaii.edu/allen/>) as well as in dinoflagellates, and the “inner membrane complex” (IMC) in Apicomplexa [33], respectively. It is reduced in *Theileria* [34] when compared to *Toxoplasma* [35], *Plasmodium* etc. Therefore, the phyla of ciliates and Apicomplexa, together with dinoflagellates, are united in the superphylum Alveolata. Recent detection in deep-sea samples of abundant gene sequences tentatively ascribable to ciliates, dinoflagellates or Apicomplexa of unknown identity [36,37] will further stimulate the interest in Alveolata.

Whereas most work on Ca^{2+} signaling has concentrated on mammalian cells, with considerable gaps also in higher plant cells, the question arises as to when in evolution specific signaling mechanisms have evolved, including Ca^{2+} signaling and the subsiding mechanisms? Alternatively, some signaling mechanisms may have been modified or eliminated during the transition to parasitism, when cells have reduced and streamlined their genome

Fig. 2. Important aspects of $[\text{Ca}^{2+}]_i$ regulation and some Ca^{2+} -regulated processes in *Paramecium*, as derived from the combination of methods outlined in the text. This includes EM analyses based quenched-flow/fast freezing (cryofixation), freeze-fracture and element analysis (EDX, ESI), as well as immuno-gold localization of proteins identified by cloning the respective genes. (A) Note the occurrence of passive or stimulated influx of Ca^{2+} via somatic influx channels and, upon depolarization, influx via voltage-dependent channels in cilia. During exocytosis store-operated Ca^{2+} -influx (SOC) occurs in concert with Ca^{2+} release from alveolar sacs (AS), as ascertained by a combination of EDX with electrophysiology and fluorochrome analysis. From the stimulation site the Ca^{2+} signal is dissipated and first rapidly bound to centrin of the infraciliary lattice (ICL) in the cell cortex; further dissociation and diffusion to acidic compartments, such as the CVC/ORS (osmoregulatory system) leads to Ca^{2+} sequestration and reduction of $[\text{Ca}^{2+}]_i$. Components most relevant for exocytosis (the emphasis of our work) and subsequent regulation steps are encircled. In the stores, ER and alveolar sacs, Ca^{2+} is enriched by a SERCA-type pump. Other potential stores, such as further acidic compartments, are not yet sufficiently analyzed in these cells and, therefore, not considered in the figure. (B) Scheme of a trichocyst (t) docking site, surrounded by alveolar sacs (as), at the cell membrane (cm), alternating with cilia (ci). (C) Electron micrograph of a trichocyst (t) just undergoing exocytosis. Alveolar sacs (as) are swollen after this standard preparation, but would appear flat after cryopreparation. Magnification $\sim 100,000\times$.

Fig. 3. Schematic and EM representation of structures relevant for Ca^{2+} signaling in Apicomplexan parasites. (A) Ca^{2+} transport mechanisms in a tachyzoite form of *T. gondii*. Ca^{2+} extrusion to the extracellular milieu occurs through a plasma membrane-type Ca^{2+} -ATPase (PMCA) (1). After influx, probably through a Ca^{2+} channel (2), the intracellular Ca^{2+} can interact with Ca^{2+} -binding proteins or be transported into different intracellular compartments, including $\Delta\psi$ -dependent transport into mitochondria (3), sequestration by the endoplasmic reticulum via a SERCA-type Ca^{2+} -ATPase (4), and delivery to acidocalcisome (5) or to the nucleus (6). (B) Electron micrograph of *T. gondii*, obtained after cryopreparation, showing the representative organelles of a tachyzoite form. Ac, acidocalcisome; Mn, micronemes; N, nucleus; arrowheads, rhoptries. Note the flat alveolar compartment, designated as the "inner membrane complex". Bar: 2 μm . Adapted from Moreno et al. [453]. Illustrated by Dirceu Esdras Teixeira, Federal University of Rio de Janeiro, Brazil.

[38,39]. This question is of top relevance with Apicomplexa (*Plasmodium*/malaria, *Toxoplasma*/toxoplasmosis etc.).

The close relationship between ciliates and Apicomplexa is undisputed (Fig. 1), as it has been established in different studies on their molecular evolution [38,40,41]. In detail, this includes analysis of a variety of components, such as small ribosomal rRNA [42,43], 268 single copy genes [44], hsp70 proteins [45], proteins derived from cyanobacterial endosymbionts [46], ribosomal proteins (including Dinoflagellates as the largely neglected third group of Alveolata [47]), and a collection of first- and second-tier marker proteins [48]. This also includes phylum-specific proteins such as "alveolin" [49] that line the alveolar compartments below the cell membrane. All contain subplasmalemmal flattened sacs, the alveoli and the IMC, respectively (Figs. 2 and 3). The rather distinct freeze-fracture appearance of docking sites of dense core-secretory vesicles (particle "rings" and "rosettes") displays striking similarity between ciliates [50–52] and Apicomplexa [53]. Docking of trichocysts proceeds along basal body associated microtubules,

in plus $\rightarrow$ minus direction [54] and, thus, resembles to positioning of lytic granules at the immunological synapse [55]. Similarly microtubules are arranged around the rhoptries and micronemes in the Apicomplexa (see above).

Another point in common is the association of cytoskeletal proteins with the alveoli and the IMC, which has been designated a membrane skeleton. Examples include the tetrins in *Tetrahymena pyriformis*, epiplasmins found in *Paramecium* and articulins in *Pseudomicrothorax dubius* [56]. In the case of *Toxoplasma gondii*, immunofluorescence microscopy has shown the presence of plateins, a subfamily of articulins. They form 10 nm thick filaments distributed throughout the protozoan body. Comparison with other protists has revealed considerable conservation [57]. In Apicomplexa the "inner membrane complex", IMC, is tightly bound to the cell membrane by a complex of proteins inserted in, or attached to the outer part of the IMC, in addition to actin and the thrombospondin-related anonymous (adhesive) protein, TRAP [58,59]. However, molecular links between alveoli and the

cell membrane are not known from ciliates, although in the EM one can clearly recognize physical links, e.g. in *Paramecium* [50].

The appearance of recent ciliate clades is estimated to ~800 [42,43] to 850 Mio years [60], with a common ancestry dating back considerably longer. This may have involved rather early signaling mechanisms on the basis of cyclic nucleotides [61] and of Ca^{2+} (this review), and thus may have developed in parallel to vesicle trafficking regulation by Rab and SNARE proteins already early in evolution [62–65]. The emergence of Apicomplexa, before becoming vertebrate parasites, has been dated to ~550 Mio years ago for early forms and 400 Mio years ago for the genus *Plasmodium* [60], but radiation of mammal-hosted *Plasmodium* species started ~13 Mio years ago [66]. With ongoing specialization the genome may have undergone reduction and adaptations and, thus, diversification from the common ciliate/apicomplexan ancestors. How much similarity has there remained?

Experimental approaches to elucidate Ca^{2+} signaling are evidently easier with the non-pathogenic, large ciliate cells, while Apicomplexa (*Plasmodium*, *Toxoplasma*) deserve considerable medical interest. This is particularly due to the exocytosis of a battery of dense core-secretory organelles (apical rhoptries, micronemes, and laterally positioned “dense granules”) [33,67]. Among them, microneme secretion definitely requires a Ca^{2+} signal for exocytosis which is prerequisite to host cell infection [20,68,69]. These organelles are reminiscent of *Paramecium*'s trichocysts and *Tetrahymena*'s mucocysts which both are positioned over the entire cell cortex. To what extent may Ca^{2+} signaling be governed in different ways in the two main groups of alveolates?

In the past decade, work with either group has profited from the ongoing genome projects. The picture emerges that these two groups of alveolates not only share some similarities, but also exhibit considerable differences, as we outline in this survey. This may reflect the considerable reduction of apicomplexan genomes [38,39] and the specific requirements for infection. However, comparison of the two main groups of Alveolata (with little data available on dinoflagellates) also reveals significant gaps of knowledge, often on specific, but widely different aspects. In practice, data obtained with one group are rarely taken into consideration for the other one. Therefore, this review is also intended to stimulate further work to fill such gaps. We want to stress, however, that it is not our intention to detail species specificities [39] or specific stages of the parasites, with their specific gene expression [70] and their specific Ca^{2+} requirements and signaling pathways [33], respectively. (These simplifications will help non-parasitologists; the facts we describe evidently refer to the stages where the respective structures and functions occur, as parasitologists will recognize.) Our main intention is to include proteomics data and experiments with cells and, thus, go beyond predictions and implications from genomic database mining. Restriction to comparative genomics can be misleading particularly when lower eukaryotes are compared with mammalian sequences; rather the analysis of specific domain sequences and protein function in situ are mandatory.

2.2. Basic $[\text{Ca}^{2+}]_i$ values and Ca^{2+} responses in Alveolata cells upon stimulation

2.2.1. Ciliates

Much of our knowledge about Ca^{2+} in ciliates comes from *Paramecium*, mainly *Paramecium tetraurelia*. Because of their large size, they were preferably used over decades for electrophysiology [71,72], mainly in the context of their swimming behavior. Another aspect analyzed to some extent in ciliates is vesicle trafficking (*Paramecium*: [64,65,73], *Tetrahymena*: [74]). All these phenomena are tightly coupled with Ca^{2+} signaling (Fig. 2).

As in mammalian and other cells, basic $[\text{Ca}^{2+}]_i$ is slightly below or near 10^{-7} molar in ciliates [75,76] and values in apicomplexan

cells are similar (Section 2.2.2). In ciliates, $[\text{Ca}^{2+}]_i$ may increase at different sites of the cell in response to different stimuli, e.g. during depolarization-induced ciliary reversal/avoidance reaction or drug-induced exocytosis [10]. This includes secretagogues such as the RyR activators caffeine and 4-chloro-meta-cresol (4CmC) as well as the polyamine secretagogue, aminoethyl dextran (AED) [10].

Remarkably, *Paramecium* cells can ward off attacks by some predators, such as *Dileptus*, by massive trichocyst exocytosis, as shown by Harumoto and Miyake [77] and confirmed by single cell analysis [78] which has revealed the function of unilaterally released (and decondensed) trichocyst contents as distance pieces. This cell–cell contact triggers Ca^{2+} signaling in the *Paramecium* cell [79] and similarly a local exocytotic response occurs with locally applied AED [80]. At the same time, ciliary beat is reversed – another aspect of the escape mechanism [78]. When *Paramecium* cells are stimulated by local heat generated by a pointed laser beam, this causes vigorous, synchronous trichocyst exocytosis and cells to move abruptly backward [81], as during chemical stimulation. Weaker heat stimulation causes ciliary reversal whose speed depends on the stimulation intensity. How this relates to Ca^{2+} remains to be established, but all this argues for a more or less restricted reaction, with limited spillover.

Normally ciliary reversal is induced by a depolarization/receptor potential generated in response to the activation of anteriorly enriched mechanoreceptor-type Ca^{2+} -channels [82]. ΔV -dependent Ca^{2+} channels are restricted to ciliary membranes [83] and intraciliary Ca^{2+} signaling does not normally reach the cell soma. For instance, the Ca^{2+} signal generated in cilia via chemical depolarization by applying 20 mM KCl does not spread into the cell soma [84]. Repolarization after depolarization involves Ca^{2+} -sensitive K^+ channels [85,86]. Exocytosis stimulation causes another, voltage-independent Ca^{2+} signal [75] which involves non-ciliary (“somatic”) channels and can entail spillover of Ca^{2+} into cilia, as shown by electrophysiological recordings [87] and by fluorescence measurements [84]. This has also been shown to induce ciliary reversal in pawn mutants (non-reactive to depolarization [88]) – a clear consequence of Ca^{2+} spillover [80]. Large scale Ca^{2+} signals can spread from the cell soma into the macronucleus. This is illustrated by the fact that exocytosis stimulation induces increased transcription of specific genes in *Tetrahymena* [89] and *Paramecium* cells (see *Paramecium* database at <http://paramecium.cgm.cnrs-gif.fr> and [90]). Finally, *Paramecium* also possesses Ca^{2+} channels sensitive to hyperpolarization [91].

Some of the Me^+ - and Me^{2+} -influx channel activities in the cell membrane of ciliates are modified by the resulting increase of local $[\text{Ca}^{2+}]_i$ or rather by formation of a Ca^{2+} /CaM complex [92,93]. As known from numerous electrophysiological studies with *Paramecium*, anteriorly enriched Na^+ and Ca^{2+} influx and posteriorly enriched K^+ efflux conductances in the somatic membrane are thus activated. This fact, in conjunction with the use of CaM inhibitors, has been exploited to register, by whole cell-patch electrophysiology, Ca^{2+} signals accompanying exocytosis stimulation [87]. Calmidazolium or W7 (n-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide), powerful CaM inhibitors also in protozoa [30], have been used in this work. Also K^+ channels responsible for chemoattractant-induced conductances are regulated by Ca^{2+} /CaM [94]. The ΔV -dependent Ca^{2+} channels in the cilia are inactivated by the intraciliary $[\text{Ca}^{2+}]_i$ increase [95]. Ironically the same mechanism has been identified, though much later, also in neuronal systems [96]. All this clearly demonstrates that basic mechanisms of Ca^{2+} signaling were invented early in evolution.

There are additional regulatory aspects of Ca^{2+} signaling in ciliates. In *Paramecium* the biogenesis of additional osmoregulatory system (=contractile vacuole complex, CVC) is induced when

increased $[Ca^{2+}]_o$ is applied in a hypoosmotic medium [97]. Also surface pattern formation in *Paramecium* depends on calcium (Section 5). All this suggests some morphogenetic plasticity in the context of “constitutive” Ca^{2+} signaling.

Encystment of some ciliates is another phenomenon which depends on an intracellular Ca^{2+} signal, as shown with dinoflagellates, where Ca^{2+} /CaM-activated adenylate cyclase may be involved [98]. *Tetrahymena* [99,100], but not *Paramecium*, can form cysts, but participation of Ca^{2+} was mostly inferred only indirectly from the abundant secretory activity accompanying this process. The Ca^{2+} requirement was endorsed by more thorough work with *Colpoda* [101,102]. Excystment, analyzed in the ciliate, *Sterkiella*, also depends on a Ca^{2+} signal [103].

In summary, just as in higher eukaryotes, Ca^{2+} governs many functions also in ciliates. Some of the plasmalemmal Ca^{2+} channel types occurring in mammalian cells are also found in ciliates. Channel activities include some modulation mechanisms, e.g. activation or inhibition by a Ca^{2+} /CaM complex, as well as intracellular Ca^{2+} release channels (Section 4.1). A Ca^{2+} signal accompanies, and is mandatory for a variety of ciliate activities, such as secretion and ciliary motion. Various aspects of Ca^{2+} signaling are rather old evolutionary achievements.

Less easy to identify is a potential Ca^{2+} -sensor in the cell membrane, of the types discussed by Hofer [104]. In mammals, this is mostly a Ca^{2+} /polyvalent cation sensing receptor (also sensing appropriate aminoacids) coupled to trimeric G-proteins, but such a sensor has not been found in *Paramecium*. However, such a sensor function can also be executed by a non-selective, high conductivity, membrane potential-independent cation channel. For instance, in hippocampal neurons, such a channel immediately responds to stepwise changes in $[Ca^{2+}]_o$, as analyzed by electrophysiology [105]. Similarly fluorochrome analysis in *Paramecium* has revealed an immediate, transient $[Ca^{2+}]_i$ increase upon abrupt increase of $[Ca^{2+}]_o$ [106], thus potentially fulfilling the criteria of a Ca^{2+} /polyvalent cation sensing receptor. See also Section 4.1 for a concise candidate.

2.2.2. Apicomplexa

In Apicomplexa, calibrated values of $[Ca^{2+}]_i$ at rest also vary between 50 and 100 nM [107,108]. After isolation, depending on $[Ca^{2+}]_o$, $[Ca^{2+}]_i$ in *Plasmodium falciparum* may rise up to 700 nM [11]. Thus, one may assume a rather high permeability, just as described for *Paramecium* (Sections 2.2.1 and 4.1). In the parasitophorous vacuole, the problem is to the opposite: here, the *Plasmodium* parasite is supplied with Ca^{2+} perhaps by the tubular connections to the outside medium and certainly by a Ca^{2+} -pump contained in the host cell membrane [109]. The value for $[Ca^{2+}]_o$ surrounding the parasite was thus determined as $\sim 40 \mu M$. When one refers to experience with *Paramecium* (Section 3.2) this may suffice to drive a SOC mechanism. This may be assumed to occur in *P. falciparum* cells during host cell penetration, based on $^{45}Ca^{2+}$ flux measurements [110].

Host cell infection depends on an intracellular Ca^{2+} signal [111], including host cell penetration [68,69,111]. The conoid contains secretory organelles (rhoptries, micronemes) surrounded by a cytoskeletal structure (microtubules); in Apicomplexa the secretory activity of this name-giving complex is important for host cell penetration. (To anticipate, egression from the host cell also requires Ca^{2+} , but from a different source [112]; see Section 3). The Ca^{2+} ionophore, A23187, by mediating secretion, can make parasite cells incapable of invasion, if incubated before seeing the host, but it also stimulate invasion and the exit of *T. gondii* tachyzoites from macrophages [113,114]. Generally, host cell penetration capacity has been probed with widely different Ca^{2+} activating and inhibitory compounds, respectively, including also the SERCA inhibitor thapsigargin (1 μM concentration) and

intracellular Ca^{2+} chelators, e.g. BAPTA-AM which is permeable and activated intracellularly by release of its acetoxymethylester moiety [115,116]. Fluorochrome recordings indicated the dependency of microneme secretion from a Ca^{2+} signal [117–119]. The molecular background, however, is poorly understood. One reason to compare the parasites with ciliates was the relatively easy accessibility of Ca^{2+} dynamics in the latter.

For the apicoplast, found as a vestigial plastid remnant in *T. gondii* [120] some plant-like functions have been suggested [112]. Abscissic acid synthesis causes cADPR formation and excystment in the infected tissue – a process requiring Ca^{2+} [112]. Evidently cyst formation reduces virulence in *T. gondii* [121,122]. For cell cycle regulation in *P. falciparum*, a complete Ca^{2+} signaling pathway has been hypothetically envisioned [108], as outlined in Section 5.2.

3. Calcium stores in alveolates and their activation

3.1. Identification of calcium stores in Alveolates

From neuronal systems, mutual interaction of Ca^{2+} signals from different sources in a cell is well known [123]. This results from interdependence of Ca^{2+} mobilization from stores and from influx, as well as the interplay between a local $[Ca^{2+}]_i$ increase and downregulation. How can we cope experimentally with such an intriguing situation?

Ca^{2+} stores may be identified by combinatorial approaches, e.g. by fluorochromes targeted to specific stores [6], energy-dispersive X-ray microanalysis (EDX- see below) in the electron microscope (EM), as well as use of activators and inhibitors. Activators are discussed in the context of the different Ca^{2+} -release channels (Section 4). Among inhibitors, those for sequestration may be typical of a store-specific uptake mechanism. For instance, the SERCA-type pump in the mammalian ER is inhibited by widely different compounds, including thapsigargin, cyclopiazonic acid, and *tert*-butylbenzohydroquinone, t-BHQ [124]. The binding sites for the latter are different from those of thapsigargin. As discussed below, sensitivity of SERCA molecules in Alveolata differs considerably from that in mammals.

3.1.1. Ciliates

In *Paramecium*, alveolar sacs have been identified as cortical Ca^{2+} stores by Stelly et al. [125]; they isolated these compartments and followed $^{45}Ca^{2+}$ sequestration in an ATP regenerating regime. These studies were extended by Länge et al. [126] to find out more details about the kinetics of uptake and release. In fact, the activation mechanism remained elusive until very recently. Alveolar sacs were shown in situ EM studies using calibrated EDX [127] and, in addition, by electron-spectroscopic imaging, ESI [128] to contain Ca^{2+} . Toward this goal, cells were rapidly inactivated by cryofixation, i.e. by shooting into a cryogen [129]. Since this was combined with timed stimulation (quenched-flow analysis), store activation and refilling could be monitored. Note that EDX and ESI register local total concentrations (free and bound) of calcium, $[Ca]$, while fluorochrome analyses register free Ca^{2+} , $[Ca^{2+}]$.

Some other compartments harboring calcium were detected early on, with calcium deposited in specific electron dense or crystal-bearing vesicles, not only in *Tetrahymena* [130] but also in *Paramecium*. Here, crystals were identified by chemical and X-ray diffraction analysis as struvite (Mg/Ca-ammonium phosphate) [131], a mineral also known from kidney stones.

In *P. tetraurelia*, it was recently possible to identify and localize a broad spectrum of different Ca^{2+} release channels (CRC) related to IP_3R/RyR -type channels (*PtCRC*) [132–134]. Different types of such *PtCRCs* occur not only in dedicated stores, such as ER and alveolar sacs, but also in the CVC. Some occur in several of the manifold vesicle types which are known to participate in vesicle trafficking, based

either on morphological analyses [73] or on analyses of molecules relevant for the trafficking process. These include SNAREs (soluble N-ethylmaleimide attachment protein receptors), specific actin isoforms and subunits of the H^+ -ATPase [64,65]. The multitude of such vesicles with specific PtCRCs suggests a rather local role for Ca^{2+} signaling in ciliates (Section 4.1). Evidence from mammalian cells also revealed that many, though not all, such vesicles are known to store Ca^{2+} [135] and – at least in part – to be endowed with a Ca^{2+} -sensor [136]. Such local Ca^{2+} signals may be prerequisite to the capacity of organelle specific membrane interaction and fusion. In summary, there is an intriguing mutual connection between local $[Ca^{2+}]_i$ and vesicle trafficking. Not only can Ca^{2+} modulate Ca^{2+} channel delivery; properly positioned CRCs in turn can regulate further vesicle trafficking. Furthermore, formation of trans-SNARE complexes per se can induce Ca^{2+} release in metazoan cells [137].

3.1.2. Similarities and dissimilarities between ciliates and Apicomplexa

In *T. gondii* immuno-gold EM localization of the SERCA-type Ca^{2+} -ATPase has identified the ER as a prominent Ca^{2+} store [138]. Considering vesicle trafficking in Apicomplexa, one may also expect a spectrum of Ca^{2+} release channels to mediate the formation of local $[Ca^{2+}]_i$ signals. However, no data on the molecular identity of such CRCs are available at this time despite repeated efforts ([139,140]; our own unpublished research). This makes clear identification and characterization of stores difficult. In particular no data are available on Ca^{2+} based signaling mechanism involving the IMC [141], the equivalent of ciliate alveolar sacs.

Is the IMC of the parasites a calcium store similar to alveolar sacs of ciliates? After preparation by standard EM protocols, the latter appear swollen and empty (Fig. 2C), but after fast freezing/freezing-substitution they appear flat [129], much like IMCs (Fig. 3B). The inner part of the IMC of *P. falciparum* and *T. gondii* is endowed with six-pass membrane proteins termed GAPM (glideosome-associated protein with multiple-membrane spans) [142] and in freeze-fracture replicas these membranes are studded with 9 nm-sized intramembranous particles/proteins arranged in register with underlying microtubules (*P. gallinaceum*: [143]; *T. gondii*: [35,144]). Therefore, GAPMs may cause this arrangement. In contrast, the inner part of the alveolar sacs of *P. tetraurelia* has no such microtubules attached, displays a rather different freeze-fracture appearance and houses densely clustered SERCA molecules [145]. (Note that SERCA is a 10-pass integral protein [32].) The inner part of the alveoli, therefore, is widely different between the two groups of Alveolata.

EDX analysis has been applied to cryofixed *T. gondii* cells [146]. There was no indication of a cortical store with any remarkable enrichment of Ca signals, in contrast to experiments conducted by the same group under comparable conditions with *Paramecium* cells [147], although the spatial resolution achieved did allow only to monitor calcium in the cell cortex, without assignment specifically to alveolar sacs. In contrast, EDX and ESI performed with *T. gondii* after Ca^{2+} precipitation by pyroantimonate resulted in reaction product not only in intracellular vacuoles and over the nuclear envelope (probably also in the ER), but also in association with the IMC [148]. Similar staining of IMC, ER and nuclear envelope was also found with this (less reliable) cytochemical approaches in *T. gondii* by others [149]. Thus, Ca^{2+} storage in the IMC has remained unsettled and repetition with cryomethods appears advised.

Alveolar sacs of *Paramecium* contain a SERCA-type Ca^{2+} -pump [145,150,151], a luminal calsequestrin-(CaS)-like protein [152], and Ca^{2+} -release channels related to RyRs [133]. Pumps and channels are located at opposite sides in a functionally useful manner: pumps facing the cell center, channels the periphery. No such components are known from the apicomplexan IMC (Fig. 3) whose dominant components are the gliding motor complex, the

“glideosome” [33,67]. Considering that the relevant molecular components of both alveolar sacs and the IMC are well established, one is tempted to conclude that, in Apicomplexa, the original function as a Ca^{2+} store has been either reduced or abandoned in favor of a machinery dedicated to the mechanics of host cell penetration. To repeat, EDX analysis with advanced methodologies appears to be needed.

Recently, involvement of Rab11A [153] and Rab11B in the biogenesis of the IMC of *T. gondii* has been reported, assuming delivery of Golgi-derived vesicles by Rab11B [154]. In general terms, this GTPase is assumed to be associated with recycling endosomes [155] and also from the trans-Golgi network [156]. According to the intracellular distribution of a GFP fusion protein in *Tetrahymena thermophila*, Rab11B is associated with the oral apparatus [74] and, thus, possibly involved in some membrane recycling activities. As far as the biogenesis of alveolar sacs in *Paramecium* is concerned, we have evidence only for vesicles derived from the ER or downstream [151] and this could include the trans-Golgi network (not analyzed). Currently the dynamic processes described for *T. gondii* are unique indications of vesicular biogenesis of the IMC similar to that of alveolar sacs in *Paramecium*. Here, silencing of the SNARE-specific chaperone, NSF, results in vesicle attachment to alveolar sacs which otherwise has not been recognized [157]. Basically we assume vesicle trafficking for the biogenesis of alveolar sacs, rather than maturation, and a similar pathway may underlay ICM biogenesis.

A Ca^{2+} storing organelle much better known in Apicomplexa than in ciliates is the acidocalcisome. These correspond to electron dense vesicles, as seen in whole mount EM preparations, containing a number of channels and transporters responsible for ion uptake, namely Mg, K, Ca, Fe and Zn (Fig. 4). They are endowed with a Ca^{2+} -ATPase, H^+ -ATPase and a H^+ -pyrophosphatase, H^+ -PPase, characteristic (though not unique) for this organelle [19–21]. In the apicomplexan genus *Eimeria*, acidocalcisomes have been described as metachromatic volutin granules, as seen in the light microscope [158]. Acidocalcisomes store Ca^{2+} as insoluble pyro- and polyphosphates [20]. Ciliates probably contain acidocalcisomes, though up to now these have not been characterized, in contrast to the parasites. In *Tetrahymena*, chemical analysis of metachromatic volutin granules has documented the presence of Ca,Mg-pyrophosphate [159], thus resembling apicomplexan acidocalcisomes. They appear different from the crystal vacuoles found in *Paramecium* cells whose molecular characteristics remains to be elucidated. A H^+ -PPase has been identified by polymerase chain reaction (PCR) not only in Apicomplexa, but also in ciliates [160]. In Apicomplexa, chemical and cell biological analyses have assigned this pump to acidocalcisomes [19,20,161]. Acidocalcisomes exploit formation of a ΔH^+ for Me^{2+} enrichment by H^+/Me^{2+} or similar antiporter activity, as ascertained for Apicomplexa. Here, together with a Ca^{2+} -ATPase (below), such exchangers serve the enrichment of Ca^{2+} largely in insoluble form (polyphosphate) and, thus, to control $[Ca^{2+}]_i$ homeostasis [20,162].

The Ca^{2+} -ATPase (TgA1) occurring in the acidocalcisomes (and reportedly in the cell membrane) of *Toxoplasma*, when compared with mammalian homologs, appears special with regard to several aspects: it has a size resembling that of mammalian PMCA, despite the reported absence of a CaM-binding domain; it is insensitive to thapsigargin (1 μ M) [20]. In fact, recent comparative genomic analysis classifies it as a P-type ATPase [163], similar to the established P-type ATPases, such as PMCA and SERCA. TgA1 knock-out *T. gondii* cells exhibited reduced pyro- and polyphosphate contents, altered Ca^{2+} homeostasis, and consequently reduced microneme secretion and host cell invasion capacity [20]. This may indicate a role of acidocalcisomes in Ca^{2+} signal transduction during host cell penetration, but further work appears to be required for more detailed specification, to exclude indirect effects of

Fig. 4. Transport mechanisms across the acidocalcisomal membrane in Apicomplexa. Calcium uptake and release is mediated by a Ca^{2+} -ATPase and $\text{Ca}^{2+}/\text{H}^+$ exchanger, respectively. A H^+ gradient is established by a V-H^+ -ATPase and a V-H^+ -PPase. An aquaporin channel is also present and a Na^+/H^+ exchanger accounts for H^+ release. High concentrations of PP_i and polyphosphates (Poly P) are found in the matrix, requiring enzymes involved in their metabolism, such as exopolyphosphatase (PPX) and pyrophosphatase (PPase). In other organisms, the presence of a vacuolar transporter chaperone (VTC) presumably involved in synthesis and translocation of Poly P has been suggested. The transport of basic aminoacids and other cations (Mg^{2+} , Fe^{2+} , K^+ and Zn^{2+}) is still unclear. Adapted from Docampo and Moreno [21]. Illustrated by Dirceu Esdras Teixeira, Federal University of Rio de Janeiro, Brazil.

knock-out and to achieve more direct correlation with microneme exocytosis.

In Apicomplexa, Ca^{2+} -ATPase activity is reported to be paralleled by $\text{Ca}^{2+}/\text{H}^+$ counterflux [164]. However, this may not be an exchanger activity in the classical definition, but rather it may indicate electrogenicity of this pump, as known for the SERCA of mammalian cells [165,166]. A Ca^{2+} -ATPase with rather similar properties also occurs in *P. falciparum*, but its localization is not indicated by the authors [167]. Clearly *P. falciparum* has a Ca^{2+} -ATPase that is sensitive not only to thapsigargin ($\text{EC}_{50} \sim 25 \mu\text{M}$, with some effects from micromolar concentrations on) but also to $5 \mu\text{M}$ cylopiazonic acid [168]. Ultrastructural localization would be helpful, in such cases. Note that no equivalent is known from ciliates where the two SERCA molecules detected are of “standard” size, $\sim 100 \text{ kDa}$ [150,151], and insensitive even to $100 \mu\text{M}$ thapsigargin [169]. Also the ciliate PMCA contrasts by the presence of a CAM-binding domain and a size of $\sim 130 \text{ kD}$ [170,171] as typical of PMCA in higher eukaryotic, including mammalian cells [31].

In the *Paramecium* DB – but not in Apicomplexa – TPC sequences can be found (unpubl. data), but they have not yet been assigned to any members of the endo-lysosomal axis. Since Ca^{2+} release can be activated by binding of NAADP^+ to TPCs of acidic stores [25,26,172] we performed such binding studies with *Paramecium* homogenates (Section 4.2). The data obtained further support the occurrence of such channels in ciliates. However, for the following reasons this may concern acidic stores different to acidocalcisomes, of a type yet to be identified. Acidocalcisomes have been excluded as TPC-endowed organelles in protozoa [26,173] and sea urchin eggs [174]. Moreover, no consistent TPC sequences are found in the Apicomplexan databases (Section 4.2). Acidocalcisomes can participate in trafficking and merge with other organelles, so that theoretically widely different CRC types could coexist. In trypanosomes, fusion between acidocalcisomes and the CVC has been suggested [175].

There is also evidence that Ca^{2+} is released by InsP_3 from apicomplexan acidocalcisomes [176,177], but this effect and organelle biogenesis is poorly understood. The molecular biology of the Ca , Mg -phosphate containing compartments found in *Tetrahymena* [130] still has to be established. Altogether, in ciliates, putative acidocalcisomes still require much more detailed analysis to achieve a level of knowledge comparable to that in Apicomplexa.

Additional Ca^{2+} storage organelles have been identified in apicomplexans. *T. gondii* possesses a “plant-like vacuole” endowed with a plant-like vacuolar H^+ -PPase, aquaporin and $\text{Ca}^{2+}/\text{H}^+$ as well as Na^+/H^+ exchange activity [178]. It serves to overcome stress in recently egressed tachyzoites when they reach their new extracellular medium. There are no indications of any Ca^{2+} activation mechanisms.

In *Toxoplasma*, acidocalcisomes [19] and the plant-like vacuole [178] are not only established Ca^{2+} stores, but they are also assumed to interact with each other [178]. Both compartments are able to store Ca^{2+} thanks to – among other mechanisms – a ΔH^+ generated by H^+ -ATPase and H^+ -PPase activity, connected to a $\text{Ca}^{2+}/\text{H}^+$ exchanger system [179]; Fig. 4. Whereas acidocalcisomes are widely distributed among Apicomplexa [19,21,161,180], the plant-like vacuole does not belong to the general repertoire. In the latter, the storage form of Ca^{2+} is not yet clear.

Altogether there are large blackspots in either one of the protozoan groups under consideration here, not only regarding the Ca^{2+} storage compartments, but also Ca^{2+} signaling (Section 4.1).

3.2. Activation of Ca^{2+} stores

3.2.1. Ciliates

AED, the established secretagogue [80] activates Ca^{2+} release from alveolar sacs [127] and also causes an influx of $^{45}\text{Ca}^{2+}$ in *Paramecium* [181]. AED is without any effect on the isolated cell

Table 1
Characteristics of Ca^{2+} dynamics pertinent to stimulated exocytosis in *Paramecium*.

		Reference
Cytosolic free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, before stimulation	0.065–0.100 μM	[75]
Total calcium concentration, $[\text{Ca}]$, in alveolar sacs	~40 mM	[127]
Ca^{2+} released from alveolar sacs upon stimulation within 1 s	~80%	[127]
Local $[\text{Ca}^{2+}]_i$ at stimulation sites	~5 μM	[75]
$[\text{Ca}^{2+}]_o$ required for store-operated Ca^{2+} -influx (SOC)		
Threshold value (no SOC observed)	0.25 μM	[183]
Half-maximal effect (50% exocytosis)	0.7 μM	[183]
Maximal effect (threshold for 100% exocytosis)	~5 μM	[75,183]
Time required for alveolar sacs refilling with Ca^{2+}		
Energy-dispersive X-ray microanalysis	~60 min	[127]
Timed double trigger experiments	~65 min	[436]
$^{45}\text{Ca}^{2+}$ uptake in vitro	~60 min	[126]

cortex fragments or with purified alveolar sacs [126], thus indicating the requirement of a kind of unknown signal transduction mechanism for store activation. A combination of timed activation (e.g. of exocytosis) with quenched-flow/cryofixation [129,182] allowed the analysis of Ca^{2+} fluxes with variable $[\text{Ca}^{2+}]_o$ [183] within the subsecond time scale and EDX with high spatial resolution of ~60–70 nm [127,184]. As summarized in the scheme, below, and in Table 1, EDX revealed that mobilization from stores is the first step, causing a Ca^{2+} influx as a second step. This mechanism of store-operated Ca^{2+} -influx, SOC, was further ascertained in different ways. (i) When during the subsecond stimulation time extracellular Ca^{2+} , Ca^{2+}_o , was chelated to ~30 nM, i.e. slightly below $[\text{Ca}^{2+}]_i$ at rest, ~60% of the alveolar Ca^{2+} was released. (Note that total absence of Ca^{2+}_o would increasingly damage *Paramecium* cells [128] and, therefore, was always avoided.) When Ca^{2+}_o was replaced by SrCl_2 , again release of stored Ca^{2+} was visible, while Sr^{2+} followed from the outside [127]. Note that the $K\alpha$ energy lines of Sr and Ca are easily discernible. (ii) The response to La^{3+} in the medium and Mn^{2+} -induced Fura-2 signal quenching, respectively, both characteristic of a SOC mechanism, also supported this mechanism [185]. (iii) Double mutants devoid of any Ca^{2+} influx could equally well be triggered by AED, with a similar decay of $[\text{Ca}]$ in alveolar sacs as in wildtype cells [186]. Therefore, a Ca^{2+} -induced Ca^{2+} -release (CICR mechanism) from alveolar sacs can be excluded. The Ca^{2+} -release channels involved are of the RyR-type, as discussed in Section 4.1. How mobilization from alveolar sacs and superimposed influx through the cell membrane is generated remains unknown. We refer to Section 1 for Orai/Stim linkers identified between cortical ER and the cell membrane, but none has been verified as yet (Stim and Orai being absent from the *Paramecium* DB). Remarkably, upon stimulation, a mass-transport of Ca^{2+} from the outside medium to the ER/SR (as we reported for alveolar sacs) was later described in an immune and a skeletal muscle cell line by totally different methods [187], just as in *Paramecium* [127].

Not only (unknown) chemical coupling but also conformational coupling between Ca^{2+} release channels in cortical compartments and plasmalemmal Ca^{2+} influx channels should be envisaged, in principle similar to – though not identical with – that known from skeletal muscle [17]. Physical linkers between cortical Ca^{2+} stores and the cell membrane different from Orai/Stim are also known. According to current knowledge, this includes (i) the protein Ist2p, known from yeast as an osmotic stress tolerance protein, which resembles coatamer protein type COPI [188]; when expressed in human cells it produces close ER-cell membrane attachments. (ii) In yeast cells, this is also achieved by the oxysterol-binding homology (Osh) protein family member Osh3, endowed with a pleckstrin

homology domain [189]. (iii) Juncate protein is another candidate [190].

In *Paramecium*, activation of exocytosis and exocytosis-coupled endocytosis takes place according to the following flow chart:

Non-permeable stimulus (AED) or permeable secretagogue (caffeine, 4CmC)
RyR activation in outer alveolar sacs membrane
→ release of Ca^{2+} from alveolar sacs
→ influx of additional Ca^{2+} (SOC-type mechanism)
→ activation of Ca^{2+} -sensor (not yet identified)
→ SNARE mediated membrane fusion
→ Ca^{2+} from outside medium causes trichocyst contents expulsion through exocytosis opening
Membrane resealing and endocytic ghost detachment accelerated by increasing $[\text{Ca}^{2+}]_i$ (depending on $[\text{Ca}^{2+}]_o$)

Whether crystal vacuoles of *Paramecium* represent acidic organelles related to acidocalcisomes in Apicomplexa (see above) has not been analyzed as yet, as is the case with the numerous acidic vesicles endowed with a H^+ -pump [191]. An exception is the contractile vacuole complex (CVC) which is also endowed with a H^+ -ATPase, as shown in *Paramecium* [192–194]. In the absence of any overt Ca^{2+} -pump, Ca^{2+} is probably sequestered by exploiting the chemiosmotic ΔH^+ using an exchanger system. During systole this organelle releases, in a cyclic exocytotic process, substantial amounts of Ca^{2+} to the outside medium [195]. This may be a major mechanism of extruding an excess of Ca^{2+} after exocytosis stimulation [132]. Remarkably, according to $^{45}\text{Ca}^{2+}$ flux measurements [181,196] and fluorochrome analyses [106], Ca^{2+} rapidly leaks into these cells. Such diffuse influx, even when provoked by pipetting high concentrations of Ca^{2+} onto cells, does not trigger any recognizable response. However, its downregulation will require permanent, efficient extrusion, notably by the CVC, which therefore does not act as a source during stimulation.

As in many other cells, the ER represents a significant calcium store also in ciliates, as outlined in Section 4.1.

3.2.2. Apicomplexa

Contact with a host cell is assumed to be the trigger for rapid Ca^{2+} -dependent release of dense core vesicles and for penetration of the host cell by the parasites [119,197]. This recalls triggering of trichocyst exocytosis by contact with a predatory cell in *Paramecium* [77,78]. An essential difference is that, in Apicomplexa, material from the micronemes mediates intense contact [68,198] (Section 6.1), whereas trichocysts keep attacking predatory cells at a distance [78].

The Ca^{2+} requirement for host cell penetration was originally derived from increased host cell interaction during Ca^{2+} ionophore treatment [68]. It was shown that incubation of tachyzoites of *T. gondii* in a solution containing A23187 or ionomycin leads to protrusion of the conoid [115,199]. Depletion of extracellular Ca^{2+} reduces tachyzoite invasion [200]. Ca^{2+} channel blockers, such as verapamil (whose channel effect in these cells is not established), and calmodulin antagonists also inhibit cell invasion [201]. As mentioned in Section 2.2, depletion of the parasite's intracellular calcium with the ionophore, A23187, inhibits invasion [115]. Tachyzoites preloaded with the Ca^{2+} chelator BAPTA/AM were unable to penetrate host cells [107]. Thus, Ca^{2+} requirement appears established for micronemes, but less for rhoptries, and not at all for constitutive dense granule secretion [202]. The requirement of extracellular Ca^{2+} for erythrocyte invasion has been documented with *P. falciparum*, as depletion of Ca^{2+}_o inhibits infection [203] and other bivalent cations cannot substitute for Ca^{2+}_o [204].

There is evidence of a SOC-type mechanism also in Apicomplexa, e.g. in *P. falciparum* [205], but the precise origin of Ca^{2+} and its mode of mobilization is not so clear, particularly since the IMC as the equivalent of alveolar sacs is more or less unlikely to be a

Ca²⁺ store (Sections 3.1, 4.1 and 4.2). With *T. gondii*, a Ca²⁺ signal merely from internal sources, even without enforcement by a SOC-type mechanism, suffices for microneme activation [119]. A further complication is the heterogeneous sensitivity of microneme secretion [206]. Clearly, precisely timed [Ca²⁺]_i signaling studies relative to the host cell penetration process would be of high value, but these are much more difficult to perform than with non-parasitic relatives.

To identify the origin of the Ca²⁺ signal, drugs which inhibit Ca²⁺ sequestration into specific stores may be useful; for Ca²⁺ mobilization, different activators have been probed with the two apicomplexan genera. The ER, but hardly acidocalcisomes have been envisaged in pilot studies by Billker et al. [12]. They found PLC-effects to activate IP₃Rs, or the effects of cADPR to activate RyR-like Ca²⁺-release channels in the context of secretion; in parallel experiments were done with thapsigargin. Microneme release is induced by cADPR in *T. gondii* [207] and in *P. falciparum* [208]. Ca²⁺ stores have been characterized in *P. falciparum* as sensitive to cyclopiazonic acid at usual concentrations, but not to thapsigargin [11], unless applied at concentrations much higher than efficient in mammalian cells [109,168]. Considering the difficulties in discriminating effects on ER and acidocalcisomes, there remains some additional uncertainty. Consider that, in apicomplexan cells, InsP₃ is assumed to act not only on the ER, but also on acidocalcisomes (reviewed in [176,177]). Moreover, both organelles are functionally linked. Ca²⁺ released from the ER is cleared from the cytosol by uptake into acidocalcisomes [11] which, thus, may exert a function, beyond signal formation, as a sink.

Little is known about NAADP⁺ effects and no useful TPC sequences can be found in the apicomplexan DBs. Although acidocalcisomes are abundant, particularly in *Plasmodium* and *Toxoplasma* [111,179,180,209] the release channels have not been identified unambiguously as yet.

Such attempts do not necessarily identify these organelles as the only stores, since the *Plasmodium* food vacuole has also been considered a thapsigargin and cyclopiazonic acid sensitive store [210]. However, when fluorochrome trapping was eliminated, the Ca²⁺ concentration in this organelle was rather low, thus disqualifying it as a major source [211]. Judged on this basis, the ER and the acidocalcisomes may be the major Ca²⁺ stores in Apicomplexa. However, the latter contain Ca²⁺ mainly as polyphosphates and, thus, may not be easily mobilized [212]; accordingly a mechanism for rapid hydrolysis in the organelle lumen is discussed, as outline in the legend to Fig. 4.

Future work may profit from more subtle approaches. For instance, in yeast, homotypic vacuole fusion has been shown to depend on CaM and on Ca²⁺ released from the organelle [213]. This has been established by inhibition with the very fast Ca²⁺ chelator BAPTA (1,2-bis-[o-aminophenoxy]ethane tetraacetate). BAPTA binds Ca²⁺ with a $\tau = 0.5 \mu\text{s}$, i.e. within a very short distance from its release site, as previously summarized [10]. This recalls binding of CaM to food vacuoles in ciliates, as reported repeatedly, for *Paramecium* [214,215] and *Tetrahymena* [216]. Using different chelators, including membrane permeable derivatives as well as CaM inhibitors, one may scrutinize local effects also in Apicomplexa and other Alveolata more reliably.

4. Calcium release channels in Alveolates

4.1. IP₃R- and RyR-type Ca²⁺ release channels

In unicellular organisms, including *Dictyostelium* and apicomplexan parasites, as well as in flowering plants, physiological effects by the application of the activators, InsP₃ and caffeine, have been repeatedly reported. However, although Ca²⁺ channels in general are considered among the oldest channels emerging in evolution

[217], paradoxically CRCs have not yet been identified on a molecular scale to any sufficient degree, neither in multicellular plants [218] nor in any protozoan. Recent critical reviews, in fact, advocate for a more stringent characterization of the respective receptors (IP₃R, RyR) on a molecular level. Current reviews agree that this is not available for any protozoan species [139,140] (except IP₃Rs *Paramecium* [132] to which we could recently add RyRs [133]) and some related CRC forms [134].

The characteristic domain structure of IP₃Rs and RyRs [219–222] should make them amenable to identification also in lower eukaryotes. Beyond this, unequivocal identification has to include, on the one hand InsP₃ binding assays and in situ analysis of the effects of InsP₃ uncaging, and on the other hand the analysis of effects of CRC-activating messengers and drugs. Both receptor types are different in their molecular pharmacology [220,223,224], although considerable deviations occur at different levels of evolution [30]. Also manyfold modulatory effects exerted by interacting molecules and receptor modifications ([220,225]; for the RyR, see [24]; for the IP₃R, see [23,226]) would have to be taken into account in future analyses with protozoa. Considering the different pharmacology of some RyR mutations and concentration dependency of ryanodine effects, analysis by the alternative standard drugs, caffeine [227] and 4CmC [228], appears advised. With RyRs, the most likely physiological activator, cADPR [229], whose effect has been definitely established only quite recently [24], should also be probed. So far, the most essential criteria have been fulfilled only for some aspects, and only with the CRCs of *Paramecium*, *PtCRC*.

The compartment-specific delivery of all CRCs, as described for *Paramecium* below, is assumed to start in the cytosol, with incorporation into the ER membrane and subsequent “shipping” in vesicles to the respective membranes, based on poorly understood cues [225].

4.1.1. Ciliates

The *Paramecium* DB contains many scaffolds showing sequence similarities with all the types of Ca²⁺-release channels mentioned. According to the above criteria, IP₃R- and RyR-type sequences have been analyzed in more detail [132–134]; see Fig. 5. Mainly the presence of some specific domains has been evaluated. This includes: (i) a near-aminoterminal InsP₃-binding domain (eventually with InsP₃-binding signatures, occurrence of InsP₃ binding and physiological InsP₃ effects). (ii) Regions homologous to established RyRs. (iii) RIH (ryanodine-InsP₃-homology) domains homologous to both types of receptor channels. (iv) Organization of transmembrane domains including a pore domain with a selectivity filter. (v) Spotting a binding signature for 4CmC in the case of RyRs [230], whereas activation sites for caffeine are less well defined [224]. (vii) Domains appropriate for tetramerization [17,220]. Note that for IP₃Rs and RyRs binding sites for activators other than InsP₃ and 4CmC are less useful because of considerable variability [224].

Typically the IP₃R contains in its InsP₃-binding domain 10 residues for InsP₃ binding of which three are essential [231]. Also typically, the selectivity filter has the signature sequence GGGIGD in the RyR and GGGVGD in the IP₃R [232,233]; variations in these change ion conductance. In *P. tetraurelia*, we have combined these molecular analyses (Fig. 5) with AB localization and gene silencing studies in conjunction with functional tests. Due to their great number only individual *PtCRC* isoforms have been scrutinized paradigmatically.

We found in *Paramecium* six subfamilies of Ca²⁺-release channels (*PtCRC*) with more or less pronounced similarities to IP₃Rs and RyRs, numerated I–VI [132–134]. To be considered for further analysis as putative Ca²⁺ release channels only those sequences were taken into account where the carboxy terminal part contains a channel domain corresponding to the criteria observed in mammalian cells. This includes six and four transmembrane

Fig. 5. Molecular characterization of gene sequences encoding *PtCRCs*, type IP_3R and RyR and related types. Only subfamily characteristics are depicted. Dark line: More (thick) or less (thin) conserved parts outside specific domains; red boxes: IP_3 -binding domain (dotted where variable within one subfamily). Turquoise boxes: RIH (RyR/IP_3R) homology domain (dotted when variable). Blue box: Domain with transmembrane regions, including the pore (yellow bar). Note large similarity of subfamilies I and II as well as of IV and VI; most subfamilies deviate in their overall similarity. For more details, see text. Based on data from Ladenburger and Plattner [134]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

domains in the IP_3Rs and $RyRs$, respectively, together with a putative pore domain [220,224]. (Note that for $RyRs$ higher numbers of transmembrane domains are also discussed in the literature [24] – a matter to be settled even for mammals). Most of the subfamilies of *PtCRCs* have several ohnologs [132–134] representing paralogs from whole genome duplications, including a quite recent one [234]. From a total of 34 *PtCRCs*, mostly only one or two characteristic subfamily members have been analyzed in more detail. Actually subfamily members may serve gene amplification, rather than individual differentiation for distinct effects. This presumption is in line with results obtained with other systems ([235], though in this work *Paramecium* evolution is plotted with much too short a time scale; see Section 2.1) and several studies with *Paramecium* [64,65,236]. The domain structure of the different paralogs/ohnologs is presented in Fig. 5, their subcellular distribution in Fig. 6, based on Ladenburger and Plattner [134]. Note that they predominantly possess a rather conserved carboxyterminal channel domain, while the other domains may be more or less variable.

Values of luminal $[Ca^{2+}]$ to be expected in organelles along the endo-lyso-phagosomal pathway are discussed in Section 4.2.

PtCRC-I-type channels are characterized by a putative $InsP_3$ -binding domain and by sequences homologous to $RyRs$ and an RIH domain. They are localized to the ER and, thus, correspond to the general distribution of IP_3R and RyR channels in higher eukaryotes where such types are also found in the ER [1–4]. So far, only *PtCRC* subfamilies II and IV have been characterized in more detail according to the above criteria. *PtCRC-II* is a genuine IP_3R , which is unexpectedly localized to the CVC [132]. When the $InsP_3$ -binding domain is overexpressed in *Paramecium* cells and probed with 3H - $InsP_3$, it clearly binds $InsP_3$, in competition with cold $InsP_3$. The three aminoacids considered most crucial for $InsP_3$ binding [231] are conserved. $InsP_3$ uncaging generates a distinct Ca^{2+} signal. Its localization and the general effects of gene silencing suggest a role in general $[Ca^{2+}]$ homeostasis regulation. Under steady-state conditions, the CVC serves to balance the considerable Ca^{2+} influx by excretion of Ca^{2+} (Section 7.1). *PtCRC-II* channels may serve fine-tuning by allowing partial reflux into the cytosol.

Fig. 6. Intracellular distribution of Ca^{2+} -release channels in *Paramecium* (*PtCRCs*). Only selected representatives of the different groups are depicted. Abbreviations: a = ampullae (part of the CVC), as = acidosomes (acidic recycling vesicles, different from acidocalcisomes), ci = cilia, cp = cytoproct (site of release of spent phagosome contents), cv = contractile vacuole (CVC), ds = decorated spongione (CVC part with H^+ -ATPase), dv = discoidal vesicles (special recycling vesicles), ee = early endosomes, er = endoplasmic reticulum, fv = food vacuole (phagosome), ga = Golgi apparatus, gh = "ghosts" (from released trichocysts), oc = oral cavity, pm = plasma membrane (with closely attached alveolar sacs and nearby vesicles at lower left), ps = parasomal sacs (clathrin-coated pits), rv = recycling vesicles, ss = smooth spongione (CVC), tr = trichocysts, trpc = trichocyst precursor. For further details, see text. According to Ladenburger and Plattner [134].

Concomitantly, fluorochrome analyses in *Paramecium* under resting conditions reveal spontaneous $[Ca^{2+}]$ fluctuations along the extensions of the CVC [132]. Since this occurs without any overt stimulus, the phenomenon observed corresponds to what has more recently been described as constitutively active IP_3R activity [237] in a chicken lymphocyte cell line where it is linked to basic bioenergetics. Compatible with this notion would be the observation that, in *Tetrahymena*, $PlnsP_2$ displays considerable turnover without any stimulation [238]. Besides an RIH domain, these channels surprisingly also contain two or three regions homologous to RyRs, but they strictly fulfill the criteria for IP_3Rs .

In type *PtCRC*-III molecules no RIH domain could be identified, in contrast to types *PtCRC*-I and *PtCRC*-II. The $InsP_3$ -binding domain is present in *PtCRC*-III-1, -2 and -4, but missing in subtype -3; the occurrence of RyR-related sequences is restricted. *PtCRC*-III-4 channels are contained in shuttle vesicles [134] that are different from discoidal vesicles and acidosomes [73]. These vesicles serve the transport of membrane materials from different stages of food vacuoles (phago[lyso]somes) to the site of nascent food vacuole [phagosome] formation; the channels evidently recycle from a secondary stage of the food vacuole development and from the expulsion site of old food vacuoles, the cytoproct (Fig. 6). Accordingly only some vacuoles are labeled by ABs against *PtCRC*-III-4. The following aspects support the feasibility of the occurrence of *PtCRC*-III-4 on some of the phagosomes. Synaptotagmin and, thus, Ca^{2+} are required for phagosome formation in human neutrophils [239] and macrophages [240]. Synaptotagmin is also required for lysosome/phagosome fusion [241]. Near-range Ca^{2+} signals will be required, as established for homotypic vacuole fusion in yeast [213], although, in *Paramecium*, the Ca^{2+} -sensor will be different from synaptotagmin (Section 5.1).

Type *PtCRC*-IV-1 molecules are of the RyR-type; they are localized to the peripheral side of alveolar sacs [133]. Their sequence displays no RIH or $InsP_3$ -binding domain, although some restricted regions resemble RyRs. Even though they are insensitive to ryanodine, when it is applied to cells or to the isolated organelles [126] (which were also shown not to bind labeled ryanodine in vitro [unpubl. res.]), *PtCRC*-IV-1 molecules are considered RyRs for the following reasons. Stimulation with caffeine and 4CmC generates a Ca^{2+} signal in parallel to massive trichocyst exocytosis; both phenomena are almost abolished when this channel is knocked-down. Previously these RyR agonists had been shown to induce a SOC-type mechanism in response to caffeine and to 4CmC [76,185], both established RyR activators. Note that 4CmC is known to activate RyRs with a point mutation also in mammals [228]. Accordingly, in the C-terminal part a binding site for 4CmC [230] has been found also in this *Paramecium* channel, *PtCRC*-IV, which enables Ca^{2+} release from alveolar sacs [133].

One of the channels characterized by patch-clamp analysis after reconstitution of isolated *Paramecium* cell cortex fragments (containing alveolar sacs) into lipid layers [242] showed properties compatible with the channels involved in AED stimulated Ca^{2+} release (and exocytosis), as analyzed by whole cell-patch analysis [87]. These channels were cation-nonspecific, but with highest permeability for Ca^{2+} , and they did not respond to ligands then known [242]. In retrospect, they appear as the equivalent of *PtCRC*-IV-1, i.e. RyRs whose poor Me^{2+} selectivity is notorious [243]. The fact that RyRs and IP_3Rs have high conductance and sufficiently long open times is prerequisite for efficient signaling. The reason is requirement of ion counter-currents to clamp the store membrane potential above the equilibrium/Nernst potential [244]. Low ion selectivity does not play a role for the selective release of Ca^{2+} as the organelle specific SERCA makes Ca^{2+} by far the predominant ion in the stores.

PtCRC types V and VI (of which subfamily members *PtCRC*-V-2 and *PtCRC*-VI-3 have been analyzed) are most heterogeneous and

most widely distributed among intracellular compartments. (This may suggest selective localization of individual ohnologs, but the ABs used did not allow us to ascertain this possibility). Type V channels vary with regard to the presence/absence of an $InsP_3$ -binding domain and a RIH domain, and both these domains are absent from Type VI channels. *PtCRC*-VI-4 is truncated. The wide intracellular distribution of different CRCs, also in higher eukaryotes, reflects requirement of local Ca^{2+} signaling and presence of a Ca^{2+} -sensor in many membranes participating in membrane flow in mammalian systems [136]. Evidently this is also the case in ciliates.

The localization of *PtCRC*-V-4 encompasses the parasomal sacs (clathrin-coated pits near ciliary basal bodies) and alveolar sacs. Here, these channels are confined to contact sites between adjacent sacs, as seen by immuno-gold EM. Since both alveolar sacs and coated pits also line the region around the cytostome, this explains labeling of this cell region, as has also been scrutinized by immuno-EM analysis. This channel subpopulation also occurs in vesicles near the clefts between alveolar sacs and near ciliary basal bodies. Interestingly the latter sites do not coincide with early endosomes ("terminal cisternae") which in turn, contain *PtCRC*-VI-3. Just like *PtCRC*-II-1, *PtCRC*-V-4 is present in the contractile vacuole and the emanating radial canals of the CVC. Its porous region contains *PtCRC*-VI-2 and *PtCRC*-VI-3. (The porous is a preformed site of exocytosis for the contractile vacuole and is also surrounded by alveolar sacs; the precise localization of these CRCs with regard to the vacuole extrusion site has not yet been identified). ABs against *PtCRC*-V-4 also stains the cleavage furrow, a site to where vesicles are delivered for cytokinesis, also in ciliates (*Tetrahymena* [245,246]). Heterogeneity of the ER is indicated by the enrichment of *PtCRC*-VI-2 type channels selectively in cortical structures, vesicles and/or ER, whereas we localized *PtCRC*-I-1 in the ER throughout the cell. Functional heterogeneity is known from mammalian cells [247–249] and has been observed in *Paramecium* during silencing of the SNARE-specific chaperone, NSF [65]. Thus, the distribution pattern of *PtCRCs* [134] is not really surprising.

How does this intriguing situation compare to higher eukaryotes? Here, within one cell, different parts of a single storage compartment, e.g. the ER, may contain different types of CRCs; this has also been found in sensory neurons [250] and in smooth muscle cells [251]. Another possibility is the generation of different Ca^{2+} responses by two isoforms of IP_3Rs , e.g. in HeLa cells [252]. As far as cleavage furrow formation is concerned, this is paralleled by Ca^{2+} waves, as shown, for instance, with cleavage-stage *Xenopus* embryos [253]. Thus, similar findings with *Paramecium* are not without precedent.

The occurrence of *CRC*-V-4, endowed with a putative $InsP_3$ -binding domain, in parasomal sacs is a challenge. In some mammalian cells, the presence of only about two $InsP_3$ receptor molecules in the cell membrane has been reported [254] and similarly only a small number of ryanodine receptors could be found in the plasma membrane of some cells [255]. The immuno-gold EM-labeling density we achieved (attached to the cytosolic side) on individual parasomal sacs is much higher than previously reported for any such channel in the cell membrane of any cell type. Is this the explanation of Ca^{2+} leakage conductances which has hardly been analyzed so far in *Paramecium* [71]? It recalls non-selective, high conductance, membrane potential-independent cation channels in hippocampal neurons; these can be activated by a stepwise increase of extracellular $[Ca^{2+}]$ [105]. Under similar conditions, fluorochrome analyses in *Paramecium* indicate an abrupt increase of $[Ca^{2+}]_i$ [106]. Remarkably this induces no exocytosis – again indicating requirement of a focused $[Ca^{2+}]_i$ increase (Section 1). Clearly it would now be worth while analyzing *CRC*-V-4 in the *Paramecium* cell membrane by electrophysiology.

CRC-V-4 harbors still another secret: it localizes selectively to the micronucleus, but not to the envelope of the macronucleus

Fig. 7. Effect of injected cADPR on CVC contraction activity in *Paramecium* cells. In the experiments shown on the left, cells respond to cADPR concentrations from ~ 100 nM on, but higher concentrations cause increased reaction. Note that, for unknown reasons, the response is more pronounced in the anterior CVC, irrespective of the site of cADPR injection. Block diagram (right): statistical evaluation shows progressive decrease of the reaction, already within the four first activity cycles ($\pm$ SEM). Injection volume: $\sim 10\%$ of cell volume, in all cases. Note that values before injection are identical in both CVCs, i.e., ~ 8 s. The effect on the CVC may be indirect (see text), but does not occur with mock-injections. Unpublished results.

[134]. Considering the multiple effects of Ca^{2+} signaling in the mammalian nucleus [256], one may now envisage a variety of functions, beyond the most discussed modulatory effect on transcription of genes encoding proteins since this does not take place in the micronucleus.

In summary, the plethora of differentially positioned *PtCRCs* (Fig. 6) strongly suggest a role of these Ca^{2+} channels in the regulation of basic Ca^{2+} housekeeping including Ca^{2+} homeostasis (*PtCRC-I-1*, *PtCRC-II-1*), of trichocyst biogenesis (*PtCRC-II-1*), exocytosis (*PtCRC-IV-1*) and trafficking of widely different vesicles (*PtCRC-III-4*, *PtCRC-V-4* and *PtCRC-VI-2/3*) etc. Considering delivery of membrane components from different compartments to subsequent ones, one might expect occurrence of the very same channels in vesicle and target membranes. Since this is not always the case in our localization studies one may assume either organelle specific retention, or local enrichment within certain membranes, that remain undetected after dilution in a larger membrane area. This could conceal certain channels, so it would not be easily visible in all fusion partners.

Another challenge to be solved is the occurrence of two different types of CRCs in one organelle, e.g. of *PtCRC-II-1* and *PtCRC-V-4* in the CVC, and of *PtCRC-IV-1* and *PtCRC-V-4* in alveolar sacs. Possibly very local functional needs have to be matched, even within one organelle, by different types of CRCs. Such a situation is known, for example, from the ER of mammalian cells [249].

Several restrictions to our *PtCRC* analysis should be stressed at this point. Mostly only one or two isoforms have been localized per subfamily (except group VI) and similarly functional analyses also have been restricted so far. Analyses of regulatory domains, such as the Ca^{2+} - and CaM-dependent gatekeeper function in the IP_3R [220] and of putative activator compounds would be advised. It should also be mentioned that not all of the *PtCRCs* display a typical pore signature and a selectivity filter mentioned above. Possibly this may vary in evolution. Work with mammalian cells suggests no pronounced selectivity of RyRs as well as of IP_3Rs and their isoforms for different cations [220,224]. In summary, at this time some of the molecules described above as *PtCRCs* may be considered only *bona fide* as Ca^{2+} -release channels, whereas this appears rather certain for the *PtCRC-II* and *PtCRC-IV* types which have been analyzed in more detail [132,133].

Taking into account that in animal cells RyRs are activated by cADPR [24] one may ask whether *PtRyRs*, e.g. those contained in alveolar sacs (*CRC-IV-1*), could be activated by cADPR? Up to now we have carried out only pilot experiments which suggest that some unspecified *PtRyRs* are activated. As Fig. 7 shows, cADPR microinjection causes a delay of the CVC contraction frequency, particularly of the anterior vacuole. (Note that this also occurs in response to NAADP $^{+}$; Figs. 8 and 9, below). This effect, which occurred independently of the injection site, can be considered an indirect response to Ca^{2+} release at remote sites inside the cell. Taking into account the dominant role of the CVC in $[\text{Ca}^{2+}]_i$ regulation [132] this response somehow reflects a physiological response to cADPR. The concentration values required for full activation are well comparable to those required for maximal Ca^{2+} release from cardiac microsomes [257]. More detailed analyses are required in order to find out which Ca^{2+} stores are activated by cADPR in ciliates.

Fig. 8. ^{32}P NAADP binding in homogenate of whole *P. tetraurelia* cells under ionic conditions occurring in living *Paramecium* cells [279]; c.f. Bak et al. [257] for functional relevance. 200 pM ^{32}P NAADP was incubated with 0.1 mg homogenate for 20 min before filtration. The K_d was found to be 3.31 ± 0.05 nM and the B_{max} , 335.4 ± 9.9 fmol/mg; ($n=6$). Unpublished results.

Fig. 9. Examples of the effect of microinjected NAADP⁺ on the contraction activity of the CVC in *Paramecium* cells. Injection volume: ~10% of cell volume. Note the different reaction in the anterior and posterior CVC, respectively. Evidently NAADP⁺ can yield more or less pronounced reactions when applied at a threshold concentration of 100 nM concentration which eventually can reach already the same level as higher concentrations. Note that, for unknown reasons, the response is more pronounced in the anterior CVC, irrespective of the site of cADPR injection. Values before injection are identical in both CVCs, i.e., ~8 s. Consider the possibility of an indirect effect of NAADP⁺ on CVC activity (see text). Unpublished results.

One may discuss which type of CRC is the oldest. From genomic analysis of the flagellate, *Monosiga brevicollis*, the impression emerges that this group may be rather close to ciliates as they are to metazoans also with respect to Ca²⁺ signaling. Remarkably *Monosiga* is reported to lack a RyR [16]. In contrast, we have found the occurrence of CRCs with features of either type, IP₃R and RyR, in *Paramecium* [134]. All this points toward a common origin. The mechanisms appear primeval, but they have been largely obliterated in the apicomplexan parasites (below).

4.1.2. Apicomplexa

Although IP₃- and RyR-related effects have been frequently described for apicomplexan parasites (Section 3.2) their molecular identity and even their occurrence remains enigmatic.

Addition of InsP₃ in physiological concentrations to permeabilized *Plasmodium chabaudi* cells yields a Ca²⁺ signal [258]. Inhibitors of PLC, as applied with higher eukaryotes, inhibit stimulated [Ca²⁺]_i increase in *P. falciparum* [205]. Functional analyses of *Toxoplasma* with InsP₃ and with different drugs suggested the occurrence of a mixed type of CRC [118]. In the databases available for *P. falciparum* and *T. gondii*, sequences with weak similarity to those in *Paramecium* have been found, but they lack transmembrane domains required for channel function and, therefore, do not fulfill any of the criteria outlined above. They do not allow us to recognize any characteristic domain structure. Moreover, only some sequences may indicate transmembrane regions of sufficient length and some of these are located at the aminoterminal side (unpublished data). Considering long lasting and ever increasing evidence of a physiological function of InsP₃, recently demonstrated by InsP₃ uncaging and Ca²⁺/fluorochrome imaging in *P. falciparum* [259], one would

definitely expect a genuine receptor for InsP₃. The same holds for the effects observed with the RyR activators, caffeine and cADPR [207,208]. Specifically *Toxoplasma* cells produce the abscission hormone, abscisic acid, which activates, via cADPR, a Ca²⁺ signaling pathway required for host cell egression [112,122]. However, neither an IP₃R nor any RyR homolog could be detected in any of the Apicomplexa even in extensive search ([139,140,212]; own unpublished observations).

The general question arises as to what extent sequences lacking specific domains would qualify for IP₃R and RyR function. Without any doubt, an IP₃R has to qualify for this function by binding IP₃. In reality, up to now, only one other protozoan, *Dictyostelium*, has been assumed to possess an IP₃R-like protein (iplA) that has been identified by partial sequence homology and a number of transmembrane domains. However, no InsP₃-binding domain has been characterized and also the putative selectivity filter region shows an aberrant amino acid composition [260]. Deviation of iplA from *Paramecium* and animal CRCs is surprising also considering the close relationship of *Dictyostelium* to the animal kingdom [261]. As outlined above, even considerably less is known from Apicomplexa. This statement can be extended to candidates for RyRs and IP₃Rs in all protozoa [262] as well as to multicellular plants [263,264] – a surprising uncertainty considering the frequent reports on effects of physiological activators and of drugs in both these systems.

4.2. Two-pore Ca²⁺ release channels and acidic stores

Among other channels, the two-pore channels (TPCs) are widely distributed from mosses to flowering plants and from *Cenorhabditis elegans* to man [26,172,265]. They owe their name

to the tandem molecular structure consisting of 2×6 membrane-spanning regions in one molecule [26]. TPCs may be considered ancestral types of CRCs because of their structural similarity to voltage-gated Ca^{2+} - and Na^{+} -channels [25,26,265] which in turn possess four such domains, also each with six membrane-spanning regions. (Recall that RyRs are mostly assumed to have four membrane-spanning regions, in contrast to IP₃R which possess six such regions [266]; see Section 4.1). Amazingly information about their possible occurrence in protozoa is practically absent. There is general agreement, however, that TPCs are absent from acidocalcisomes of the protozoa analyzed, including Apicomplexa [26,174]; we could also not find such sequences in the respective databases (unpublished data).

TPCs are activated by nicotinic acid adeninedinucleotide phosphate (NAADP⁺), a metabolite formed by ADP-ribosyl cyclase activity from NADP [26,172,229]. This cyclase is widely distributed and it can also produce cADPR from NAD [229]. The target of NAADP⁺ is clearly different from that of cADPR [267]. Concomitantly, recent analysis of mammalian cells transfected with either RyRs or TPCs clearly revealed selectivity of these Ca^{2+} pool-activating second messengers [268]. Overall levels of NAADP⁺ required to trigger Ca^{2+} release via TPCs in mammalian cells are only 1.5–8-fold above basal levels [26], but local concentrations will be higher. Just as with Ca^{2+} and InsP_3 , overall changes should not be considered a diagnostic of activation processes.

In all non-protozoan species analyzed TPCs are associated with acidic compartments, from early endosomes to lysosomes [22,26], possibly including some unidentified vesicles [26]. TPCs have a selective, though not strict, conductivity for Ca^{2+} . The Ca^{2+} released by TPCs can subsequently activate RyRs and IP₃R [26,229]. In mammalian cells, stimuli somehow resulting in TPC activation can be widely different and concomitantly this can contribute to signal transduction in a widely different context [26,269]. For ciliates and for Apicomplexan parasites [21], such analyses are not available.

Which specific functions may be envisaged? In “higher” animal cells, Ca^{2+} is required for vesicle trafficking and fusion, also along the endo-lysosomal pathway [270]. Here, a transient Ca^{2+} release is assumed to regulate membrane fusion/fission events in metazoans [137]. TPCs are known to contribute to these processes [26]. However, luminal $[\text{Ca}^{2+}]$ (or $[\text{Ca}]$) is generally reported to decrease already at the early endosome stage [271]. If one assumes a requirement of $[\text{Ca}^{2+}]$ between 0.3 and $\leq 1 \mu\text{M}$ for progressing in the endosomal pathway [272], would this Ca^{2+} have to come from other sources? This is not very likely the case, as in mammalian cells TPC1 channels are found in late endosomes, TPC2 in lysosomes, and – interestingly – TPC3 in the recycling endosomal compartment; only in early endosomes the presence of TPCs is less clear [273]. Also to be noted are reports on relatively high $[\text{Ca}^{2+}]$, 37 μM , in endosomal compartments of some mammalian cells, such as pancreatic acinar cells [274]. In *Arabidopsis thaliana*, TPCs are localized to the big vacuole membrane [275]. Lysosomes in animal cells, e.g. in macrophages, contain substantial concentrations of Ca^{2+} (0.6 mM, [276]) which theoretically could be made available for organelle interaction via TPCs whose occurrence in lysosomes of mammalian cells has been documented by patch-clamp analysis [269,277]. Whatever the relevant CRCs are, a measurable $[\text{Ca}^{2+}]$ increase is long known to be required also for phago-lysosome fusion in macrophages [278]. What is the situation in alveolates?

4.2.1. Ciliates

In unpublished work with *P. tetraurelia* we find evidence for the presence of TPCs according to the following criteria. (i) The *Paramecium* DB contains sequences corresponding to TPCs; this is supported by partial cloning. (ii) We have used *Paramecium* whole cell homogenates to determine the K_d for NAADP⁺ under physiological intracellular ionic conditions (which is important for such

measurements [257]), as specified by Lumpert et al. [279]. We found a K_d of $3.31 \pm 0.05 \text{ nM}$ (Fig. 8), i.e. somewhat above that in sea urchin eggs (c.f. 200 pM [280]) and almost identical to TPC type 2 in mouse liver [265]. This binding site was selective for NAADP⁺ over NAADPH and NADP(H). Also to be considered is that mammalian TPC2 possesses high and low affinity NAADP⁺ binding sites [269]. (iii) We microinjected NAADP⁺ into *Paramecium* cells. We observed that it alters the activity of the CVC (Fig. 9) which is known to be involved in the regulation of $[\text{Ca}^{2+}]$ homeostasis [132,195]. Although *Paramecium* cells recognize NAADP⁺ the effect achieved may be due to Ca^{2+} released from stores other than the CVC. The concentration values we had to use for full activation compare well with those required for maximal Ca^{2+} release from cardiac microsomes [257].

There are numerous acidic compartments in a *Paramecium* cell [191]. Nevertheless, their endowment with TPCs remains to be established. Some of the acidic compartments contain CRCs of the RyR-/IP₃R-type (Section 4.1). They could possibly coincide with TPCs within one organelle, accounting for different signaling pathways. (Recall that we find two types of IP₃R alone in the CVC and two RyR-types in alveolar sacs.) So far, only for *Dictyostelium* is a cursory note on TPCs available [273]. Considering the change in luminal pH within the phagosomes in *Paramecium* during cyclosis [191] and the capability of TPCs to sense the luminal pH [269,277] the endowment of vesicles with TPCs, if occurring, and their function may also change during intracellular trafficking. Since luminal pH does change in *Paramecium* food vacuoles during cyclosis [73], one may expect different CRCs in different functional stages and possibly different types even in one stage.

Clearly more detailed studies are mandatory to establish in ciliates the occurrence of true TPCs, the type of Ca^{2+} stores which contain TPCs and which stores are activated by NAADP⁺.

4.2.2. Apicomplexa

Their acidocalcisomes are endowed with different means of sequestering Ca^{2+} and protons (Fig. 4). A ΔH^{+} supports sequestration of Ca^{2+} , as outlined in Section 3.1. Sequences resembling TPC1 of the *A. thaliana* vacuole reportedly occur in the genome of *T. gondii*, but not of *Plasmodium* or *Cryptosporidium* [212]. This may indicate progressive loss during evolution to parasitic life. Due to binding of Ca^{2+} as pyro- and polyphosphate [281] one could argue that Ca^{2+} mobilization could not take place sufficiently within adequate time, e.g. for serving host cell penetration (a rapidly hydrolyzing pyro-/polyphosphatase is required). However, in *Toxoplasma*, depletion of acidocalcisomes of their Ca^{2+} storage capacity by TgA1 knock-out disturbs $[\text{Ca}^{2+}]$ homeostasis and micronemal secretion, and finally infectivity [20]. Nevertheless it would be useful to demonstrate that this is a direct, rather than a systemic effect of acidocalcisomes.

In *P. falciparum*, the food vacuole has previously been considered a significant Ca^{2+} store on the basis of fluorochrome analyses [210]. However, this view has been corrected, as Ca^{2+} fluorochrome signals collected from this organelle could be attributed to pH effects [211].

Altogether, in protozoa some aspects of vesicle trafficking and dynamic distribution of CRCs are not well understood. The interaction of acidocalcisomes with contractile vacuoles in *Dictyostelium* [282] and in trypanosomatids [180] may serve transfer of important membrane components. Interaction of acidocalcisomes with “lysosome-related organelles” is also reported [180], as it has been suggested for the plant-like vacuole in *T. gondii* [178]. In that case, vesicles with IP₃R/RyR-type Ca^{2+} -release channels and TPCs (if they exist) could merge. All this might imply multiple interactions of organelles somehow involved in $[\text{Ca}^{2+}]$ regulation.

The basic questions with apicomplexan parasites remain: which types of CRCs do they have, how much do they deviate from

sequences known from other species, in which organelles are they located and how are they activated? These questions are of paramount importance and, hence, deserve detailed investigation.

5. Ca^{2+} -binding and Ca^{2+} -activated proteins

Ciliates contain a number of Ca^{2+} -activated and of Ca^{2+} -binding proteins (CaBPs), particularly in their cilia and in the cell cortex. Centrin and calmodulin (CaM) are among the most prominent CaBPs containing EF-hand Ca^{2+} -binding loops [283–285]. As known from higher eukaryotes, each molecule has four such loops made of 12 aminoacids. In CaM these are suitable for high affinity/low capacity Ca^{2+} -binding with hierarchical occupancy (loop I → IV). Recent high time resolution studies confirmed the aminoterminal loop as by far most efficient [286]. With centrin the situation is less clear as it also displays low affinity/high capacity Ca^{2+} -binding capability [287]. (Related proteins in the SR lumen [288] are not known from Alveolates.) Therefore, CaM may more likely act as a Ca^{2+} -sensor for dynamic processes [286,289], whereas centrin can also act as a sink for Ca^{2+} due to low affinity/high capacity binding, as demonstrated with *Paramecium* [290]. CaM also activates many proteins, for instance, the mammalian [291] and ciliate [170] PMCA-type Ca^{2+} -pump. The efficiency of Ca^{2+} pumping activity in downregulation of $[\text{Ca}^{2+}]$ after stimulation may lag far behind inactivation of Ca^{2+} by binding to CaBPs [286], as we found with *Paramecium* [290]. In summary, CaBPs exert widely different, important functions, from activation to deactivation.

5.1. Ciliates

CaM has been characterized in *Paramecium* and *Tetrahymena*, but unexpectedly, *P. tetraurelia* contains 14 genes encoding CaM (R. Kissmehl, unpubl. res.). Light microscopic localization of CaM has also been studied in *Tetrahymena* [292] and in *Paramecium* [92,293]. In EM immuno-localization studies CaM is posted at many strategic sites: in cilia, in the cell membrane-alveolar sacs interface, at trichocyst docking sites, at the CVC [214], as well as along different regions of microtubule guided vesicle transport [215].

Paramecium cells defective in CaM are unable to assemble functional exocytosis sites [294]. In fact, CaM can interact with specific SNARE proteins or SNARE-associated proteins, as has been ascertained for mammalian cells [295,296]. Thus, CaM, though frequently judged inappropriate to serve as a Ca^{2+} -sensor for rapid signal transmission and membrane fusion during exocytosis, may at least indirectly serve this process by favoring the assembly of SNAREs. In neurons, the Q-SNARE, syntaxin 1A, together with Qb/c SNARE, SNAP-25, associates with Ca^{2+} influx channels and thus can favor exocytosis [297]. Formation of a Ca^{2+} /CaM/Munc13 (auxiliary SNARE-interacting protein) sensor complex has also been reported, including neurons [298]. A more direct effect facilitating membrane fusion by transferring the lipid-binding domain of a v-SNARE from the cis- to the trans-side of juxtaposed membranes has also been shown [299]. The actual role of CaM precisely at exocytosis sites may differ from one cell type to another. In fact, in *Paramecium*, established CaM antagonists, such as W7 and trifluoperazine, do not inhibit AED-induced trichocyst exocytosis [87]. Therefore, Ca^{2+} -sensors with C2 domains (see below), rather than EF-hand type CaBPs, remain favorites for future investigations along these lines.

As known from neurotransmitter release, CaM can serve as a Ca^{2+} -sensor for exocytosis-coupled endocytosis [300]. Whether this is also true of endocytotic retrieval of trichocyst membranes is not known (although this step is also accelerated by increasing the Ca^{2+} gradient between the cell interior and the outside medium [183]). However, in ciliates involvement of CaM appears

feasible for constitutive endocytosis via the parasomal sacs. In higher eukaryotes this is known to require dephosphorylation of dynamin by the Ca^{2+} /CaM activated protein phosphatase 2B (PP2B, CaN=calcineurin) [301]. In fact, CaN is heavily enriched at such preformed sites of endocytosis in *Paramecium* [302]. In summary, different endocytotic processes in ciliates are Ca^{2+} -dependent, just as in higher eukaryotes up to mammals [303–305].

A number of CaM-binding proteins (CaM-BPs) occur in *Paramecium* cells; among them are Ca^{2+} -dependent and Ca^{2+} -inhibitable CaM-binding proteins [306]. In whole cilia, $^{45}\text{Ca}^{2+}$ overlays revealed CaBP bands of 126, 96/95, 63 and 36 kDa; axonemes displayed bands of 126, 96–105 and 63 kDa, in ciliary membrane fractions bands are 120, 70 and 63 kDa in size, whereas the 36 kDa band was shared by axoneme and ciliary membranes. Cell bodies contained bands of about 120, 85 and 16–44 kDa [306]. In isolated *Paramecium* cell cortices, a ~120 kDa large band [125] as well as bands of 126, 63 and 58 kDa have been recognized [10]. (Their molecular identity has not been established.) Other candidates are the PMCA (by its CaM-binding site [171]), the CaN-A subunit of ~55 kDa [307] and possibly the nd7 gene product with a sequence-derived size of 59 kDa [308]. The manyfold cation conducting channels in the somatic and ciliary membrane of *Paramecium* also bind CaM. Here, CaM acts as a Ca^{2+} -sensor [93], probably mediated by a multigene family of Ca^{2+} -dependent CaM-BPs, between 60 and 70 kDa in size and associated with the cell membrane [309]. A thorough analysis by systematic genetic screening is a task for future work.

In ciliates, phagosomes are also endowed with CaBPs. CaM-BPs [216,310] and CaM [214,215] bind already from an early stage on. Similar observations with higher eukaryotes suggest a role in the interaction with the numerous vesicle fusions and fissions during trafficking. Vacuole fusion in *Saccharomyces cerevisiae* requires CaM [213], in the absence of the Ca^{2+} -sensors of the C2-type [311], as discussed below. Similarly, in *Tetrahymena*, Ca^{2+} /CaM regulates cleavage furrow formation by vesicle delivery [312].

In mammalian cells, membrane fusion occurring during exocytosis and also at some intracellular sites is executed generally by interaction of SNAREs with the Ca^{2+} -sensor protein, type synaptotagmin [313]. The synaptotagmin molecule is endowed with two C2 domains. Binding constants for Ca^{2+} differ widely [314], depending on the isoform locally expressed, which meets local availability of $[\text{Ca}^{2+}]$ for membrane-to-membrane interactions. This can be in the (sub-)micromolar (gland cell exocytosis [315]) to sub-millimolar range (transmitter release in nerve terminals [5]). Upon Ca^{2+} -binding, its C2 domain most close to the aminoterminal membrane anchor [316] performs a lipophilic interaction with the lipid layers to be fused [317]. It thus qualifies for a Ca^{2+} -sensor function and induction of membrane fusion by perturbation of the lipids. (For more details concerning ciliates, see [65] and below.) Constitutive exocytosis is considered independent of Ca^{2+} [135,318], or rather of a $[\text{Ca}^{2+}]_i$ increase, as are some intracellular steps of membrane trafficking [136].

These proteins are different from two other families of CaBPs, the annexins and the copines, the latter endowed with C2 domains [319]. They bind to the phospholipids of biomembranes in a Ca^{2+} -dependent manner. While annexins are discussed as being relevant for various aspects of membrane dynamics, copines may be involved in cell division and growth. Using ABs against common sequences, putative annexins have been localized in *Paramecium* to exocytosis sites, including trichocyst docking sites and the cytoproct [320]. Copines were actually first detected in *Paramecium* [321] before they were found, just like annexins, on a much broader scale [319].

The $[\text{Ca}^{2+}]_i$ generated locally at trichocyst exocytosis sites is ~5 μM [75] and, thus, rather similar to mammalian cells. In the *Paramecium* DB, gene sequences homologous to synaptotagmin occur, but they possess eight C2 domains (R. Kissmehl, pers.

comm.). Similar proteins with up to six C2 domains are the exception in higher eukaryotic systems [322]. At least six proteins with C2 domains can participate in neurotransmission [323]. To account for the different Ca^{2+} sensitivities of different compartments a variety of Ca^{2+} -sensors would have to be expected [324,325]. All these aspects await further elucidation in ciliates. However, the situation is intriguing even in some mammalian cells; for instance, in cochlear inner hair cells, exocytosis can occur also in the absence of synaptotagmin and/or of otoferlin, a multi-C2 domain CaBP with Ca^{2+} -sensor properties [326].

Among the Ca^{2+} -activated proteins in ciliates we find some that are directly and others that are indirectly activated by Ca^{2+} , e.g. via a Ca^{2+} /CaM complex. Among the ~2600 protein kinases found in the *Paramecium* genome, the largest fraction is Ca^{2+} /CaM regulated [327]. This includes kinases with a CaM-like domain integrated to the kinase molecule, CDPKs [328], as described from *Paramecium* [329]. No "CaM kinases" proper, i.e. kinases binding CaM, as occurring in higher eukaryotes, are known from ciliates. Inhibitory effects achieved with "anti-CaM" drugs (Section 2.2) may be due to inhibition of CaM or of CDPKs, as found in plants [330,331], but this has not always been scrutinized in protozoa. In *Paramecium*, CDPKs phosphorylate a set of ciliary CaBPs as well as centrin of the infraciliary lattice [332] and they closely resemble CDPKs in Apicomplexa [329]. In addition, in *Paramecium*, a casein kinase type 1, CK1, was found which is unconventionally inhibited by Ca^{2+} [333].

Calcineurin (CaN) is a multifunctional Ser/Thr phosphatase, type 2B (PP2B), consisting of a ~60 kDa catalytic subunit (SU-A) and a ~20 kDa regulatory SU-B [334]. The catalytic SU requires binding of CaM and binding of the Ca^{2+} -activated SU-B for its activation. This is the situation known for metazoan cells [335]. Evidence for CaN in *Paramecium* was based on detailed enzymatic analysis [336], on effects of microinjected monospecific ABs [337] and on immunoperoxidase [338] and quantitative immuno-EM gold localization studies [302]. This work was accompanied by a long lasting dispute about its existence in *Paramecium* (negation by [13,339]), until partial sequences were found [15]. Finally, the genes of this dimeric protein could be cloned and the two SUs, PtCaN-A and -B, characterized as very closely related to their mammalian counterparts [307].

In *Paramecium*, its localization to sites of notorious PP2B activity, as known from mammalian cells, is quite evident [302]. Its role during exocytosis performance, though also observed in mammalian systems, is not settled as yet [307]. Remarkably, in mammals, CaN has been shown to regulate the phosphorylation-dependent activity of the SNARE, SNAP-25, in chromaffin cells [340] and of the Ca^{2+} -sensor, synaptotagmin VI, in spermatozoa [341]. As in mammals, apart from its other manifold activities currently discussed [334,342], CaN may also play a pivotal role during Ca^{2+} -activated gene transcription in protozoa. Interestingly, massive exocytosis stimulation enhances the transcription and translation of a large number of genes in *Tetrahymena* [89] and *Paramecium* (J. Cohen and L. Sperling, data presented in the *Paramecium* DB), thus recalling activation of transcription factors in mammals [343]. Some of the upregulated proteins are themselves engaged in Ca^{2+} -mediated effects.

Ca^{2+} is also involved in the secretion of trichocysts in *Paramecium* in a quite different way. Ca^{2+}_o is required for decondensation (severalfold stretching to needles longer than the cell diameter) [344]. Such a step is also essential for mucocyst release in *Tetrahymena* [345] where it also depends on Ca^{2+}_o [346]. A mutant affecting the Ca^{2+} -binding capacity of some trichocyst matrix proteins (derived from a pretrichynin precursor) are unable to perform exocytosis, although exocytotic membrane fusion takes place [347]. This can be mimicked with wildtype cells by stimulation at low $[\text{Ca}^{2+}]_o$ [348]. The same was shown with mast cells, using in parallel amperometry (to monitor contents release) and patch-clamp

electrophysiology to monitor membrane fusion and fission [349]. In *Paramecium*, the complex exo-endocytosis process as a whole is accelerated by increasing $[\text{Ca}^{2+}]_o$ due to increase influx [183]. Also duration of these processes is within the range known from mammalian cells [350,351].

In ciliates, centrin (= PCBP-25), like CaM, is endowed with four EF-hand Ca^{2+} -binding domains for varying intermolecular interactions and functions [352] and with an excess of negatively charged aminoacids, e.g. in *Paramecium* [332,353]. It is localized to the cell cortex where it forms the main component of the "infraciliary lattice" [354]. This is pivotal for downregulating high cortical $[\text{Ca}^{2+}]_i$ after massive exocytosis stimulation [290], as documented in Fig. 10. Centrin may be the most important immobile Ca^{2+} -buffer since knock-out cells display an over 10-fold delay in $[\text{Ca}^{2+}]_i$ down-regulation after trichocyst exocytosis stimulation. Centrin can be phosphorylated by the Ca^{2+} -dependent PK-1 [332]. The colocalization of CaN together with centrin in the infraciliary [302] on the one hand and its assembly in dependency of the phosphorylation state on the other hand [332,355] could indirectly regulate in ciliates the role of centrin in surface pattern formation, together with the different molecular interactions of the subdomains [352]. In fact, centrin is essential to achieve surface patterning in *Paramecium* [353], as it is for regulating duplication and maintenance of ciliary basal bodies in *Paramecium* [356] and *Tetrahymena* [352,357]. All this indicates long-time and far reaching constitutive effects of Ca^{2+} on epigenetically controlled surface patterning in these cells.

The enzyme required to release InsP_3 from PIP_2 , PLC, was identified only very recently in *Paramecium* [358] and *Tetrahymena* [238,359]. If InsP_3 is formed from PlnsP_2 by the activity of PLC, this implies simultaneous formation of diacylglycerol (DAG), the activator of protein kinase C (PKC). There is only spurious evidence for the occurrence of PKC from sequence interference studies [360]. (A number of studies transferring inhibitors from mammalian to protist systems tacitly presumed equal efficacy and, thus, remains controversial for reasons discussed previously [30].) What other possible functions could DAG then execute? There is a paradigmatic situation in neuronal systems. Here, DAG binds the protein Munc13, a modulator of SNARE function [361]. Knowledge of SNARE-associated proteins in ciliates is very restricted, but a homolog of Munc18/Sec1, with six genes, can be found in the *Paramecium* DB (R. Kissmehl, unpubl. data). Although all this is hypothetical, an analogous effect of DAG on local lipid composition and function would appear feasible, particularly considering its modulatory effect on protein-based membrane interactions in higher eukaryotic systems [362].

In ciliates, knowledge about the occurrence of the high capacity/low affinity CaBPs, type calsequestrin (CaS in SR) and calreticulin (CaR in ER), which also exerts a chaperone function for protein folding [363], is fragmentary. Both have been identified *bona fide* by monospecific ABs (against the respective mammalian antigens) in homogenates from *Paramecium*. Thus, a CaM-like and a CaS-like protein have been localized at the EM level to the ER and to the alveolar sacs, respectively [152]. Molecular data are still missing (see also Section 8). Alone from the high $[\text{Ca}]$ found by EDX in alveolar sacs of *Paramecium* and the rapid release upon stimulation [127] one has to postulate a high capacity/low affinity CaBP comparable to CaS in this organelle. For comparison, the ER chaperone, PDI (protein disulfide isomerase), has been cloned in *Paramecium* and clearly localized to the ER [133], just like the CR-like protein [152] and the ER tags DiOC₆ [134] and DiOC₁₈ [151].

5.2. Apicomplexa

To anticipate, the situation is principally rather similar to that in ciliates. Apicomplexan cells also contain CaM [111], CaN composed of a catalytic and a regulatory SU, CaN-A and CaN-B [364], and

Fig. 10. Relevance of centrins, contained in the infraciliary lattice, and of H^+ -ATPase activity (inhibited by concanamycin B) for downregulation of $[Ca^{2+}]_i$ in cortical and central regions of the *Paramecium* cell following exocytosis stimulation by AED. Strains used: d4-2, wildtype cells; A1, cells deficient in infraciliary lattice. The top and middle diagram are reproduced from Sehring et al. [290], the bottom diagram shows unpublished results.

centrin (*T. gondii* [365]; *P. falciparum* [366]). *T. gondii* possesses a Ca^{2+} -ATPase, described as TgA1, complemented by a paralog called Tg44.mo2812 [140]. It is localized to the cell surface and to the acidocalcisomal membrane and contributes to Ca^{2+} homeostasis [162]. TgA1 is 130 kDa large, but no CaM-binding has been reported [20] – in contrast to PMCA (Sections 3.1 and 7.1).

Altogether, the *Plasmodium* genome contains over 30 proteins with EF-hand modules [38]. Only one CaM gene may exist in *P. falciparum*, with 92% protein sequence identity to *T. gondii* and *H. sapiens* [367]. In *P. falciparum*, CaM has been localized to the cell apex by immuno-fluorescence [201] and immuno-gold EM analysis [368], just as in *Paramecium*. Anti-CaM drugs inhibit host cell invasion by *T. gondii* tachyzoites [201] and differentiation of stages of *P. falciparum* in vitro [369]. Reliability of such drugs at different levels of evolution can be attributed to high sequence conservation in CaM [30]. The situation clearly reminds that in *Paramecium*, not only regarding localization [214,338] but also the requirement for the assembly of exocytosis sites [294] and drug effects on exocytosis in *Tetrahymena* [370].

In a phylogenetic analysis, CaN-A of *T. gondii*, *Plasmodium yoelii*, and *Cryptosporidium parvum* form clusters close to, but separate from the ciliates, *P. tetraurelia* and *T. thermophila* [307]. CaN and CaN-interacting proteins have been considered important for medical use of cyclosporin A (CsA) against *Plasmodium* infection [364,371,372].

In *T. gondii*, centrins are enriched in patches near the cell surface within the conoid [365] in a situation which may qualify it as a sink for Ca^{2+} . This is comparable to the cortical centrins in the *Paramecium* cell cortex ([290]; see above).

The *P. falciparum* genome contains only ~100 sequences encoding protein kinases (PKs) or PK-related proteins [373,374] which is far below the number in ciliates [327]. Thus, diversification is very much smaller in the parasites than in their free-living relatives. As in ciliates, genuine CaM kinases have not been found in Apicomplexa [374]. *P. falciparum*, *T. gondii* and other Apicomplexa are also endowed with Ca^{2+} -dependent protein kinases, type CDPKs [12,328,375], which otherwise are considered typical of plants [330,331] and ciliates (*Paramecium* [329]). These include a CDPK4 in *Plasmodium vivax*, which displays a well conserved N-terminal kinase domain and a C-terminal CaM-like structure with 4 EF-hand motifs for Ca^{2+} -binding. This protein is expressed in the mature schizont, thus suggesting a role in the process of proliferation [376]. CDPK4 also seems to be essential for the process of exflagellation of the male gametocytes, a key event during the life cycle of malaria parasites in the insect [377]. A CDPK3 has also been identified [378]. CDPK1 in *T. gondii* is essential for its exocytotic activity. Its suppression blocks exocytotic release of micronemal contents, thus interfering with tachyzoite motility, host cell invasion and egression from the host cell at the end of the intracellular cycle [379]. It is inhibited by nanomolar concentrations of the kinase inhibitors called "bumped" – compounds which are inactive against mammalian kinases. Since these kinase inhibitors inhibit an early stage of *T. gondii* infection they open the possibility to use these compounds against toxoplasmosis [380].

In *P. falciparum*, a Ca^{2+} /CaM-activated protein kinase B-like enzyme, PfPKB, may be important for host cell penetration, by affecting the actomyosin motor of the glideosome attached to the inner membrane complex [12] (Section 6.1.2), in conjunction with upstream CaM and PLC activation [381]. Vaid and Sharma [382] were the first to identify non-GPI-specific PLC in any Alveolata member, i.e. *P. falciparum*. No protein kinase C (PKC), but putative kinase-like sequences (PKLs) with some similarity, has been detected in the *P. falciparum* genome; rather untypically, however, these lack a Ca^{2+} -binding and a phospholipid-binding domain [373]. In summary, the situation is quite similar to that in ciliates, with the exception of $InsP_3$ signaling which has been established in *Paramecium* (above), but not in any of the Apicomplexa.

Cell cycle regulation in *P. falciparum* is also reported to include several activities related to Ca^{2+} signaling: a PLC activity, an atypical Ca^{2+} /CaM-regulated protein kinase B (PKB; see above), a CDPK, and a PKG involved in Ca^{2+} mobilization; an IP_3R in the ER and

plasmalemmal Ca^{2+} influx channels are also assumed to be involved in cell cycle regulation [108], although IP_3 Rs have not been identified in any of the Apicomplexa as yet (Section 4.1). Some of these proteins are also discussed in the context of “glidosome” function for host cell penetration ([12]; Section 6.1).

The analysis of the *P. falciparum* genome does not allow one to identify the ER-specific luminal Ca^{2+} -dependent and glycosylation-sensitive chaperones, calnexin and CaR, as known from mammalian cells [363,383]. This was endorsed by chaperone network analysis [366]. The latter paper differs in its opinion about the occurrence of ER-resident glycosylation from a paper by Hirtzlin et al. [371] which was based on an analysis of cyclophilin/peptidylprolyl *cis-trans* isomerase. If the Ca^{2+} -dependent chaperones would prove to be absent, other proteins with highly negative charge may bind Ca^{2+} sequestered in the ER lumen which is considered a rapidly releasable pool in these cells (Section 3.1). For instance, *Pf*ERC/reticulocalbin, a CaBP of lower Ca^{2+} affinity than CaR family members, is assumed to be an ER-resident component due to its ER retention signal [384]. Also in *P. falciparum*, such a signal is absent from a CaBP, type hsp90; although it otherwise resembles much more CaS, e.g. by a 68 aminoacid stretch with an excess of acidic residues, it is assumed to be cytosolic [385]. In summary, ER-resident Ca^{2+} -BPs in Apicomplexa are insufficiently known and require more detailed investigation just as in ciliates.

6. Calcium for motility and bioenergetics

6.1. Ca^{2+} for cell motility and cytokinesis

These phenomena encompass rather heterogenous mechanisms, including ciliary beat regulation and parasite movement. Among them are gliding and penetration into, and liberation from host cells. Cytokinesis is also regulated by Ca^{2+} . In addition, in ciliates, local increase of cortical $[\text{Ca}^{2+}]$ also entails local cell contraction due to contraction of the centrin-based infraciliary lattice ([386]; Section 5).

6.1.1. Ciliary activity regulation

Ca^{2+} is required for basic activity of flagella [387] and cilia [388]. This also holds for ciliates [71,72]. A cyclic switch based on CaM-associated and CaM-dissociated states of target molecules may operate by cyclic changes in the concentration of intraciliary $[\text{Ca}^{2+}]$ [387]. In ciliates, accelerated forward swimming can be induced by hyperpolarization. This is accompanied by an increase in cAMP formation [389], though with some delay over several beats [390]. A drop of $[\text{Ca}^{2+}]_i$ in cilia by 3-fold may be closer to the latter basic regulation mechanism [391]. Some aspects of the underlying mechanism, though not fully established as yet, differ from those assumed for mammalian cilia [392].

In ciliates, a stimulated increase of $[\text{Ca}^{2+}]$ in cilia induces a change in ciliary beat direction (ciliary reversal [389]), as shown by injection of EGTA-buffered Ca^{2+} solutions into *Paramecium* cells [393] and also with Triton-extracted *Paramecium* models [394]. In both cases ciliary reversal was complete at $[\text{Ca}^{2+}] = 10^{-6}$ M. In vivo, this reaction takes place as an avoidance reaction when a cell hits an obstacle (see scheme, below). Then, mechanoreceptors with Ca^{2+} -channel characteristics, concentrated in the anterior somatic membrane, are activated [82]. Database searches suggest Piezo as a candidate for mechanosensitive Ca^{2+} channels, also in *Paramecium* [395]. The receptor potential is mediated by depolarization due to Ca^{2+} influx [396] and activates voltage-dependent Ca^{2+} -channels that are localized exclusively in the cilia [83]. Their activation is regulated by ciliary centrin [397]. Depolarization is counterbalanced by K^+ efflux [396].

In ciliates, the intraciliary $[\text{Ca}^{2+}]$ increase thus produced activates a Ca^{2+} /CaM-stimulated guanylate cyclase, so that cyclic guanosine monophosphate (cGMP) levels increase [13,14,398]. This Ca^{2+} -activated step is followed by activation of protein kinase G (PKG) activity. PKG mediated phosphorylation of some ciliary proteins [399] is reversed by a protein phosphatase type 1, PP1 [339]. Since the cGMP increase goes on over considerably more time than the rise of the Ca^{2+} signal [390] the coupling of the two processes does not appear very clear. In summary, the situation seems as follows: whereas Ca^{2+} appears as the first second messenger, cGMP may exert a modulatory effect over longer times [400].

In *Paramecium*, ciliary reversal takes place according to the following flow chart:

Anterior mechanical stimulus (hitting an obstacle) on the somatic (non-ciliary) cell membrane
 → Depolarization by activation of anterior mechanosensitive Ca^{2+} channels, repolarization by K^+ -efflux
 → Effect of depolarization on cilia
 Activation of voltage-dependent Ca^{2+} influx channels in ciliary membrane
 → Guanylate cyclase activation
 → cGMP formation → PKG activation
 → Phosphorylation of target proteins in ciliary axoneme in parallel: activation of axonemal CaM → effects?
 → Ciliary reversal
 Inactivation by
 Closing of ciliary ΔV -channels by a Ca^{2+} /CaM complex
 And binding of excessive Ca^{2+} to immobile buffer (CaM, centrin etc.)
 No Ca^{2+} spillover into cell soma

The immediate reversal response may be induced by the swift rise of intraciliary $[\text{Ca}^{2+}]$ in interaction with CaM. This is associated with the axoneme in *Paramecium* [214,215] and *Tetrahymena* [401]. How may CaM be integrated in the machinery of ciliary activity? The hierarchical occupancy of the four Ca^{2+} -binding loops of CaM is well established; considering increased liability for Mg^{2+} binding toward the C-terminal loop IV, Machemer and Teunis [402] hypothesized a Ca^{2+} /CaM switch for ciliary activity, i.e. for back- and forward swimming. As recently found, differential occupancy of the four binding loops by Ca^{2+} in fact suffices to significantly change the effects on target molecules [403]. Biochemical experiments have shown the occurrence in cilia of further candidates for the interaction with Ca^{2+} , including CaBPs and Ca^{2+} -activated proteins [404]; Section 5. One of them is a heteropolymeric radial spoke protein, as found in *Chlamydomonas*; it associates with CaM in a Ca^{2+} -dependent manner and disposes of a phosphate transfer capacity as well [405]. Despite all these details, the precise mechanism of Ca^{2+} -based regulation of ciliary activity in ciliated protozoa is not yet fully understood.

Subsequent to activation, further Ca^{2+} influx is abolished by a feedback exerted by the very same Ca^{2+} that has activated the ciliary reversal process [95]. This involves a Ca^{2+} /CaM complex [92], just as found much later in different neuronal channels [96]. In general terms, CaM is an established Ca^{2+} -sensor also in ciliates [92,93], in conjunction with Ca^{2+} -dependent CaM-BPs associated with the cell membrane [309].

The number of Ca^{2+} ions per cilium required for ciliary reversal activation may be of the estimated order of only $\sim 10^3$ [84]. These data are compatible with values estimated from the concentration required for activating ciliary reversal in permeabilized models [394], from Ca^{2+} /EGTA injected cells [393], and from electrophysiology. For further discussion, including electrophysiology, see Plattner and Klauke [10].

As shown by EDX, Ca^{2+} -mediated activation of ciliary reversal in *Paramecium* entails no Ca^{2+} spillover into somatic regions underneath [84], unless cells are overstimulated [406]. This makes a cilium a functionally defined compartment with regard to Ca^{2+} signaling. By contrast, exocytosis stimulation can entail Ca^{2+} spillover from the soma, where the signal is generated by a SOC mechanism

(Section 3.2), deep into cilia [84]. This has been recognized not only in EDX analyses but also by fluorochrome analysis. By this mechanism *P. tetraurelia* pawn mutants (which are defective in ciliary Ca^{2+} influx channels [88]) are also enabled to perform ciliary reversal [84].

Could centrin play a role in Ca^{2+} signaling in cilia? Using immuno-gold labeling centrin is found not only in ciliary basal bodies but also in the basis of cilia of human nasal cells [407]. No such data are available for ciliates and proteome analysis of isolated ciliary fractions gave no evidence along these lines [408]. If more scrutinized analysis would detect centrin in the ciliary basis of ciliates, it could contribute to the phenomenon often cited in electrophysiological recordings, that is a hypothetical “pump” which should be basally located and which would normally impede Ca^{2+} spillover from cilia into the soma. Alternatively the equivalent of this “pump” could be centrin associated with basal bodies (Section 5). In contrast, the PMCA-type pump is very likely too slow to produce such effects.

In *Paramecium*, exogenously added guanosine triphosphate (GTP) can trigger alternating forward and backward swimming in ~8 s periods without the characteristics of ciliary reversal [409]; see scheme, below. Electrophysiological recordings have documented oscillating depolarizations which do not occur in response to other nucleotides. A role for Ca^{2+} has been verified with a microinjected fluorochrome which showed a strong first peak, followed by smaller ones [410]. When $[\text{Ca}^{2+}]_0$ was quenched to near resting $[\text{Ca}^{2+}]_i$ levels, the first peak was reduced to the size of the subsequent ones, but otherwise signaling and behavior was as with normal $[\text{Ca}^{2+}]_0$. The fact that signaling was altered by application of thapsigargin was considered as evidence of Ca^{2+} release from alveolar sacs [411]. Considering that this store is definitely insensitive to thapsigargin ([150,185]; see also Section 7.2) and that depletion of this store from Ca^{2+} by other, more specific means had no effect on the GTP response [410], one may well have to consider one of the other manyfold intracellular stores [134].

Cyclic backward/forward swimming induced in *Paramecium* by exogenous GTP follows the following scheme:

Exogenous GTP (15 μM)

- The first $[\text{Ca}^{2+}]_i$ peak, larger than subsequent ones, requires Ca^{2+}_0 .
 - Further (smaller) cyclic activity peaks in ~8 s intervals supported by Ca^{2+} from internal stores (type of store for internal Ca^{2+} mobilization during GTP activation is unknown)
 - Periodic activation and downregulation, followed by desensitization (mechanism unknown), decaying Ca^{2+} signal etc.
-

6.1.2. Gliding, host cell penetration and parasite liberation

Ca^{2+} plays a fundamental role in the process of motility of tachyzoites, merozoites, sporozoites and ookinetes of members of the Apicomplexa phylum. This special type of cell motility is initiated by the secretion of rhoptry and microneme proteins. In this motility process the parasite membrane is captured unilaterally, the parasite itself is pushed forward. Initially a “moving junction” complex is formed by rhoptry exocytosis, RON protein being integrated into the host cell membrane and then serving as a receptor for micronemal protein AMA1, all arranged in a ring [412]. Gliding then depends on the binding of thrombospondin-like proteins (TRAP) to the protozoan surface where it interacts on the one hand with a host cell-receptor and on the other hand, via aldolase, with an actin-myosin complex. All this is located in the parasites sub-plasmalemmal space in close association with the IMC. Examples of TRAPs include TgMIC2 in *T. gondii*, circumsporozoite protein (CSP) of *Plasmodium*, CTPR of *Plasmodium* ookinetes and the merozoite TRAP homolog in *Plasmodium* (reviewed in [67]). Some of these steps depend on Ca^{2+} in an intriguing manner.

A $[\text{Ca}^{2+}]_i$ increase in the parasites is considered crucial for host cell penetration [68,69,111]. How precisely does Ca^{2+} act and from

which pools is it mobilized in the parasites? In the *T. gondii* cell cortex, CaM interacts with myosin light chains (MLC) and, thus, forms part of the invasion machinery. TgMLC1 is associated with the inner membrane complex, in contrast to TgMLC2 which is associated with the cell membrane [413]. Some of the “glidosome” components are probably a target for Ca^{2+} , as one may assume for MLC activation. However, recently the involvement of a Ca^{2+} signal in activating glidosome function has been disputed [208]. If so, the function of Ca^{2+} could be more associated with the effects of secretory activity accompanying host cell penetration ([68,119,414]; see also Section 3). As mentioned above, inhibition of TgCDPK1 inhibited microneme secretion and inhibited progress in the life cycle [379]. Ca^{2+} imaging during *T. gondii*-host cell interaction has shown the following. First, Ca^{2+} is required for the exocytotic processes in the context of establishing of parasite-host contact (prerequisite to formation of the “moving junction”; above). Then, still during invasion, $[\text{Ca}^{2+}]_i$ goes down abruptly [119], possibly to avoid an overshooting reaction which otherwise could entail egression from the host cell.

With *Plasmodium*, Ca^{2+} is also involved in the formation of microgametes [415]. Release of microgametocytes of *P. falciparum* for transformation into mature microgametes (“exflagellation”) requires Ca^{2+} , as it is inhibited by the CaM inhibitor, W7 [415].

Interestingly *T. gondii* has recently been reported to contain voltage-gated Ca^{2+} -channels (VGCCs). Three genes encoding VGCCs have been detected in *T. gondii*, clustering with a group of three, out of a total of eight, VGCCs in *T. thermophila* [140]. They were found neither in *P. falciparum*, nor in *C. parvum*. For *T. gondii* the authors suggest a role for Ca^{2+} influx upon Ca^{2+} release from internal stores. Concomitantly, a SOC-type mechanism has also been described in *P. falciparum* [205]; see Section 3.2.2.

6.1.3. Cytokinesis

In vertebrates, cell division requires a $[\text{Ca}^{2+}]_i$ increase near the cleavage furrow on a time scale of minutes, as seen during early cell divisions in zebrafish and *Xenopus* development [253,416]. In both cases Ca^{2+} was from internal, InsP_3 -sensitive pools. InsP_3 formation is essential also for the progression of cytokinesis in *Drosophila* spermatocytes [417]. This coincides with the local enrichment of recycling endosomes characterized by Rab11 and FIP3 (family of Rab11-interacting proteins) [418,419]. In HeLa cells, such vesicles also contain the R-SNARE, VAMP8; some other vesicles associated with the cleavage furrow are devoid of the endosomal markers and are considered as constitutive secretory vesicles [420]. Which one of these vesicles contributes the Ca^{2+} required for cytokinesis progression is not known. Among them, recycling endosomes are known to contain channels for Ca^{2+} release (Section 4.2). What is known about alveolates?

In *Tetrahymena*, the situation may be comparable. Similar to HeLa cells, two types of vesicles containing Cda12p and Cda13p were seen to be engaged in cytokinesis [246]. They are functionally related to Rab11-interacting proteins and, thus, connected to recycling endosomes (Sections 3.1 and 4.2). Both vesicle types travel along microtubules to the cleavage furrow. Antisense studies revealed the relevance of Cda12p vesicles for endocytosis and cytokinesis, and of Cda13p vesicles for conjugant separation and for cytokinesis [246]. As in higher eukaryotes, a Ca^{2+} signal is likely needed for the progress of cytokinesis, but its origin remains to be determined. In *Paramecium* we found PtCRC-V-4, associated with the cleavage furrow ([134]; Fig. 6).

6.2. Ca^{2+} for bioenergetics

Upon stimulation, mitochondria of mammalian cells take up Ca^{2+} very rapidly, as has been established using Rhodamine-2 as an organelle specific fluorochrome. Rapid Ca^{2+} uptake into

Fig. 11. $[Ca^{2+}]$ increase in cortical mitochondria after exocytosis stimulation in *Paramecium* cells by AED, analyzed with Rhodamine-2. Unpublished results.

mitochondria depends on a locally generated $[Ca^{2+}]_i$ increase [421] and requires a uniporter. Release into the cytosol is mediated by a Na^+/Ca^{2+} exchanger, but other mechanisms are also discussed [422]. An increase in mitochondrial $[Ca^{2+}]$ activates mitochondrial dehydrogenases and consequently ATP production [423].

Ca^{2+} interferes with energetics also in *Paramecium*. A 63 kDa-phosphoprotein, pp63, is dephosphorylated during synchronous trichocyst exocytosis in parallel with ATP decay (≤ 5 s) and rephosphorylated during ATP recovery (≥ 20 s), as summarized previously [424]. This protein was identified in *Paramecium* as phosphoglucosyltransferase, PGM [425]; it was also found in *Toxoplasma* [426]. Based on its sensitivity to PP2B/CaN and to a Ca^{2+} -inhibitable protein kinase we derived a Ca^{2+} -sensitive de-/rephosphorylation cycle for pp63/PGM in connection with the mobilization of glucose for glycolysis [424,427] in spatially restricted cortical areas where pp63/PGM is enriched [428]. Since AED stimulation, by Ca^{2+} spillover, also induces ciliary reversal [84,182] some of the increased ATP production may be consumed in cilia. Considering the structural and functional similarity of alveolar sacs with muscular SR it is interesting to see that, in human skeletal muscle, a considerable proportion of glycogen mobilization, that also involves PGM activity, is significantly correlated with Ca^{2+} release from the SR [429].

During AED-induced synchronous trichocyst exocytosis, analyzed by quenched-flow/cryofixation [129,430], local total calcium, $[Ca]$, also increases in mitochondria. By EDX analysis, mitochondrial $[Ca]$ increase was detected within a subsecond time scale, followed by decay within seconds [127]. In contrast, when mitochondrial $[Ca^{2+}]$ was followed by the fluorochrome, Rhodamine-2, a rapid increase was registered, to ~ 7.5 -fold within a second time scale, but decay was much slower, lasting ≥ 5 min (Fig. 11). Thus, according to the comparison of $[Ca]$ and $[Ca^{2+}]$ in mitochondria, only a small fraction of Ca^{2+} is retained after stimulation. As mentioned, this may serve in the activation of dehydrogenases in the mitochondrial matrix and, thus, increase mitochondrial energy supply, as known from mammalian cells [423,431,432].

The frequently observed enrichment of mitochondria in *Paramecium*'s cell cortex could also exert a "vacuum cleaner" effect, enforcing the influx of Ca^{2+} , when mitochondria sequester much of it upon stimulation. Such an effect has been discussed for the immune synapse [433].

In a genomic survey of *Paramecium*, neither a mitochondrial Ca^{2+}/H^+ exchanger nor a Ca^{2+} -sensor regulating the mitochondrial Ca^{2+} uniporter was indicated, and no data were presented for such a uniporter [434], probably because its sequence had not yet been determined. It remains open whether these molecules differ too

much from known sequences or whether other molecules are in use.

7. Downregulation of calcium

7.1. Extrusion of Ca^{2+} from the cytosol and from the cell

As mentioned, *Paramecium* cells have a PMCA-type Ca^{2+} -pump in their cell membrane [171,435], probably not only in the somatic region, but also in cilia. Not only its molecular size (~ 130 kDa), but also occurrence of a CaM-binding site helps identifying this pump [171], as does inhibition by calmidazolium [170]. (Note: Partial sequences of another Ca^{2+} -pump which we identified as a SERCA-type [150] have been erroneously annotated as a PMCA in the *Paramecium* DB). According to our balance calculations, PMCA activity may be much too slow to account for the rapid decay of $[Ca^{2+}]_i$ observed, e.g. after exocytosis stimulation [10,132]. Also the SERCA localized to the inner area of alveolar sacs [145,151] sequesters Ca^{2+} with a $t_{1/2}$ of only ~ 60 min ([436]; Table 1).

Downregulation of $[Ca^{2+}]_i$ under steady-state conditions (passive diffusion) and after exocytosis stimulation in *Paramecium* can be summed up as follows (see also Fig. 2A):

$[Ca^{2+}]_i$ increase due to
Ca^{2+} leakage from outside medium or stimulus $\rightarrow [Ca^{2+}]_i$ increase
In parallel: Ca^{2+} flux into mitochondria
$[Ca^{2+}]_i$ downregulation by
$\rightarrow$ Binding to CaBPs (mainly centrin, but also CaM, CaN etc.)
$\rightarrow$ Gradual dissociation of Ca^{2+} from CaBPs and dissipation of Ca^{2+}
$\rightarrow$ Extrusion by PMCA (cell membrane), sequestration by SERCA (alveolar sacs, ER) or by other mechanisms into other stores; most important: Ca^{2+} extrusion via CVC (H^+ -ATPase $\rightarrow H^+/Ca^{2+}$ antiporter etc.)

As an alternative, rapid extrusion by a Na^+/Ca^{2+} exchanger in the cell membrane (as operating in cardiomyocytes between heart beats [437]) has been proposed, based on experiments with the ciliate, *Euplotes crassus* [438]. This was founded on the application of an exchanger inhibitor, although the specific efficacy of the drug effect has not been analyzed directly with a ciliate. In fact, one has to consider the low specificity of many drugs borrowed from mammalian cell biology for use in ciliates [30]. Restricted specificity is particularly a general problem with antiporter inhibitors [439]. Therefore, more direct evidence of any antiporter systems in the ciliate cell membrane, as well as anywhere else, appears mandatory.

By contrast, non-mitochondrial antiporters are well established functionally in Apicomplexa, particularly in their acidocalcisomes [21,179]; Fig. 4. Much less is known about antiporters in the cell membrane. Scrutinized analysis of apicomplexan databases suggests the occurrence of several Ca^{2+}/H^+ antiporter orthologs, in the absence of Na^+/Ca^{2+} exchangers [140]. In contrast, a Na^+/Ca^{2+} exchanger is known from choanoflagellates [16] – protists that are much closer to higher eukaryotes than any other unicellular organism.

Currently we judge, in ciliates under steady-state or stimulation conditions, the recovery of $[Ca^{2+}]_i$ as follows (Fig. 2). Cytosolic CaBPs, such as cortically enriched centrin, rapidly bind and, thus, inactivate Ca^{2+} in a first step, followed by desorption and dissipation. Pilot calculations evidenced that the CVC may be the crucial organelle guaranteeing recovery within a short time period [132]. The CVC possesses a H^+ -ATPase for sequestration of water and ions [192–194]. Here, extrusion of Ca^{2+} can be operated by the chemiosmotic ΔH^+ -based H^+/Ca^{2+} (or similar) exchange system in a second step. In fact, using microelectrodes Stock et al. [195] have measured extrusion of substantial concentrations of Ca^{2+} by the organelle, in the absence of a Ca^{2+} -pump (our data). Concomitantly, application of the established inhibitor of the *Paramecium* H^+ -ATPase, concanamycin B [440], before stimulation delays the recovery of $[Ca^{2+}]_i$ to resting levels after AED stimulation of exocytosis by

~10-fold (Fig. 10). Therefore, in *Paramecium*, a ΔH^+ -based secondary active Ca^{2+} transport in acidic compartments, notably the CVC, is a most realistic mechanism for rapid downregulation of $[Ca^{2+}]_i$.

As mentioned, *T. gondii* possesses a plasmalemmal-type Ca^{2+} -ATPase, described as TgA1, complemented by a paralog called Tg44.mo2812 [140]. It is localized not only to the cell surface, but also to the acidocalcisomal membrane and, thus, contributes to Ca^{2+} homeostasis [162].

7.2. Store (re)filling

In mammalian cells, re-uptake of Ca^{2+} by the ER, mediated by its SERCA pump, takes place on the scale of minutes, as summarized by Verkhratsky [441]. For mammalian cells, thapsigargin is the diagnostic of SERCA activity; its binding site has been specified by affinity labeling [442]. In protists, this pump may work much more slowly and several additional uptake mechanisms have been established for non-ER type stores in Apicomplexa. Also drug sensitivity can differ from mammalian cells.

7.2.1. Ciliates

Sequestration of $^{45}Ca^{2+}$ by alveolar sacs isolated from *Paramecium* cells in an ATP-regenerating system [125] was the first hint to the occurrence of an organellar Ca^{2+} -pump. Two genes encode closely related paralogs of a sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase, SERCA, in *Paramecium* [150,151]. The green fluorescent protein-(GFP)-tagged SERCA protein passes from the ER, by vesicle flow, selectively to the alveolar sacs [151]. The fact that hardly any SERCA-GFP remains visible in the ER can be explained by rapid throughput. The PtSERCA-type pump is not sensitive to the classical inhibitor, thapsigargin, and this drug does not cause a SOC in *Paramecium* [10,30]. This failure can be attributed to the lack of conservation of eight aminoacids in the putative binding site [150]. *Paramecium* is also little, if at all, sensitive to the other standard SERCA inhibitors, cyclopiazonic acid [10,30] and TBHQ when used in concentrations which are effective in mammalian cells [30]. The SERCA of *Tetrahymena* has also been cloned [443], but inhibitor studies are lacking. Ca^{2+} storage by the ER in ciliates must be important also for some signaling events because of the presence of PtCRC-I channels in the ER [134].

The CVC can also be considered a Ca^{2+} store because of the sequestration of Ca^{2+} into the CVC lumen [195]. As outlined above, in the absence of a Ca^{2+} -pump, a Ca^{2+}/H^+ or similar exchanger system may lumenally enrich Ca^{2+} . As mentioned in Section 4.1, there also occurs a partial reflux via constitutively active IP_3 Rs, thus producing stochastic Ca^{2+} sparks along the outlines of the organelle [132]. For all other components endowed with CRCs, the mechanism of Ca^{2+} sequestration has not been analyzed in ciliates.

7.2.2. Apicomplexa

The SERCA molecule is encoded by only one gene in *T. gondii* [167] and in *P. falciparum* [140]. The SERCA activity of Apicomplexa is reported to be inhibited by thapsigargin at concentrations applied to mammalian cells [138,258], although the thapsigargin binding site in *P. falciparum* also differs – though less than in *Paramecium* – from that in mammals. Other studies refute thapsigargin sensitivity in the apicomplexan parasites, but indicate a response to usual TBHQ concentrations in *Plasmodium* [258]. With cyclopiazonic acid, a 10 times higher concentration has to be applied to inhibit PfATP6 [444]. Such differences may also be a problem of accessibility and the actual concentration “seen” by the target [445]. Furthermore, there is some polymorphism of the SERCA molecule concerning sensitivity to inhibitors in natural *P. falciparum* populations, as well as in some laboratory lines [446]. See also Section 3.1.2. Artemisinin has been considered a SERCA inhibitor of therapeutic

interest for *Plasmodium* and *Toxoplasma* infection [138,445,447]. Apart from its usefulness, artemisinin was recently found not to bind to the *Plasmodium* SERCA molecule, PfATP6, and not to inhibit its activity. Concomitantly it may exert another effect on these parasites [444,448].

7.3. Ca^{2+} oscillations

Spontaneous $[Ca^{2+}]_i$ oscillations have been analyzed only with *P. tetraurelia*, though not systematically. Prajer et al. [449] were the first to recognize spontaneous $[Ca^{2+}]_i$ oscillations with a period of $t = \geq 2$ min ($[Ca^{2+}]_o = 1.5$ mM; fluorochrome: calcium Green-1). Oscillations with much shorter periods of ~8 s were seen by Plattner and Klauke [10] to emanate spontaneously from the oral region ($[Ca^{2+}]_o = 0.05$ mM; fluorochrome: Fluo-3). Ladenburger et al. [132] observed spontaneous oscillations with a period of 8–20 s ($[Ca^{2+}]_o = 1$ mM; dextran-coupled Fluo-4). These oscillations were significantly altered upon uncaging $InsP_3$ (though this does not indicate the source of Ca^{2+}). For unknown reasons, in other extensive experimental series, we did not recognize any spontaneous $[Ca^{2+}]_i$ oscillations.

Oscillations of a periodicity of ~8 s can be induced by exogenous application of GTP ($[410]$; $[Ca^{2+}]_o = 1$ mM; fluorochrome: Fura Red), as first recognized by electrophysiological recordings [409]. Exogenous BAPTA, calculated to reduce $[Ca^{2+}]_o$ to slightly below $[Ca^{2+}]_i$ at rest [75,185], reduced the first peak which otherwise was larger than the subsequent ones. Over some minutes, the Ca^{2+} response to GTP runs in a self-sustaining manner until it decays [410].

Since all fluorochromes used in these experiments have comparable K_d values of $\leq 1 \mu M$ and time constants of around 1 μs (summarized in [10]), data appear roughly comparable, even though obtained in a different context under variable experimental conditions ($[Ca^{2+}]_o$, fluorochrome). The $[Ca^{2+}]_i$ oscillations observed must involve internal stores. Since we could not normally observe such $[Ca^{2+}]_i$ oscillations, even under comparable conditions [75], the oscillations described may collectively suggest different physiological states of the cells, with unknown parameters, except GTP stimulation. Prajer et al. [449] discussed their data hypothetically in the context of surface structure modulation. Specifically for GTP induced oscillations we believe that they can serve near-range signaling between cells [410]. More detailed analyses will have to consider that the Ca^{2+} involved may come from different stores, with different kinds of mobilization as well as feedback mechanisms [450,451]. See also doi:10.4249/scholarpedia.1576 for mechanisms currently discussed for Ca^{2+} oscillations in mammalian cells.

8. Conclusions

In Table 2 we summarize some of the essential similarities and differences currently known about Ca^{2+} stores and Ca^{2+} signaling in ciliates and in some of the evolutionarily closely related apicomplexan parasites.

– *Cell membrane channels.* Over a long time period, ciliates, notably *Paramecium*, have served electrophysiologists as a model to analyze plasmalemmal ion fluxes. A plethora of channels has been characterized, some conducting Ca^{2+} , other with Ca^{2+} -regulated conductances. ΔV -dependent Ca^{2+} channels are clearly restricted to cilia which, thus, represent a specific Ca^{2+} sensitive compartment. ΔV -dependent Ca^{2+} channels are known from Apicomplexa [212], their precise role requiring further work. To our knowledge nothing is known about transient receptor potential (TRP) channels in alveolates, though sequences occur in the

Table 2Ca²⁺-stores and Ca²⁺-signaling in Ciliates (*Paramecium*, *Tetrahymena*) and Apicomplexa (*Plasmodium*, *Toxoplasma*).

	Ciliates	Reference (selection)	Apicomplexa ^a	Reference (selection) ^a
Basic [Ca ²⁺] _i	50–100 nM	[75]	50–100 nM	[107,108]
Cytosolic CaBPs	CaM	[92,292]	Ditto	[111]
	Centrin	[354]	Ditto	[365,366]
	CaN-B	[307]	Ditto	[364]
PMCA	Present	[170,435]	Ditto	[140,162]
			TgA1 (mainly classified as acidocalcisomal)	[20]
Ca ²⁺ -influx channels	Many types	[71,72,93]	No details available	–
Cortical Ca ²⁺ -stores	Alveolar sacs	[10,87,106,125–127,150]	Inner membrane complex? (Unknown Ca ²⁺ -storage) ^a	–
Type of Ca ²⁺ -pump	SERCA	[150,169]	SERCA, <i>T. gondii</i> <i>P. chabaudi</i> <i>P. falciparum</i>	[138,167] [258] [140]
CaBP	Calsequestrin-LP ^b	[152]	Not clear; see Section 5.2	–
Mobilization by	Caffeine	[76]	Unknown	–
	4-Cl-m-cresol	[185]	Unknown	–
Type of release channel	Ryanodine receptor	[133,134]	Unknown	–
Type of structural coupling	Unknown (no Orai/Stim)	–	For IMC/cell membrane Connection, see Section 6.1	–
Type of functional coupling	SOC/trichocyst release	[10,127,185]	–	–
SERCA-type Ca ²⁺ -ATPase				
Ca ²⁺ -ATPase activity	SERCA-type	[151]	Various species	See subsequent refs.
Drug sensitivity	TG insensitive	[30,150,169]	<i>P. chabaudi</i> : insensitive <i>P. falciparum</i> : TG insensitive <i>T. gondii</i> : TG sensitive tBHQ sensitive <i>P. falciparum</i> : more CPA sensitive	[258] [444] [12] [258,444] [444]
	tBHQ insensitivity	[169]		
	CPA insensitivity	[169]		
Localization	ER and alveolar sacs	[150,151]	ER	[138]
CaBP (chaperone)	CaR-LP ^b	[152]	Not detectable in PfDB	[374]
Dense core vesicles	Trichocysts		Micronemes, rhoptries	
Stimulation by	AED	[80,129]	Ethanol	[117]
	Predator contact	[77,78]	Host cell contact	[119,197]
Ca ²⁺ _o -requirement	Multiple steps	See Section 3.2	<i>P. falciparum</i> None: <i>T. gondii</i>	[203,205] [119]
Source	Release from alveolar Sacs + influx (SOC)	[127,185,186]	<i>P. falciparum</i> : SOC assumed Ca ²⁺ _i source uncertain	[205]
Plant vacuole-like comp.	Unknown	–	<i>T. gondii</i>	[178]
Ca ²⁺ -storage	Unknown	–	<i>T. gondii</i>	[178]
Other acidic compartments	Many	[191]	Acidocalcisomes	[453]
Ca ²⁺ uptake mechanism	Unknown	–	Ca ²⁺ -ATPase (TgA1), Ca ²⁺ -PPase, Ca ²⁺ /H ⁺ and other exchangers	[179]
Ca ²⁺ -release mechanism	NAADP ⁺ (not localized)	Binding studies (Fig. 9)	Not known	
Crystal vacuoles	Struvite (Ca,Mg phosphate)	[131]	Not known	
TPCs	Present in <i>Paramecium</i> DB (Not localized)	Unpubl. data	Acidic compartments Except acidocalcisomes No TPCs in databases	[173] [212]
InsP ₃ effects	Yes (InsP ₃ uncaging)	[132]	<i>P. falciparum</i>	[259]; also see text
IP ₃ -R	Cloned (subfamilies)	[132]	Unknown	–
RyR-LP ^b	Cloned (subfamilies)	[133]	Unknown	–
cADPR effects	Yes (not localized)	Microinjection (Fig. 7)	<i>T. gondii</i> <i>P. falciparum</i>	[207] [208]

^a For more details, see text.^b -LP = -like protein.

Paramecium DB. Their localization could be plasmalemmal and/or intracellular [452].

- *Ca²⁺-dependent and Ca²⁺-binding proteins*. CaM is largely conserved in the two groups of Alveolata. Similarly CaN and centrin appear to play important roles. Among protein kinases, both groups contain CDPKs with an inherent CaM-like domain, rather than kinases activated by CaM as a separate molecule.
- *Overall Ca²⁺ regulation*. In both groups of Alveolata, molecular mechanisms underlying intracellular Ca²⁺ sequestration and Ca²⁺ extrusion are probably largely comparable to those in metazoans. This includes participation of PMCA, SERCA, and exploitation of ΔH⁺ (generated either by H⁺-ATPases) in the context of antiporter systems. ΔH⁺-dependent mechanisms of Ca²⁺

regulation have been analyzed in much more detail in the parasites. In detail, acidocalcisomes abound in Apicomplexa, whereas they are not identified as yet in ciliates, where they probably also occur. In the parasites they may serve to clear an excess of Ca²⁺ from the cell, and some evidence suggests a role in Ca²⁺ signaling during host cell penetration.

- *Ca²⁺ stores*. In ciliates, Ca²⁺ stores are represented by the alveolar sacs, the ER and probably by the numerous endo-lysosomal and phagosomal compartments (as one may derive from the distribution of putative CRCs). The CVC also contains Ca²⁺ which it regularly extrudes from the cell during each systole in ~10 s time intervals. Release of Ca²⁺ from alveolar sacs via RyR-type CRCs during exocytosis stimulation is superimposed by a SOC

mechanism. In contrast, the inner membrane complex of Apicomplexa apparently has lost – fully or partially – the capability of storing substantial amounts of Ca^{2+} – if any – and the SOC mechanism recently described [205] would have to originate from another store. Ciliates contain numerous types of RyRs and IP_3 Rs – two of them are now molecularly and functionally clearly defined according to criteria valid for mammalian cells. Ciliates very likely also contain TPCs in compartments yet to be identified. In the genomic databases available for Apicomplexa, there occur only short fragments of sequences from similar channels, RyRs, IP_3 Rs and TPCs. Nevertheless, when InsP_3 is introduced into the parasites, it clearly exerts some effects. However, the molecular equivalents of these potential channels have resisted identification in a variety of attempts. Altogether, with the exception of *Paramecium*, information about such CRCs in protozoa is only fragmentary. For instance, gene sequences attributed to IP_3 Rs in the amoeba, *Naegleria* [41], and in *Dictyostelium* [260] have never been supplemented by proteomic analysis, localization and functional studies. As far as lower eukaryotes are concerned, one speculation says that, wherever InsP_3 effects have been ascertained in the absence of a true IP_3 R, such effects may operate in a (not yet identified) way different from receptor activation. An exception would be the true IP_3 Rs described in *Paramecium* [132,134]. Therefore, the identification of Ca^{2+} release mechanisms and the underlying channels in the stores of Apicomplexa remain a problem of paramount importance. Here – in contrast to ciliates – acidocalcisomes are well analyzed [453], although InsP_3 effects remain enigmatic.

- *Evolutionary aspects of signaling.* According to comparative genomics, IP_3 Rs are assumed to have emerged in evolution before RyRs [434]. However, in contrast to this view derived from database information then available, the *Paramecium* cell clearly contains RyRs and IP_3 Rs, as we have shown [132–134]. (Accordingly a PInsP_2 hydrolyzing PLC occurs in ciliates [238,358,359].) The temporal sequence of the emergence of RyRs and IP_3 Rs, as well as of TPCs (like RyRs not identified in the genomic analysis by Collins and Meyer [434]) requires more detailed analysis. No comparable data are available from Apicomplexa. Considering the clear identification of RyR- and of IP_3 R-type CRCs in *Paramecium*, the argument that in Apicomplexa they may be undetectable because of evolutionary distance is no longer maintainable. Rather, these may have lost such channels from a common ancestor during transition to parasitic life. In the course of genome reduction, they may have switched to other mechanisms more appropriate for such life style. Many genes pertinent to Ca^{2+} handling and signaling, which are contained in the *Paramecium* DB have been annotated by the group in Konstanz. In their recent comparative genomic analysis using the *Paramecium* DB Collins and Meyer [434] find a mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger and a polycystic Ca^{2+} -channel of the transient receptor potential- (TRP-)type Ca^{2+} channel. Any other TRP-type channels also await elucidation in *Paramecium*. In their evolutionary survey, these authors detected no sequences of a plasmalemmal-type of $\text{Na}^+/\text{Ca}^{2+}$ (whose existence we questioned in Section 7.1) or $\text{Na}^+/\text{K}^+/\text{Ca}^{2+}$ exchanger, and neither plasmalemmal Orai-related or -associated Ca^{2+} channels, nor Stim-related ER-type sensors involved in sensory signal transduction can be found (as we confirm by our own data mining).
- *A set of basic questions awaiting solutions.* Interestingly, no trimeric G-proteins, e.g. for the regulation of PLC activity, have been safely established as yet in alveolates (except fragmentary information for ciliates; see [30]). However, PLC for the hydrolysis of PInsP_2 occurs in ciliates [238,358,359]. We confirm the absence of CaR and CaS sequences in the *Paramecium* DB, as indicated by Collins and Meyer [434], in contrast to our immuno-localization to the ER and alveolar sacs of *Paramecium*, respectively, using

monospecific ABs (see Sections 3.1 and 5.1). However, in this case, when so widely different evolutionary levels are compared, an excess of acidic aminoacids may be more important than primary sequence. We consider this question open for further work. If this assumption is correct similar reasons may have impeded the identification of the ER-type Ca^{2+} -dependent chaperones in Apicomplexa, although other work suggests core glycosylation (Section 6.1) which in higher eukaryotes is normally paralleled by such chaperone functions. Similar uncertainty exists in both groups concerning annexins, as characterized in higher eukaryotes [454,455].

- *Consequences of parasitic life.* In summary, it appears that components relevant for Ca^{2+} handling and signaling are much more complex in ciliates than in Apicomplexa. These may have reduced several functions inherited from common evolutionary precursor forms or transformed them into other functions. This concerns the alveoli, where the equivalents (inner membrane complex) in the parasites cannot be safely considered as overt Ca^{2+} stores. They may rather serve as an abutment for glidosome activity during host cell entry. In the parasites, the main store of Ca^{2+} amenable to rapid mobilizations, e.g. during host cell penetration, could be the acidocalcisomes and/or the ER, although the molecular mechanism of Ca^{2+} release is not yet sufficiently clear for neither one of these compartments. In addition, acidocalcisomes may serve disposing of an excess of Ca^{2+} , or storage for eventual availability over longer times. While characteristic TPCs cannot be found and apparently have been lost, mechanisms for rapid Ca^{2+} handling via H^+ gradients and exchanger mechanisms have become preponderant in Apicomplexa. Prerequisites are H^+ -ATPases, Ca^{2+} -ATPases and H^+ -pyrophosphatase, as well as several Ca^{2+} exchanger activities. Acidocalcisomes as a whole and some of their transport activities have not been characterized in ciliates as yet, where they may also occur.

Generally the number of proteins involved in Ca^{2+} regulation increases during evolution. This is true not only for the comparison of Archaea with Eubacteria, but also for the transition to higher plants and to mammals [456]. It is also evident for the transition from protozoa to metazoa [434]. Nevertheless, we realize that a great part of the standard repertoire available in “higher” organisms can already be identified in ciliates. This is also in contrast to the younger branch of alveolates represented by the apicomplexan parasites whose Ca^{2+} regulation appears secondarily greatly reduced. The finding in *Paramecium* of CRCs closely related to the RyR is striking, considering their reported absence from choanoflagellates, the roots of metazoan evolution [16,457]. Here, however, detailed domain analyses are still missing.

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