Erschienen in: Cell Calcium ; 57 (2015), 3. - S. 174-185

Molecular aspects of calcium signalling at the crossroads of unikont and bikont eukaryote evolution – The ciliated protozoan *Paramecium* in focus

Helmut Plattner*

Department of Biology, University of Konstanz, P.O. Box M625, 78457 Konstanz, Germany

ARTICLE INFO

Keywords:
Ca^{2+}
Calcium
Channel
Ciliate
Paramecium
Protozoa

ABSTRACT

The ciliated protozoan, *Paramecium tetraurelia* has a high basic Ca^{2+} leakage rate which is counteracted mainly by export through a contractile vacuole complex, based on its V-type H^+-ATPase activity. In addition *Paramecium* cells dispose of P-type Ca^{2+}-ATPases, i.e. a plasmamembrane and a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (PMCA, SERCA). Antiporter systems are to be expected, as inferred from indirect evidence. Among the best known cytosolic Ca^{2+}-binding proteins, calmodulin activates Ca^{2+} influx channels in the somatic cell membrane, but inactivates Ca^{2+} influx channels in cilia, where it, thus, ends ciliary reversal induced by depolarization via channels in the somatic cell membrane. Centrin inactivates Ca^{2+} signals after stimulation by its high capacity/low affinity binding sites, whereas its high affinity sites regulate some other functions. Cortical Ca^{2+} stores (alveolar sacs) are activated during stimulated trichocyst exocytosis and thereby mediate store-operated Ca^{2+} entry (SOCE). Ca^{2+} release channels (CRCs) localised to alveoli and underlying SOCE are considered as Ryanodine receptor-like proteins (RyR-LPs) which are members of a CRC family with 6 subfamilies. These also encompass genuine inositol 1,4,5-trisphosphate receptors (IP_3Rs) and intermediates between the two channel types. All IP_3R/RyR-type CRCs possess six carboxyterminal transmembrane domains (TMD), with a pore domain between TMD 5 and 6, endowed with a characteristic selectivity filter. There are reasons to assume a common ancestor molecule for such channels and diversification further on in evolution. The distinct distribution of specific CRCs in the different vesicles undergoing intracellular trafficking suggests constitutive formation of very locally restricted Ca^{2+} signals during vesicle–vesicle interaction. In summary, essential steps of Ca^{2+} signalling already occur at this level of evolution, including an unexpected multitude of CRCs. For dis-/similarities with other bikonts see "Conclusions".

1. Introduction – why study Ca^{2+} in ciliates?

Why are ciliated protozoa interesting for Ca^{2+} signalling in the context of evolution? *Paramecium* and *Tetrahymena* are among the protozoan models most frequently used in cell and molecular biology [1]. Recent clades of ciliates have emerged between ~800 and 850 million years (Myr) ago according to small mRNA analysis [2], evaluation by more extended molecular biology [3] eventually combined with microfossils [4]. The data suggest that the phylum as such is considerably older.

Assuming that the Ur-eukaryote arose probably between ~1900 and 1700 Myr ago [5,6] and soon developed an elaborate and dynamic internal membrane system [7–9], vesicle trafficking must have required adequate signals. For example, for the Ur-eukaryote, 20 types of SNARE (*s*oluble *N*-ethylmaleimide *a*ttachment protein *re*ceptor) proteins [10] have been extrapolated, about half or less of the number occurring in present day species including *Paramecium* [11]. The number of small GTPases rose even considerably more during eukaryote evolution from *Tetrahymena* [12] to man [13]. From unequivocal experience with modern species and from the physicochemical corollaries underlying membrane interaction and fusion, some form of Ca^{2+} signalling must have been prerequisite already to early development of vesicle trafficking. See contribution by Plattner and Verkhratsky [14]. Thereby early eukaryotes were able to profit from an inventory of Ca^{2+} binding proteins (CaBP), influx channels, pumps etc. which they

* Correspondence to: Department of Biology, University of Konstanz, Room M1132, P.O. Box M629, 78457 Konstanz, Germany. Tel.: +49 7531 88 2228; fax: +49 7531 88 2168.
E-mail address: helmut.plattner@uni-konstanz.de

inherited from prokaryotes (see chapter by Domínguez et al. [15]).

Choanoflagellates as well as myxamoebae on the one hand and ciliates on the other hand stand for the two main lineages, unikonts and bikonts, leading to the evolution of the most highly evolved groups: from unikonts metazoa and from bikonts flowering plants (angiosperms) have evolved [16,17]. Therefore, it appears rewarding to have a closer look on the ciliate, *Paramecium*, particularly since this is one of the unicellular organism best analysed with regard to Ca^{2+} signalling by molecular biology and by a series of experimental approaches [18].

Focussing on ciliates this review also recalls the close evolutionary relationship to pathogenic forms of the phylum Apicomplexa. It is interesting to see changes in evolution due to parasitic lifestyle [19]. See also chapter by Moreno [20]. Precursors leading to *Plasmodium* have evolved since ~400 Myr [3], the mammalian parasites since ~13 Myr [21]. What these parasites have "forgotten" and "learned" to survive under their specific environment has been outlined previously [19]. A crucial question concerns the possible functional transformation of the alveolar sacs into the "inner lamellar complex" as pointed out in the chapter by Plattner and Verkhratsky [14] in the current issue.

2. Paramecium as a model and methodologies available

Over decades *Paramecium* has been frequently used as a unicellular model by electrophysiologists and by cell biologists [1]. It was the subject of extensive and innovative electrophysiology, yielding basic new insight into signalling processes [22–25], as to be specified in Section 6.1. The highly regular design, together with epigenetic determination of cell surface structure, made *Paramecium* and *Tetrahymena* a widely used model also for cell surface pattern formation [26]. The high number of cilia in regular arrangement made ciliates interesting for biogenetic studies on basal body and cilia formation [27] and the numerous, also regularly arranged dense core-secretory organelles, trichocysts, with the possibility of triggering synchronous exocytosis gave a handle to the study of some basic aspects of membrane fusion (exocytosis) and resealing (exocytosis-coupled endocytosis) [28,29]. As these structural redundancies are epigenetically controlled in *Paramecium* [26] it was possible during evolution to link the two aspects, regular design of the cells with very locally confined Ca^{2+} signalling by distinctly arranged Ca^{2+} influx and release channels (Plattner [30]).

About 10 years ago databases became available from a *Paramecium* and a *Tetrahymena* genome project [31,32], respectively. This enabled many detailed analyses of Ca^{2+} signalling particularly in the larger species, *Paramecium*, that previously were very much dependent on spontaneous and experimentally generated mutations. This explains why functional and molecular data on Ca^{2+} signalling in ciliates are rather stochastically distributed over the years, for different aspects and with widely different methodologies. First, there was a strong focus on electrophysiology, then on cell biology and more recently on molecular biology. Some of these aspects can now be combined and reconciled.

Paramecium has ~1.8times more protein encoding genes than man, i.e. nearly 40,000, due to several rounds of whole genome duplications [31]. Many paralogues (also called "ohnologues" when generated by whole genome duplication) have been lost after each round. But whenever very similar ohnologues have been maintained they usually are all transcribed, as we found with a variety of protein encoding genes [11]. Therefore, most ohnologues are thought to serve for gene amplification. There are also examples of neofunctionalisation [33]. In the extreme, two ohnologue genes, e.g. for calcineurin B, result in an identical translation product [34]. The absence of such whole genome duplications in *Tetrahymena* may be considered an advantage in some regards. In contrast, the situation in *Paramecium*, much more than in *Tetrahymena*, allows us to have a glance at Ca^{2+} signalling during ongoing evolution at the basis of bikonts. One may also recognize steadily ongoing parallel evolution at the level of protozoa. This situation lets us expect to see ancestral forms of molecules and further developments similar to those in higher eukaryotes. It is much less likely that ciliates would have acquired elements of Ca^{2+} signalling by vertical gene transfer [35], as these cells have a very broad and robust inventory of elements serving for Ca^{2+} signalling [18].

In the 1990s electrophysiological work with *Paramecium* has largely run dry for several reasons. First, patch-clamp analysis is not applicable because these cells are rather large and possess a pronounced egg case-like, rigid surface, thus precluding tight sealing. Only whole cell patch analysis has been possible to register Ca^{2+} currents and Ca^{2+}-activated currents along the cell membrane during exocytosis stimulation [36,37]. Second, the registration of Ca^{2+} transients, i.e. changes in intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, by f/f_0 ratio imaging during stimulation was hampered by several problems. One was that these cells do not easily take up fluorochromes even as esters, and when taken up they are not easily activated and if so, fluorochromes are rapidly sequestered into vacuoles. Another problem was the high mobility of these cells. These difficulties have finally been tackled [38], eventually in combination with fast opto-acustic confocal laser scanning microscopy [39]. Ca^{2+} signalling was, thus, analysed during synchronous ciliary beat, i.e. during normal beat and during ciliary beat reversal, respectively, as well as during synchronous exocytosis stimulation.

Fluorochrome analyses were complemented by quenched-flow/cryofixation (fast freezing) and energy-dispersive X-ray microanalysis, EDX [40,41]. Each of these methods provided us with a temporal resolution of ~30 ms [29]. This is comparable to the time required for these processes because ciliary beat operates with ~20 Hz and since exocytosis, though individual events (e.g. of membrane fusion) are much more rapid, is not absolutely synchronous when all events are collectively evaluated in a population of cells. The combination of fluorochrome analysis and EDX allowed us to analyse changes in $[Ca^{2+}]_i$ and in total calcium [Ca] (predominantly in bound form), respectively, and, therefore, to register Ca^{2+} fluxes. Higher spatial resolution of the intracellular distribution of Ca^{2+} was achieved by electron spectroscopic imaging, ESI, which enabled us to clearly identify alveolar sacs as cortical Ca^{2+} stores [42].

More recently we have identified and partially characterised at a molecular level, and finally localised in *Paramecium* at the light and electron microscope level SNAREs and other elements relevant for vesicle trafficking [11,43]. More recently this has been extended to Ca^{2+} release channels (CRC) [44–46].

A survey of the handling of Ca^{2+}, of its regulation and of Ca^{2+} regulated processes in the *Paramecium* cell is presented in Fig. 1.

3. Ca^{2+} leakage, sequestration and extrusion and estimation of Ca^{2+} fluxes

3.1. Ca^{2+} leakage, sequestration and extrusion

The *Paramecium* cell has an unexpectedly high leakage rate for Ca^{2+} [47,48]. How is this counteracted to maintain $[Ca^{2+}]_i$ homeostasis?

The cell membrane of *Paramecium* possesses a PMCA of ~130 kDa [49,50], i.e. of the usual size. Calmodulin binding has not been assessed and it is also unknown whether the PMCA extends to

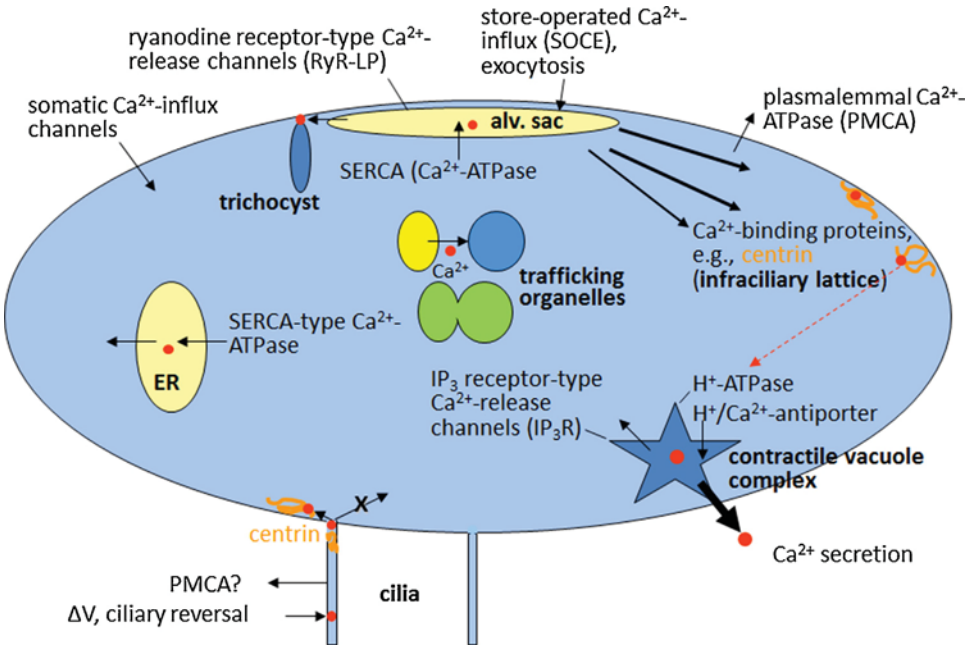

Fig. 1. Expanded scheme of vesicle trafficking, based on Plattner [18] and a basic trafficking concept outlined by Allen and Fok [107], with sites of Ca^{2+} influx, pumps, stores and local release. Red dots symbolise Ca^{2+}. Ca^{2+} influx can occur via widely distributed channels, including voltage-dependent influx channels (in cilia only) and a SOCE type mechanism for exocytosis, operated by non-ciliary channels. PMCA can extrude Ca^{2+} (though with restricted capacity), its occurrence in the ciliary membrane requiring confirmation. Inside the cell, a SERCA type pump sequesters Ca^{2+} into the ER and into alveolar sacs. Any uptake mechanisms in other organelles, e.g. of the endo-/phago-/lysosomal apparatus are not known, but Ca^{2+} content is concluded from the CRCs found in these organelles. Ca^{2+} is also sequestered into the contractile vacuole complex; here, occurrence of a PMCA (as in some other protozoa) is not known, but strong evidence argues in favour of Ca^{2+} sequestration and extrusion via secondary active transport coupled to the organellar H^+-ATPase. IP_3Rs and RyR-LPs have been analysed specifically in the contractile vacuole complex and in the alveolar sacs, respectively. IP_3Rs serve for partial reflux of Ca^{2+} after sequestration. Globally the contractile vacuole complex extrudes Ca^{2+} constitutively and particularly also following stimulated $[Ca^{2+}]_i$ increase and, thus, is a substantial control mechanism for $[Ca^{2+}]_i$ homeostasis. Ca^{2+} release from alveolar sacs via RyR-LPs serves as ignition for SOCE during stimulation of trichocyst exocytosis. Centrin is shown as a representative of cytosolic CaBPs with activation effects in cilia and downregulation effects in the cortical filament system, the infraciliary lattice, as described in the text. This centrin-containing filament lattice serves as a potent immobile buffer system from where Ca^{2+} can dissipate for extrusion, again mainly by the contractile vacuole complex. See Fig. 5 for details.

the ciliary membrane. Furthermore, the cells contain two isoforms of a SERCA, ~105 to 110 kDa in size and devoid of a calmodulin binding domain [51]. The domain usually accounting for thapsigargin binding greatly differs from mammalian SERCA and no inhibitory effect is achieved [51,52]. Carboxyterminal attachment of green fluorescent protein revealed formation of SERCA molecules in the ER and vesicular transport to alveolar sacs where, according to immune-electron microscopy, it localises to the inner part facing the cell centre [53]. Theoretically both, PMCA and SERCA (in the ER and in alveolar sacs), could keep $[Ca^{2+}]_i$ low and also contribute to downregulation after stimulation.

However, kinetics of both these P-type ATPases is rather slow [54]. For instance, refilling of alveolar sacs after massive exocytosis stimulation has a $t_{1/2}$ = 60 min, as determined by three independent methods [55]. Even in mammalian cells PMCA has been recently envisaged less for household functions, i.e. overall $[Ca^{2+}]_i$ regulation, but rather for maintaining local subplasmalemmal $[Ca^{2+}]$ low [56]. As extensively discussed previously, primary active Ca^{2+} transport cannot explain rapid downregulation of $[Ca^{2+}]_i$, e.g. after synchronous exocytosis stimulation [54]. A much more efficient way is secondary active transport via the contractile vacuole complex [46]. The contractile vacuole secretes Ca^{2+} in unexpectedly high concentration even in resting cells [57]. Ca^{2+} extrusion by this organelle works on the basis of its extensive endowment of the organelle with a V-type H^+-ATPase [58–60]. Its relevance can easily be seen after a Ca^{2+} load (Section 6.5).

Fig. 2 presents signals recorded during spontaneous exocytosis by electrophysiology [36,37] and by confocal fluorochrome imaging [39]. Fig. 3 summarises results obtained during caffeine stimulated exocytosis [61].

3.2. *Ca^{2+} fluxes during stimulation*

In *Paramecium*, exocytosis stimulation entails Ca^{2+} mobilisation from cortical stores, the alveolar sacs, paralleled by Ca^{2+} influx via the somatic (non-ciliary) cell membrane. Such *s*tore-*o*perated *Ca*$^{2+}$ *e*ntry, SOCE, has been documented by widely different methods [39,40,62]. In *Paramecium*, from [Ca] in alveolar sacs and the percentage of its mobilisation one can calculate that, referred to the entire cell, a total $[Ca^{2+}]$ of 0.25 mM would result if Ca^{2+} would not be rapidly downregulated by binding to *Ca*$^{2+}$-*b*inding *p*roteins, CaBPs [54], as characterised in Section 4.1. These remove Ca^{2+} from the cytosol considerably faster than any pump activity. In combination with SOCE, exocytosis stimulation would cause a global increase to ~0.85 mM in the entire cell, if not rapidly counteracted. In contrast, only ~5 μM $[Ca^{2+}]$ are locally required to drive exocytosis and only ~0.7 μM are seen with fluorochromes along the cell boundary [38]. How can this be reconciled? First, only a fraction of Ca^{2+} is seen with fluorochromes and real local values can be determined only by injection of Ca^{2+} chelators with different time constant [38]. Second, $[Ca^{2+}]$ is rapidly downregulated in *Paramecium* by centrin, as ascertained with mutants devoid of centrin-binding proteins and concomittantly of centrin filament assembly in the cortex [63] (See Fig. 5C).

Is such excessive Ca^{2+} flux in the course of stimulation restricted to ciliates? Comparable phenomena were registered by electrophysiology with endocrine cells [64]. Also here an excess is required to reach local activation levels for a subsecond time period, while Ca^{2+} rapidly diffuses and is rapidly bound to immobile buffers. Similar balance calculations, based on electrophysiology with mammalian cells, yield a similar picture as with ciliates, thus

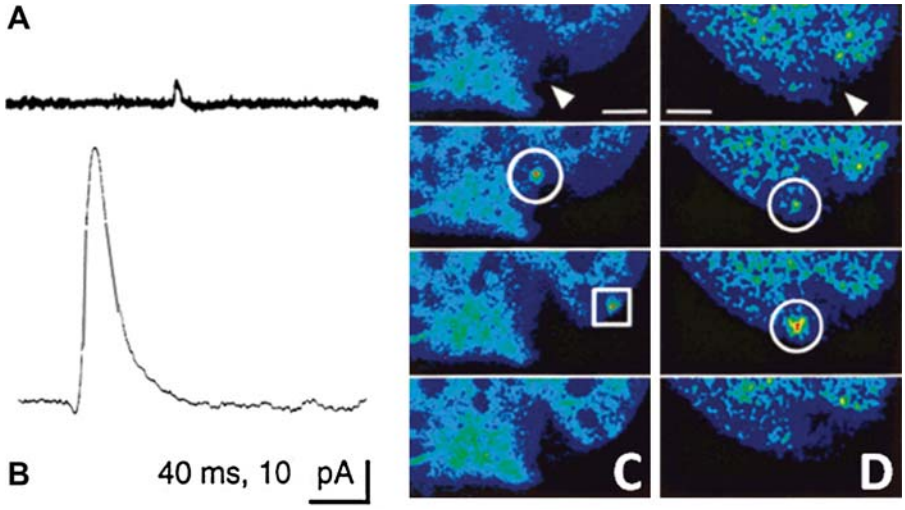

Fig. 2. Mini-Ca^{2+} signals accompanying exocytosis of trichocysts. (A) Electrophysiological whole cell-patch recording of Ca^{2+}-activated currents paralleled by individual spontaneous exocytotic events (only one shown). (B) Averaged curves from recordings as in (A), showing a duration of Ca^{2+}-activated currents of ~80 ms, with $t_{1/2}$ = 21 ms. (A, B) are from Erxleben et al. [37]. (C, D) Ca^{2+} signals recorded by fluorochrome during release of individual trichocysts by locally (arrowheads) applied small doses of trigger agents, i.e. AED (C) and 4CmC (D), respectively, visualized by confocal microscopy. (C, D) are from Klauke et al. [39]; scale bars = 5 μm.

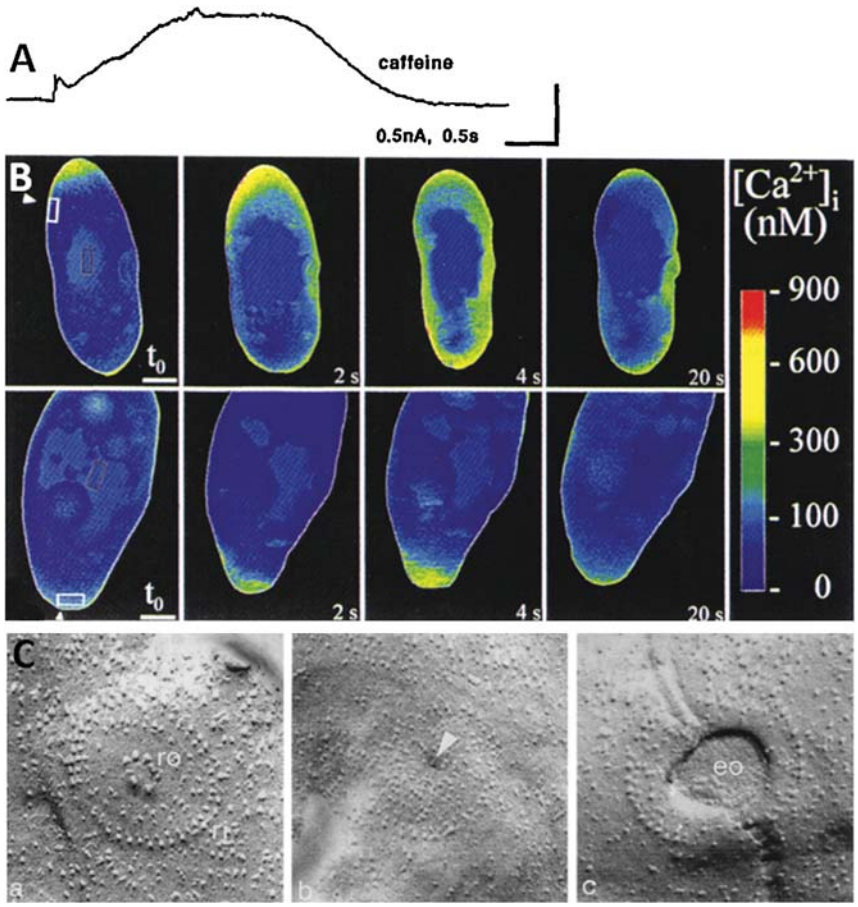

Fig. 3. Caffeine as a trigger for trichocyst exocytosis. (A) Electrophysiological whole cell-patch recording of Ca^{2+}-activated current generated by massive exocytosis of trichocysts. (Note that exocytosis induced by caffeine is much less synchronous than that induced by AED.) From Erxleben and Plattner [36]. (B) Fluorochrome imaging of Ca^{2+} signals generated during exocytosis induced by caffeine (applied at arrowhead), with $[Ca^{2+}]_o$ = 50 μM (top row) and $[Ca^{2+}]_o$ shortly reduced to ~0.03 μM during stimulation (bottom row), respectively, at times from t_o (before application) to 20 s. Scale bars = 10 μm. Squares are the areas evaluated by f/f_o ratio analysis, calibrated under the assay conditions at the right; at t_o, basic cortical $[Ca^{2+}]_i$ varied between 0.05 and 0.08 μM. Note the occurrence of a reduced signal at $[Ca^{2+}]_o$ lowered to a level slightly below $[Ca^{2+}]_i$ at rest, thus excluding a CICR and rather suggesting a SOCE mechanism. (C) Freeze-fracture demonstration of exocytotic membrane fusion produced by caffeine, as seen after rapid freezing within 1 s of stimulation. In (a) the resting state is presented, showing a ring (ri) of protein particles delineating the exocytosis site, 0.3 μm wide, and an aggregate of larger protein particles in the form of a rosette (ro). Image (a) is before stimulation, (b) shows focal (point fusion [28,29]) at the arrowhead and (c) the expansion to a large exocytotic opening. (B, C) are from Klauke and Plattner [61].

stressing the importance of signalling by an excess of Ca^{2+} because of rapid $[Ca^{2+}]$ downregulation below a minimal local level. Over longer times, after synchronous exocytosis induction, inhibition of the H^+-ATPase by concanamycin A considerably slows down reestablishment of $[Ca^{2+}]_i$ in *Paramecium* [19]. On the one hand this stresses the importance of secondary active Ca^{2+} transport over longer times and on the other hand assigns the main regulatory function over short times to CaBPs, i.e. centrin in ciliates.

Not only exocytosis, but also exocytosis-coupled endocytosis depends on $[Ca^{2+}]_o$. Thus, both are driven by SOCE [65]. Again this is well comparable with mammalian cells [66] where the C2-type CaBP synaptotagmin is involved also in this second step of exo-endocytosis coupling [67]. Remarkably, in the calyx of Held, a large synapse in the mammalian auditory central nervous system, two different Ca^{2+} influx channels, localised side-by-side, contribute to the two phenomena, exocytosis and exocytosis-coupled endocytosis [68]. Thus, this tight temporal coupling of exocytosis and endocytosis on the basis of a local Ca^{2+} signal is a widely distributed phenomenon.

In cilia of *Paramecium*, under our standard conditions, induction of Ca^{2+} influx by depolarization would cause an increase of $[Ca^{2+}]$ to an estimated 50 μM, corresponding ~700 Ca^{2+} molecules per organelle, if free Ca^{2+} were not downregulated immediately [41]. The range of estimations based on electrophysiology is between 10 μM and 1 mM, depending on $[Ca^{2+}]_o$ [69,70]. Again centrin may be involved in rapid inactivation (Section 4.1). Under standard conditions no spillover of Ca^{2+} from cilia into the cytosol can be recognized [41].

In summary, the high leakage rate of Ca^{2+} is compensated more by secondary, rather than by primary active transport activity. This is paralleled by rapid binding to immobile buffers, i.e. CaBPs, from where Ca^{2+} diffuses, also for extrusion mainly by the contractile vacuole complex.

4. Ca^{2+}-binding proteins

Principally CaBPs contain different motifs enabling binding of Ca^{2+}. First, this can take place with low specificity/affinity, but high capacity, as is the case with calreticulin and calsequestrin in the lumen of Ca^{2+} stores. (Here, Ca^{2+} specificity is guaranteed by the selectivity of the uptake mechanism.) Such molecules are rich in acidic aminoacids. Second, there are low capacity/high affinity CaBPs endowed with EF-hand motifs for coordinative binding of Ca^{2+} via loops of 12 aminoacids, such as calmodulin [71] and centrin, although centrin also contains a stretch with abundant acidic residues also in *Paramecium* [72,73]. Third, some other proteins bind Ca^{2+} with low capacity/high affinity by C2 motifs, i.e. β-barrels with a Ca^{2+} binding loop [74]. Moreover, there are still other CaBPs in the cytosol, such as annexins and copines (Section 4.1).

4.1. Cytosolic Ca^{2+}-binding proteins

Calmodulin (CaM) is enriched at several sites of the *Paramecium* cell [75,76]: along the cell membrane, in cilia and on vesicles of the food vacuole (phagolysosome) system as well as along the contractile vacuole and its extensions [75]. Here, CaM is associated with microtubules [76], so one can expects a stabilizing effect [77]. At the cell membrane CaM activates Ca^{2+}/CaM-activated cation influx channels (Section 6.1) and the PMCA has a CaM binding domain for its activation [50] (although CaM binding still remains to be ascertained with *Paramecium*). This may also be the case with the contractile vacuole, provided it contains PMCA as in other species – a currently open question [77]. At the ciliary membrane of *Paramecium* Ca^{2+} occupancy of specific EF-hand loops in the centrin molecule is required to activate voltage-dependent Ca^{2+} influx channels [78] whereas a Ca^{2+}/CaM complex deactivates these channels (Section 6.1). A Ca^{2+}/calmodulin complex activates guanylate cyclase [79] and cyclic GMP in turn activates protein kinase G (PKG) [80]. Both, the cyclase and the PKG are localised to cilia [81] where the cascade of events controls ciliary reversal. Finally the assembly of functional trichocyst docking sites with exocytosis competence depends on CaM [82] and CaM is also localised to these sites [75]. CaM is required for the assembly of exocytosis sites up to mammalian nerve terminals [83,84].

CaM-like domains are integrated in *C*a^{2+}-*d*ependent *p*rotein *k*inases, CDPK, of ciliates [73] and higher plants [85]. Ca^{2+}/CaM-activated protein kinases ("CaM kinases") proper are rare in plants (see contribution by Edel and Kudla [86]) and evidently absent from ciliates. Beyond that a Ca^{2+}-inhibitable casein kinase-type protein kinase has been cloned in *Paramecium* [87]. It antagonizes dephosphorylation of an exocytosis sensitive phosphoprotein of 63 kDa, pp63 (phosphoglucomutase), by the Ca^{2+}/CaM-activated protein phosphatase 2B, calcineurin [34,88]. This process has been discussed in detail by [89] and is considered important for ATP regeneration during rapid synchronous trichocyst exocytosis.

Centrin has in part EF-hand motifs and in part a domain with a surplus of acidic aminoacids [73]. Whereas the first aspect is interesting for the interaction with the voltage-dependent Ca^{2+} channels in cilia (see above) which thus are activated [78], the second aspect efficiently adsorbs an excess of free Ca^{2+}. Centrin is not only a main component of the infraciliary lattice of *Paramecium* [63,72,73], but also occurs at the basis of cilia in *Tetrahymena* [90]. Here it is enriched also in the transition zone [91] and, therefore, can act like a pump. Activation of the infraciliary lattice by a Ca^{2+} load causes cell contraction. Both functions are downregulated in mutants devoid of a centrin-binding protein [63,72] (Fig. 5C). Centrin is also engaged in morphogenetic processes in *Paramecium* [74] and in *Tetrahymena* [90]. Thus, differential duties can be assigned to the centrin molecule, as is the case also in human epithelial cells [92].

The C2-type CaBP, synaptotagmin, has two C2 domains and, due to its fast kinetics, enables membrane fusion in connection with SNAREs [94]. In the *Paramecium* database only an equivalent with eight C2 domains is found (R. Kissmehl and H. Plattner, unpubl. observ.), as confirmed by others (pers. commun.). Such multiple C2 domain CaBPs are also present in some intracellular membranes of mammalian cells [93] and here such extended-(e-)synaptotagmins can substitute for synaptotagmin proper [94]. Remarkably, the actual local $[Ca^{2+}]_i$ at exocytosis sites during stimulation is ~5 μM in *Paramecium* [38] and, thus, in the range of C2-domain proteins. Unfortunately universality of CaBPs with C2-domains and their role in membrane fusion have not been analysed consistently in many systematic groups including ciliates.

Copines, another group of cytosolic C2-domain proteins, have been detected and cloned in *Paramecium* [95]. Annexins are cytosolic and membrane binding CaBPs with alternative Ca^{2+} binding motifs [96]. In *Paramecium*, using antibodies against common (mammalian) sequences, putative annexins have been found associated with preformed exocytosis sites, specifically with trichocyst docking sites and with the cytoproct (the site of release of spent food vacuoles) [97]. Both, copines and annexins are considered important for establishment of membrane-to-membrane links [96,98] and both occur in animals as well as in plants where they deal with Ca^{2+}-dependent developmental and defence processes [99,100].

In an evolutionary context CaBPs can be summarised as follows. Many of the functions of CaM are maintained up to the top of multicellular uni- and bikonts. However, there also occurs some diversifications between uni- and bikonts. The occurrence of voltage-dependent Ca^{2+} influx channels in unikonts is in contrast to

higher plants where they are absent, as discussed by Edel and Kudla [86]. Their regulation by CaM in mammalian pyramidal neurons [101] is as occurring in ciliates. CaM-like sequences integrated in CDPKs are typical of bikonts, i.e. ciliates and plants (Edel and Kudla [86]). No such dual distribution is found with annexins, centrin and copines, as they occur in both lineages.

4.2. Lumenal Ca^{2+}-binding proteins

Genes encoding calreticulin and calsequestrin have not been found in the *Paramecium* database. However, monospecific antibodies provided by a leading expert, H.-D. Söling (Göttingen), clearly differentiated between ER and alveolar sacs, the cortical Ca^{2+} stores resembling very much the SR of muscle [102]. Difficulties in finding gene sequences can be explained by the importance of the abundant acidic aminoacid residues, rather than of specific motifs [103].

Different dense core-secretory organelles of mammalian cells also store Ca^{2+}, occasionally in tens of millimolar concentrations. This is in striking contrast to trichocysts of *Paramecium*. These contain no Ca^{2+} detectable by EDX [40]. Whereas this could be due to low sensitivity of the method, biological arguments support the absence of any remarkable Ca^{2+} in the organelles. First, in vitro, Ca^{2+} in (sub)millimolar concentrations causes vigorous decondensation (stretching) of the trichocyst matrix [104], an effect normally coupled with formation of an exocytotic opening which gives Ca^{2+} access to the lumen. Second, the relevance of Ca^{2+} binding to the trichocyst contents just during exocytosis is supported by the finding of CaBPs in the trichocyst matrix in wildtype cells [105]; in contrast, a mutant unable decondense trichocysts in vitro upon addition of Ca^{2+} is also unable to bind $^{45}Ca^{2+}$ to trichocyst contents on electrophoresis gels. In such a mutant, trichocysts also do not decondense in vivo even though an exocytotic fusion pore has been formed upon stimulation with a secretagogue [105]. Evidently, in *Paramecium*, membrane fusion and contents release are two distinct steps of exocytosis which, however, are automatically coupled by the access of Ca^{2+} to the secretory contents. This aspect would deserve detailed analysis also in metazoan secretory cells.

5. Dedicated Ca^{2+} stores and Ca^{2+} in trafficking organelles

5.1. The endoplasmic reticulum, alveolar sacs and trafficking organelles

There is no continuity between the two stores and the sacs must, therefore, be formed by vesicle trafficking [53,106]. They share the SERCA [53] and in part also CRCs [45]. [Ca] in the sacs is ~40 mM according to calibrated EDX analysis [40].

Much more elaborate is trafficking via the endocytotic, phagocytotic and exocytotic route as well as along different membrane recycling routes, for instance to and from the food vacuoles (phagosomes and phagolysosomes), as established by Allen and Fok [107]. Surprisingly the contractile vacuole complex which undergoes cyclic kiss-and-run exocytosis contains SNAREs as molecular attributes of vesicle trafficking not only at the pore (exocytotic outlet), but also within the organelle [77]. This reflects membrane trafficking by marching on the spot, meaning restructuring of the complex spongiome-like membrane network [77]. Its Ca^{2+} content can be seen in the electron microscope after appropriate preparation [18] and by Ca^{2+} selective electrode recordings [57,108].

No data are available for the Ca^{2+} content in the trafficking organelles of *Paramecium*. This must be assumed, however, considering their endowment with distinct CRCs and the known Ca^{2+} content in equivalent structures in higher eukaryotes, as outlined in Section 6.

5.2. Crystal vacuoles

According to X-ray diffraction analysis the membrane-bounded crystals contain Ca^{2+} as $(Mg,Ca)NH_4PO_4.6H_2O$, struvite, one of the minerals also contained in kidney stones [109]. Their number increases with cell age and they may, therefore, serve as a waste basket, but their role in Ca^{2+} regulation is unexplored.

5.3. Mitochondria and Ca^{2+} handling in ciliates

A *m*itochondrial *C*a^{2+} *u*niporter, MCU serves for rapid uptake of Ca^{2+} into the organelle. A homolog occurs in *Tetrahymena* [110]. In contrast, the *e*ssential *M*CU *re*gulator, EMRE, has not been found in the *Tetrahymena* database [111].

During exocytosis stimulation, [Ca] is seen by EDX to rise in mitochondria within 30 ms and then to decay already within 80 ms [40]. With the mitochondrial fluorochrome, Rhodamine, $[Ca^{2+}]$ rise in mitochondria is also very fast, but the decay of $[Ca^{2+}]$ is by orders of magnitude slower, $\geq$5 min [19]. This indicates that only a fraction of Ca^{2+} is retained in the mitochondria. Interestingly, in *Paramecium*, mitochondria are enriched in cortical regions (unpubl. observ.). This may not only contribute to the regulation of $[Ca^{2+}]_i$ homeostasis, but it may also contribute to energetics by activation of matrix dehydrogenases [112]. Such cortical localisation is considered a principal aspect of energetics in many unicellular organisms [113].

6. Ca^{2+} influx and intracellular Ca^{2+} release channels in ciliates

A *Paramecium* cell contains numerous Ca^{2+} influx and release channels which all are distributed over the cell in a rather precise pattern (see chapter by Plattner [30]). This allows for precise local Ca^{2+} signalling.

6.1. Ca^{2+} influx channels

Paramecium cells dispose of an ample assortment of Ca^{2+} influx channels and of Ca^{2+}/calmodulin-activated channels in their somatic (non-ciliary) cell membrane [22,23,24]. Different channels have to cooperate for a specific ciliary/behavioural response. In part, this is guaranteed by their specific localization. For instance, voltage-gated Ca^{2+} channels do not occur outside cilia [114]. They are activated by depolarization of the cell membrane potential which in turn occurs in the course of mechanical stimulation of the cell in its anterior part where mechanosensitive channels are found in the somatic cell membrane [115]. Thus, a receptor potential is generated and depolarisation beyond a threshold induces a graded action potential by the voltage-dependent Ca^{2+} channels [116]; the membrane potential is reversed by Ca^{2+}-activated K^+ efflux. The activity of voltage-gated Ca^{2+} channels is supported by associated centrin [78] and rapidly counteracted by the forming Ca^{2+}/calmodulin complex which inactivates the voltage-dependent Ca^{2+} channels [117]. This inactivation mechanism is maintained in evolution up to pyramidal cells of the human brain [101]. When hyperpolarising K^+ channels are activated in the posterior part of the cell surface, hyperpolarization-sensitive Ca^{2+}-channels in the somatic cell membrane activate another signalling cascade resulting in accelerated forward swimming [118]. This channel distribution has been summarized in more detail in an accompanying article by the author [30]. Voltage-dependent Ca^{2+} influx and its regulation in ciliates seem to be an old heritage which has been lost in the bikont lineage, i.e. plants (see Edel and Kudla [86]).

6.2. Inositol 1,4,5-trisphosphate receptors

A broad variation in the collection of CRCs of the type inositol 1,4,5-trisphosphate ($InsP_3$) receptors (IP_3R) [46] and Ryanodine receptor-(RyR-)like proteins (RyR-LPs) [45], respectively, together with intermediate forms [44], has been detected in *Paramecium*. Epigenetically determined intracellular distribution of many of these CRCs is discussed in a separate article by the author in this issue [30]. Only a few of these channels have been followed up in more detail experimentally.

IP_3Rs of *Paramecium*, type CRC-II, contain a genuine $InsP_3$-binding domain according to ^{3}H-inositol 1,4,5-trisphosphate binding to the homologously overexpressed and isolated protein, $InsP_3$ uncaging experiments paralled by $[Ca^{2+}]_i$ recording, and molecular modelling [46]. The finding of IP_3Rs in the contractile vacuole complex/osmoregulatory system was insofar surprising as these complex organelles extrude Ca^{2+} with every pulsation [57,108]. However, a stochastic, non-stimulated Ca^{2+} reflux is clearly seen with fluorochromes. This indicates constitutive activity of the IP_3R, as then also seen in avian and mammalian cell cultures [119]. In *Paramecium*, this could serve for fine-tuning of $[Ca^{2+}]_i$. Since these CRCs are localised to the smooth spongiome – a widely branched tubular membrane system undergoing extensive rearrangements during systole/diastole cycles of the contractile vacuole [120,121] – and considering the presence of v- and t-SNAREs in this region [43,77], Ca^{2+} most likely also contributes to the periodic rearrangement of this extensive and dynamic membrane system during each functional cycle of ~8 s [77].

6.3. Ryanodine receptor-like proteins in Paramecium

The identification of RyRs, or rather RyR-LPs, is more difficult, particularly since these molecules are remarkably smaller in *Paramecium* than in higher eukaryotes [45]. One type analysed in more detail is important for the release of Ca^{2+} from the cortical Ca^{2+} stores, the alveolar sacs, as summarised in Fig. 4. As mentioned, this involves a SOCE mechanism. This has been concluded unanimously from whole cell-patch electrophysiology [36], fluorochrome analysis [38,39] and quenched-flow/EDX [40]. For instance, rapid substitution of Sr^{2+} for Ca^{2+} in the medium during quenched-flow stimulation clearly showed, during exocytosis stimulation, the release of Ca^{2+} and the rapid emergence of Sr^{2+} Kα energy signals in the alveolar sacs [40]. When cells were stimulated at extracellular Ca^{2+} concentrations, $[Ca^{2+}]_o$, slightly below $[Ca^{2+}]_i$ at rest, there was still some fluorochrome signal generated by stimulation [38] and, in EDX, Ca^{2+} in the alveoli decreased by ~50% [40]. To definitely exclude *C*a^{2+}-*i*nduced *C*a^{2+} *r*elease, CICR, we stimulated mutant cells devoid of Ca^{2+} influx; the result was as with wildtype cells [62] – again excluding a CICR (Fig. 5A). Originally aminoethyldextran, AED, has been used to stimulate cells, not only because this perfectly mimics the physiological situation during predator defence by massive local trichocyst exocytosis and superimposed ciliary reversal for escape [18], but also because it can be used in multiple rounds for exocytosis stimulation without any recognizable side-effects [122]. In connection with molecular biology work, the RyR agonists, caffeine and *4*-chloro-*meta*-cresol, 4CmC, have been applied [45]. In fluorochrome analyses, the results were all the same as with AED, i.e. the same signals were registered with caffeine and 4CmC when stimulated at normal $[Ca^{2+}]_o$ and at reduced $[Ca^{2+}]_o$, respectively [45]; Fig. 4A–C. Moreover, gene silencing almost abolished the signal normally seen upon stimulation (Fig. 4D).

A notoriously aberrant pharmacology of ciliates (e.g. actin isoforms insensitive to phalloidin, SNARE proteins insensitive to *Clostridium* toxins, SERCA insensitive to thapsigargin, etc. [52]) makes tests with ryanodine unreasistic. Is there any other support for the assumption of the occurrence of RyRs or RyR-LPs in *Paramecium*? Response to the alkaloid ryanodine from the South American plant, *Ryania speciosa* (Salicaceae), can hardly be considered an ultimate selection characteristic, unless specific functional/molecular clues would be evident (which is not the case). Unfortunately cADPR, a potential signal transducer for these channels [123], has not been established at the time we made our analyses. Experience with the secretagogues, caffeine and 4CmC, in general and with *Paramecium* in particular [39,61] agree with the assumption of RyRs/RyR-LPs [44,45] and SOCE in *Paramecium.* The aminoacid signature critical for 4CmC binding [124] is present in the *Paramecium* RyR-LP molecule [45]; Fig. 4E. This, together with the insensitivity to ryanodine makes it similar to a RyR in malignant insomnia patients [125]. Also the position in a subcellular compartment reminds the terminal cisternae in muscle, as does the SOCE mechanism – another characteristic of skeletal muscle cells [126]. The lumenal high capacity/low affinity CaBP found in the alveolar sacs lumen [102] is also equivalent to that in the sarcoplasmic reticulum (see above). Furthermore, the channel identified to respond to exocytosis stimulation does not respond to $InsP_3$ uncaging [46]. Finally, by electrophysiological recording of *Paramecium* surface membrane proteins reconstituted with liposomes, a non-$InsP_3$ sensitive, non-plasmalemmal channel has been seen that "may reflect a Ca^{2+} release channel of the alveolar sacs" [127]. Collectively all these arguments support the hypothesis that the channels, CRC-II, found in the cortical stores of *Paramecium* represent RyRs, or RyR-LPs.

6.4. Mixed types of CRCs and common origin

Let us now ask whether there may have been a common ancestor of IP_3Rs and RyRs, considering the intermediate forms mentioned. This follows from presence/absence of an $InsP_3$ binding domain and/or of RIH domain, variable aminoacid sequence of selectivity filter, etc., as shown in these molecules in *Paramecium* [44]. Remarkably, despite considerable difference in size between IP_3Rs and RyRs in vertebrates, both vertebrate channel forms show a coherent allosteric gating [128]. Also the conserved structural architecture of the aminoterminal part suggests common precursor [129]. Further support comes from the recent insight – based on advanced algorithms applied to the evaluation of established trans-membrane proteins as indicated by Ladenburger and Plattner [44] – that in the carboxyterminal part six trans-membrane domains occur in both channel types, rather than only four in the RyR. This applies not only to ciliates [44], but is now propagated also for mammals [130,131a,b]. Furthermore, the selectivity filter has previously been assumed to show a clear differentiation between the two channel types [132]: In mammals, the sequence GVGD has been assigned to IP_3Rs and GIGD to RyRs. In contrast, in *Paramecium* and other "lower" eukaryotes both channels can contain the GIGD or still other signatures [133]. All this is compatible with a common ancestor for both channel types and such forms show up in *Paramecium*'s collection of IP_3Rs/RyR-LPs [44].

It has been suggested, however, that CRCs in protozoa would be due to vertical gene transfer [35]. Considering on the one hand the considerable number of Ca^{2+} regulating and of Ca^{2+} regulated proteins in protozoan cells (for ciliates, see [18]), and on the other hand the general and imperative requirement of Ca^{2+} for the regulation of vesicle trafficking already in early times of evolution (Section 1), one may consider this not very likely. Both types of CRCs may also occur in a variety of organisms where, up to recently, RyRs or RyR-LPs have not been detected in broad scanning explorations [134] when they did not apply domain specific analysis. Particularly the pore domain with its filter region can be helpful for identification because this is rather well conserved and most crucial for specific channel function [133].

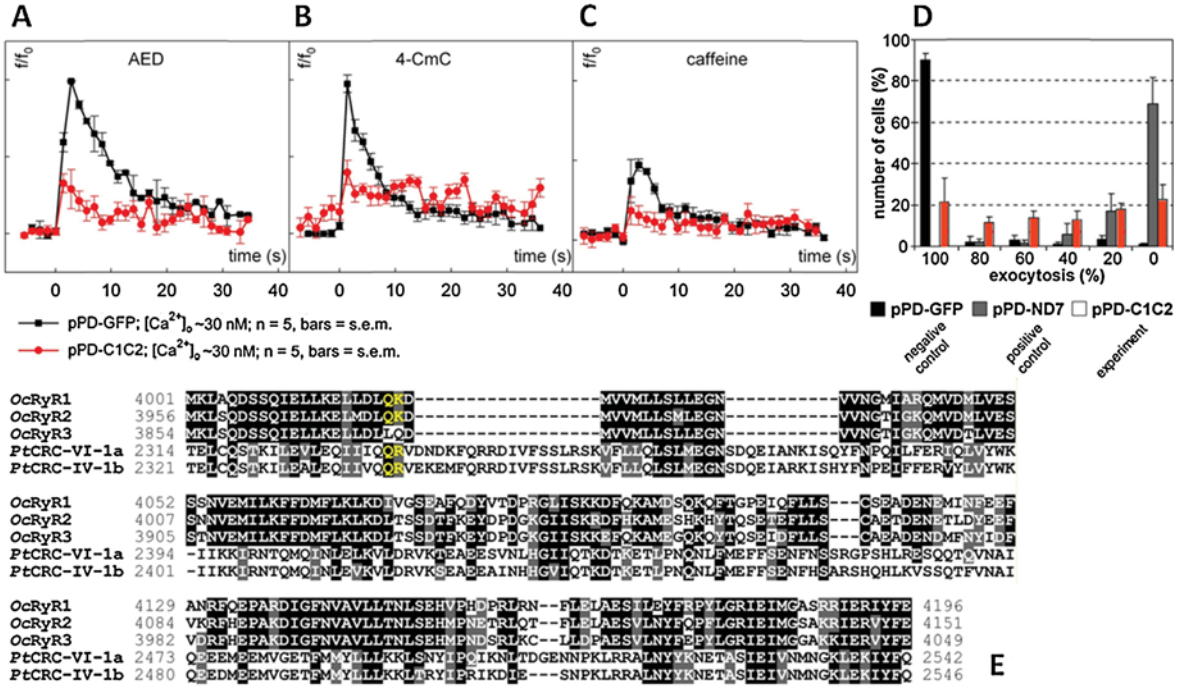

Fig. 4. Involvement of RyR-LPs in stimulated trichocyst exocytosis. (A–C) Fluorochrome analysis of Ca^{2+} signals generated by AED (A), 4CmC (B) and caffeine (C) at $[Ca^{2+}]_o$ shortly reduced to ~0.03 μM, i.e. below the level at rest. Black curves are for normal cells, red curves for cells silenced in the RyR-LP of alveolar sacs. Note that in silenced cells the signal is clearly reduced. Bars = standard errors. (D) Evaluation of trichocyst exocytosis in normal wildtype cells (black bars) and in cells silenced in the RyR-LP of alveolar sacs (red bars). Cells were assigned to six groups, from 100 to 0% exocytosis (abscissa), with the percent activity indicated in the ordinate. The reduced activity in silenced cells (red columns) becomes, thus, evident. Only exocytosis-incompetent ND7 mutant cells, used for additional controls (grey columns), show more stringent reduction of exocytosis than silenced cells because the effect of the posttranscriptional silencing used is never all-or-none. (E) Carboxyterminal part of the RyR-LP, with two isoforms (*Pt*CRC-IV-1a [erroneously labelled VI in the original work reproduced here] and *Pt*CRC-IV-1b) showing that these channels are considerably shorter than their equivalents in rabbit (*Oryctolagus cuniculus*), OcRyR types 1–3. Moreover it shows coincident position of aminoacids Q and K/R (yellow) at positions considered critical for 4CmC binding in mammals, as indicated in the text. From Ladenburger et al. [45].

Whenever analyses were not restricted to overall nucleotide similarity, similar CRCs could be detected also in some other protozoan species. In the flagellated protozoans, *Trypanosoma brucei* [135] and *Trypanosoma cruzi* [136], IP_3R channels have thus been identified, complemented by thorough experimental work comparable to that with *Paramecium*. This included activation studies, intracellular localisation, knockdown experiments and functional analysis. Similar work would now be necessary also for all of the other para-/ohnologues of IP_3R/RyR-type channels in *Paramecium*, altogether 34, grouped in 6 subfamilies [44]. Their selective arrangement in different compartments is presented in a parallel paper in this issue [30]. This multitude may be exceptional, yet all

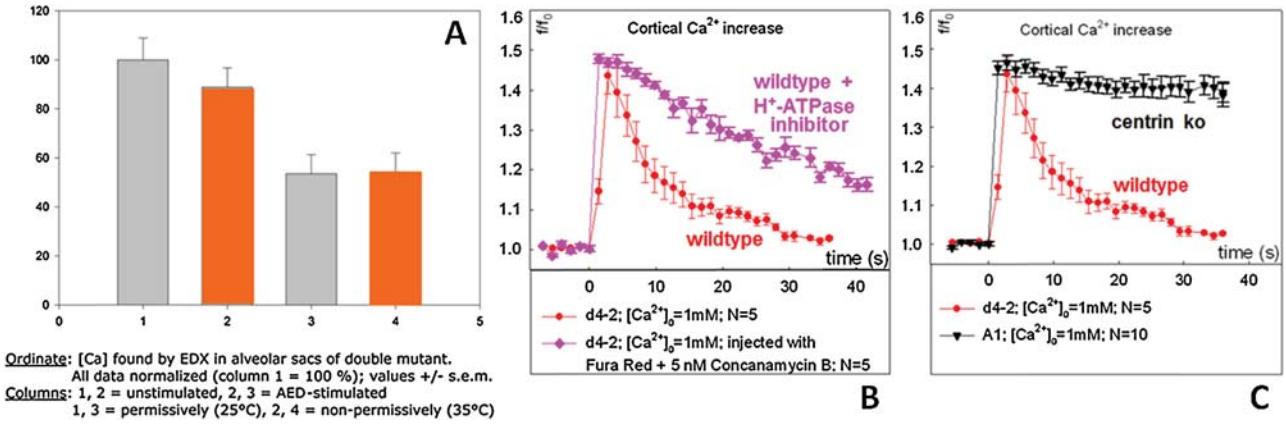

Fig. 5. Relevant aspects of signalling during stimulated trichocyst exocytosis. This includes SOCE for signalling (A) and H^+-ATPase activity (B) as well as binding of Ca^{2+} to cortical centrin (C). (B, C) both serve for signal downregulation. (A) The release of Ca^{2+} from alveolar sacs was registered by evaluating CaKα signals by EDX at the electron microscope level. Wildtype cells (grey columns) and a double mutant devoid of Ca^{2+} influx (orange columns) were analysed. Columns 1,2 are resting cells, columns 2,3 are AED stimulated cells. The amount of Ca^{2+} released during stimulation is the same, with and without Ca^{2+} influx, respectively, thus excluding CICR and supporting SOCE. Bars are standard deviations. (B) After exocytosis stimulation by AED, cortical $[Ca^{2+}]_i$ is downregulated considerably more rapidly in wildtype cells under normal conditions than after exposure to the H^+-ATPase inhibitor, concanamycin B. From the occurrence of this pump in the contractile vacuole complex, its strong Ca^{2+} expulsion activity (see text) and inhibition by concanamycin B it is concluded that Ca^{2+} is sequestered by a secondary active antiport pathway. (C) Knock-out of cortical centrin-binding protein impedes the assembly of cortical centrin that is normally contained in the infraciliary lattice. Its elimination causes a big delay in the downregulation of cortical $[Ca^{2+}]_i$ after AED stimulation. (A) is from Mohamed et al. [62], (B) from Plattner et al. [52], (B) and (C) are from Refs. [52] and [19], respectively.

(but one) are expressed. On the one hand diversification of these CRCs is evident, but on the other hand some forms probably serve for a kind of gene amplification.

Attempts toward identification of IP_3Rs appear a particularly rewarding challenge, particularly in those protozoan species where $InsP_3$ effects have been reported. This is the case, e.g. with Apicomplexan parasites; see contribution by Moreno [20]. Remarkably, for choanoflagellates Cai et al. ([137]) indicate the presence of IP_3Rs in *Monosiga* and of RyRs in *Salpingoeca*. One may now expect from scrutiny either the occurrence of both channel types, and/or of an intermediate channel type in each of the two species.

6.5. Are there other Ca^{2+} release channels and antiporters in *Paramecium*?

NAADP (*n*icotinic *a*cid *a*denine*d*inucleotide*p*hosphate) is considered the activator of two-pore channels (TPC) that are widely distributed among organisms [138]; see contribution by Patel and Cai [139]. With *Paramecium* homogenates and under intracellular ionic conditions we determined a K_D of 3.5 nM for NAADP binding and microinjected NAADP clearly caused a Ca^{2+}-based response [19] which, however, was not further specified. We expect the occurrence of TPCs, but this still awaits experimental verification. This is in contrast to what is currently assumed for higher plants (Edel and Kudla [86]).

Gene sequences suggest the presence of a Na^+/Ca^{2+} antiporter in *Tetrahymena* [140], but beyond this cursory note neither its localisation nor its function has been followed up. The occurrence of a H^+/Ca^{2+} exchanger is very likely for the contractile vacuole complex because the organelle actively sequesters protons and releases Ca^{2+} not only into the outside medium, but also into the cytosol (see above) and, because no acidification of the organelle is recognized [141]. This strongly indicates exploitation of a H^+ gradient for the secondary active transport of Ca^{2+}. Experiments with the H^+-ATPase inhibitor, concanamycin B, support this concept (Fig. 5B).

In *Paramecium*, one type of Ca^{2+} release channel, CRC-V-4, with a small $InsP_3$-binding homology domain, is associated with parasomal sacs, the clathrin coated pits [44]. A light and electron microscope picture is presented in the accompanying paper [30]. This channel is a candidate for ill-defined channels with high conductance/low selectivity described by electrophysiologists [22].

7. Conclusions

One may now try to make a balance sheet of components pertinent to Ca^{2+} signalling in ciliates, specifically for *Paramecium*. The essential mechanisms (beyond cilia) are represented by experimental data shown in Fig. 5. Work with a mutant devoid of Ca^{2+} influx used for EDX analysis strongly supports the SOCE mechanism of signalling during stimulated exocytosis. Such rapid coupling of internal Ca^{2+} mobililsation and enforcement by strictly coupled Ca^{2+} influx is vital for surviving as it wards off very efficiently predator attacks [142]. Downregulation takes place very rapidly by Ca^{2+} binding to cortical centrin and in parallel by Ca^{2+} expulsion via the contractile vacuole complex. Cilia are essentially autonomous compartments with their own Ca^{2+} influx and downregulation system.

Paramecium is currently one of the best experimentally analysed unicellular model. Comparison of the two main lineages, uni- and bikonts, reveals the following tendencies. (i) There are components shared by *Paramecium*, higher animals and higher plants. This includes CaM, centrin, PMCA, SERCA, copines and annexins. (ii) Components shared with animals are voltage-gated Ca^{2+} channels, dimeric calcineurin, IP_3Rs, RyR-LPs and possibly TPCs. (iii) Ciliates share CDPKs with plants which here dominate over Ca^{2+}/CaM-activated protein kinases. Only subunit B of calcineurin is retained in plants. Finally there is some uncertainty about lumenal high capacity/low affinity CaBPs, cation exchangers and TPCs in *Paramecium*.

Acknowledgment

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

References

[1] M. Simon, H. Plattner, Unicellular eukaryotes as models in cell and molecular biology: critical appraisal of their past and future value, Int. Rev. Cell Mol. Biol. 309 (2014) 141–198.
[2] A.-D. Wright, D.H. Lynn, Maximum ages of ciliate lineages estimated using a small subunit rRNA molecular clock: crown eukaryotes date back to the paleoproterozoic, Arch. Protistenkd. 148 (1997) 329–341.
[3] E.J. Douzéry, E.A. Snell, E. Bapteste, F. Delsuc, H. Philippe, The timing of eukaryotic evolution: does a relaxed molecular clock reconcile proteins and fossils? Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 15386–15391.
[4] C. Berney, J. Pawlowski, A molecular time-scale for eukaryote evolution recalibrated with the continuous microfossil record, Proc. Biol. Sci. 273 (2006) 1867–1872.
[5] A.H. Knoll, Proterozoic and early Cambrian protists: evidence for accelerating evolutionary tempo, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 6743–6750.
[6] L.W. Parfrey, D.J. Lahr, A.H. Knoll, L.A. Katz, Estimating the timing of early eukaryotic diversification with multigene molecular clocks, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 13624–13629.
[7] J.B. Dacks, M.C. Field, Evolution of the eukaryotic membrane-trafficking system: origin, tempo and mode, J. Cell Sci. 120 (2007) 2977–2985.
[8] M. Elias, Patterns and processes in the evolution of the eukaryotic endomembrane system, Mol. Membr. Biol. 27 (2010) 469–489.
[9] P. Forterre, The common ancestor of archaea and eukarya was not an archaeon, Archaea 2013 (2013), http://dx.doi.org/10.1155/2013/372396.
[10] T.H. Kloepper, C.N. Kienle, D. Fasshauer, SNAREing the basis of multicellularity: consequences of protein family expansion during evolution, Mol. Biol. Evol. 25 (2008) 2055–2068.
[11] H. Plattner, How to design a highly organized cell: an unexpectedly high number of widely diversified SNARE proteins positioned at strategic sites in the ciliate, *Paramecium tetraurelia*, Protist 161 (2010) 497–516.
[12] L.J. Bright, N. Kambesis, S.B. Nelson, B. Jeong, A.P. Turkewitz, Comprehensive analysis reveals dynamic and evolutionary plasticity of Rab GTPases and membrane traffic in *Tetrahymena thermophila*, PLoS Genet. 6 (2010) e1001155.
[13] A.M. Rojas, G. Fuentes, A. Rausell, A. Valencia, The Ras protein superfamily: evolutionary tree and role of conserved amino acids, J. Cell Biol. 196 (2012) 189–201.
[14] H. Plattner, A. Verkhratsky, The ancient roots of calcium signalling evolutionary tree, Cell Calcium 57 (2015) 123–132.
[15] D.C. Domínguez, M. Guragain, M. Patrauchan, Calcium binding proteins and calcium signalling in prokaryotes, Cell Calcium 57 (2015) 151–165.
[16] T. Cavalier-Smith, Protist phylogeny and the high-level classification of protozoa, Eur. J. Protistol. 39 (2003) 338–348.
[17] F. Burki, J. Pawlowski, Monophyly of Rhizaria and multigene phylogeny of unicellular bikonts, Mol. Biol. Evol. 23 (2006) 1922–1930.
[18] H. Plattner, Calcium regulation in the protozoan model, *Paramecium tetraurelia*, J. Eukaryot. Microbiol. 61 (2014) 95–114.
[19] H. Plattner, I.M. Sehring, I.K. Mohamed, K. Miranda, W. De Souza, R. Billington, A. Genazzani, E.M. Ladenburger, Calcium signalling in closely related protozoan groups (Alveolata): non-parasitic ciliates (Paramecium, Tetrahymena) vs. parasitic Apicomplexa (Plasmodium, Toxoplasma), Cell Calcium 51 (2012) 351–382.
[20] S. Moreno, The calcium toolkit of the apicomplexan parasites *Toxoplasma gondii* and *Plasmodium* spp., Cell Calcium 57 (2015) 186–193.
[21] R.E. Ricklefs, D.C. Outlaw, A molecular clock for malaria parasites, Science 329 (2010) 226–229.
[22] H. Machemer, Electrophysiology, in: H.-D. Görtz (Ed.), Paramecium, Springer-Verlag, Berlin/Heide berg, 1988, pp. 185–215.
[23] H. Machemer, Motor control of cilia, in: H.-D. Görtz (Ed.), Paramecium, Springer-Verlag, Berlin/Heidelberg, 1988, pp. 216–235.
[24] Y. Saimi, C. Kung, Calmodulin as an ion channel subunit, Annu. Rev. Physiol. 64 (2002) 289–311.
[25] C. Kung, B. Martinac, S. Sukharev, Mechanosensitive channels in microbes, Annu. Rev. Microbiol. 64 (2010) 313–329.
[26] J. Beisson, Preformed cell structure and cell heredity, Prion 2 (2008) 1–8.
[27] A. Aubusson-Fleury, M. Lemullois, N.G. de Loubresse, C. Laligne, J. Cohen, O. Rosnet, M. Jerka-Dziadosz, J. Beisson, F. Koll, The conserved centrosomal protein FOR20 is required for assembly of the transition zone and basal body docking at the cell surface, J. Cell Sci. 125 (2012) 4395–4404.
[28] G. Knoll, C. Braun, H. Plattner, Quenched flow analysis of exocytosis in Paramecium cells: time course, changes in membrane structure, and calcium

requirements revealed after rapid mixing and rapid freezing of intact cells, J. Cell Biol. 113 (1991) 1295–1304.
[29] H. Plattner, J. Hentschel, Sub-second cellular dynamics: time-resolved electron microscopy and functional correlation, Int. Rev. Cytol. 255 (2006) 133–176.
[30] H. Plattner, Calcium signalling in the ciliated protozoan model, Paramecium: strict signal localisation by epigenetically controlled positioning of different Ca^{2+}-release channels, Cell Calcium 57 (2015) 203–213.
[31] J.M. Aury, O. Jaillon, L. Duret, B. Noel, C. Jubin, B.M. Porcel, B. Segurens, V. Daubin, V. Anthouard, N. Aiach, O. Arnaiz, A. Billaut, J. Beisson, I. Blanc, K. Bouhouche, F. Camara, S. Duharcourt, R. Guigo, D. Gogendeau, M. Katinka, A.M. Keller, R. Kissmehl, C. Klotz, F. Koll, A. Le Mouel, G. Lepere, S. Malinsky, M. Nowacki, J.K. Nowak, H. Plattner, J. Poulain, F. Ruiz, V. Serrano, M. Zagulski, P. Dessen, M. Bétermier, J. Weissenbach, C. Scarpelli, V. Schachter, L. Sperling, E. Meyer, J. Cohen, P. Wincker, Global trends of whole-genome duplications revealed by the ciliate *Paramecium tetraurelia*, Nature 444 (2006) 171–178.
[32] J.A. Eisen, R.S. Coyne, M. Wu, D. Wu, M. Thiagarajan, J.R. Wortman, J.H. Badger, Q. Ren, P. Amedeo, K.M. Jones, L.J. Tallon, A.L. Delcher, S.L. Salzberg, J.C. Silva, B.J. Haas, W.H. Majoros, M. Farzad, J.M. Carlton, R.K. Smith Jr., J. Garg, R.E. Pearlman, K.M. Karrer, L. Sun, G. Manning, N.C. Elde, A.P. Turkewitz, D.J. Asai, D.E. Wilkes, Y. Wang, H. Cai, K. Collins, B.A. Stewart, S.R. Lee, K. Wilamowska, Z. Weinberg, W.L. Ruzzo, D. Wloga, J. Gaertig, J. Frankel, C.C. Tsao, M.A. Gorovsky, P.J. Keeling, R.F. Waller, N.J. Patron, J.M. Cherry, N.A. Stover, C.J. Krieger, C. Del Toro, H.F. Ryder, S.C. Williamson, R.A. Barbeau, E.P. Hamilton, E. Orias, Macronuclear genome sequence of the ciliate *Tetrahymena thermophila*, a model eukaryote, PLoS Biol. 4 (2006) e286.
[33] O. Arnaiz, J.F. Goût, M. Bétermier, K. Bouhouche, J. Cohen, L. Duret, A. Kapusta, E. Meyer, L. Sperling, Gene expression in a paleopolyploid: a transcriptome resource for the ciliate *Paramecium tetraurelia*, BMC Genom. 11 (2010) 547.
[34] D. Fraga, I.M. Sehring, R. Kissmehl, M. Reiss, R. Gaines, R. Hinrichsen, H. Plattner, Protein phosphatase 2B (PP2B, calcineurin) in Paramecium: partial characterization reveals that two members of the unusually large catalytic subunit family have distinct roles in calcium-dependent processes, Eukaryot. Cell 9 (2010) 1049–1063.
[35] J.J. Mackrill, Ryanodine receptor calcium release channels: an evolutionary perspective, Adv. Exp. Med. Biol. 740 (2012) 159–182.
[36] C. Erxleben, H. Plattner, Ca^{2+} release from subplasmalemmal stores as a primary event during exocytosis in Paramecium cells, J. Cell Biol. 127 (1994) 935–945.
[37] C. Erxleben, N. Klauke, M. Flötenmeyer, M.P. Blanchard, C. Braun, H. Plattner, Microdomain Ca^{2+} activation during exocytosis in Paramecium cells. Superposition of local subplasmalemmal calcium store activation by local Ca^{2+} influx, J. Cell Biol. 136 (1997) 597–607.
[38] N. Klauke, H. Plattner, Imaging of Ca^{2+} transients induced in Paramecium cells by a polyamine secretagogue, J. Cell Sci. 110 (1997) 975–983.
[39] N. Klauke, M. Blanchard, H. Plattner, Polyamine triggering of exocytosis in Paramecium involves an extracellular Ca^{2+}/(polyvalent cation)-sensing receptor, subplasmalemmal Ca-store mobilization and store-operated Ca^{2+}-influx via unspecific cation channels, J. Membr. Biol. 174 (2000) 141–156.
[40] M. Hardt, H. Plattner, Sub-second quenched-flow/X-ray microanalysis shows rapid Ca^{2+} mobilization from cortical stores paralleled by Ca^{2+} influx during synchronous exocytosis in Paramecium cells, Eur. J. Cell Biol. 79 (2000) 642–652.
[41] M.R. Husser, M. Hardt, M.P. Blanchard, J. Hentschel, N. Klauke, H. Plattner, One-way calcium spill-over during signal transduction in Paramecium cells: from the cell cortex into cilia, but not in the reverse direction, Cell Calcium 36 (2004) 349–358.
[42] G. Knoll, A. Grässle, C. Braun, W. Probst, B. Höhne-Zell, H. Plattner, A calcium influx is neither strictly associated with nor necessary for exocytotic membrane fusion in Paramecium cells, Cell Calcium 14 (1993) 173–183.
[43] H. Plattner, Membrane trafficking in protozoa: SNARE proteins, H^+-ATPase, actin, and other key players in ciliates, Int. Rev. Cell Mol. Biol. 280 (2010) 79–184.
[44] E.M. Ladenburger, H. Plattner, Calcium-release channels in Paramecium. Genomic expansion, differential positioning and partial transcriptional elimination, PLoS ONE 6 (2011) e27111.
[45] E.M. Ladenburger, I.M. Sehring, I. Korn, H. Plattner, Novel types of Ca^{2+} release channels participate in the secretory cycle of Paramecium cells, Mol. Cell. Biol. 29 (2009) 3605–3622.
[46] E.M. Ladenburger, I. Korn, N. Kasielke, T. Wassmer, H. Plattner, An Ins(1,4,5)P_3 receptor in Paramecium is associated with the osmoregulatory system, J. Cell Sci. 119 (2006) 3705–3717.
[47] J.L. Browning, D.L. Nelson, Biochemical studies of the excitable membrane of *Paramecium aurelia*. I. $^{45}Ca^{2+}$ fluxes across resting and excited membrane, Biochim. Biophys. Acta 448 (1976) 338–351.
[48] D. Kerboeuf, J. Cohen, A Ca^{2+} influx associated with exocytosis is specifically abolished in a Paramecium exocytotic mutant, J. Cell Biol. 111 (1990) 2527–2535.
[49] M.V. Wright, J.L. Van Houten, Characterization of a putative Ca^{2+}-transporting Ca^{2+}-ATPase in the pellicles of *Paramecium tetraurelia*, Biochim. Biophys. Acta 1029 (1990) 241–251.
[50] N.L. Elwess, J.L. Van Houten, Cloning molecular analysis of the plasma membrane Ca^{2+}-ATPase gene in *Paramecium tetraurelia*, J. Eukaryot. Microbiol. 44 (1997) 250–257.
[51] K. Hauser, N. Pavlovic, R. Kissmehl, H. Plattner, Molecular characterization of a sarco(endo)plasmic reticulum Ca^{2+}-ATPase gene from *Paramecium tetraurelia* and localization of its gene product to sub-plasmalemmal calcium stores, Biochem. J. 334 (1998) 31–38.
[52] H. Plattner, I.M. Sehring, C. Schilde, E.M. Ladenburger, Pharmacology of ciliated protozoa – drug (in)sensitivity and experimental drug (ab)use, Int. Rev. Cell Mol. Biol. 273 (2009) 163–218.
[53] K. Hauser, N. Pavlovic, N. Klauke, D. Geissinger, H. Plattner, Green fluorescent protein-tagged sarco(endo)plasmic reticulum Ca^{2+}-ATPase overexpression in Paramecium cells: isoforms, subcellular localization, biogenesis of cortical calcium stores and functional aspects, Mol. Microbiol. 37 (2000) 773–787.
[54] H. Plattner, N. Klauke, Calcium in ciliated protozoa: sources, regulation, and calcium-regulated cell functions, Int. Rev. Cytol. 201 (2001) 115–208.
[55] I. Mohamed, M. Husser, I. Sehring, J. Hentschel, C. Hentschel, H. Plattner, Refilling of cortical calcium stores in Paramecium cells: in situ analysis in correlation with store-operated calcium influx, Cell Calcium 34 (2003) 87–96.
[56] R. Lopreiato, M. Giacomello, E. Carafoli, The plasma membrane calcium pump: new ways to look at an old enzyme, J. Biol. Chem. 289 (2014) 10261–10268.
[57] C. Stock, H.K. GrØnlien, R.D. Allen, The ionic composition of the contractile vacuole fluid of Paramecium mirrors ion transport across the plasma membrane, Eur. J. Cell Biol. 81 (2002) 505–515.
[58] A.K. Fok, M.S. Aihara, M. Ishida, K.V. Nolta, T.L. Steck, R.D. Allen, The pegs on the decorated tubules of the contractile vacuole complex of Paramecium are proton pumps, J. Cell Sci. 108 (1995) 3163–3170.
[59] T. Wassmer, M. Froissard, H. Plattner, R. Kissmehl, J. Cohen, The vacuolar proton-ATPase plays a major role in several membrane-bounded organelles in Paramecium, J. Cell Sci. 118 (2005) 2813–2825.
[60] T. Wassmer, R. Kissmehl, J. Cohen, H. Plattner, Seventeen a-subunit isoforms of Paramecium V-ATPase provide high specialization in localization and function, Mol. Biol. Cell 17 (2006) 917–930.
[61] N. Klauke, H. Plattner, Caffeine-induced Ca^{2+} transients and exocytosis in Paramecium cells. A correlated Ca^{2+} imaging and quenched-flow/freeze-fracture analysis, J. Membr. Biol. 161 (1998) 65–81.
[62] I. Mohamed, N. Klauke, J. Hentschel, J. Cohen, H. Plattner, Functional and fluorochrome analysis of an exocytotic mutant yields evidence of store-operated Ca^{2+} influx in Paramecium, J. Membr. Biol. 187 (2002) 1–14.
[63] I.M. Sehring, C. Klotz, J. Beisson, H. Plattner, Rapid downregulation of the Ca^{2+}-signal after exocytosis stimulation in Paramecium cells: essential role of a centrin-rich filamentous cortical network, the infraciliary lattice, Cell Calcium 45 (2009) 89–97.
[64] A.W. Henkel, W. Almers, Fast steps in exocytosis and endocytosis studied by capacitance measurements in endocrine cells, Curr. Opin. Neurobiol. 6 (1996) 350–357.
[65] H. Plattner, C. Braun, J. Hentschel, Facilitation of membrane fusion during exocytosis and exocytosis-coupled endocytosis and acceleration of "ghost" detachment in Paramecium by extracellular calcium. A quenched-flow/freeze-fracture analysis, J. Membr. Biol. 158 (1997) 197–208.
[66] M.D. Bootman, K.W. Young, J.M. Young, R.B. Moreton, M.J. Berridge, Extracellular calcium concentration controls the frequency of intracellular calcium spiking independently of inositol 1,4,5-trisphosphate production in HeLa cells, Biochem. J. 314 (1996) 347–354.
[67] C. Li, B. Ullrich, J.Z. Zhang, R.G. Anderson, N. Brose, T.C. Südhof, Ca^{2+}-dependent and -independent activities of neural and non-neural synaptotagmins, Nature 375 (1995) 594–599.
[68] M. Midorikawa, Y. Okamoto, T. Sakaba, Developmental changes in Ca^{2+} channel subtypes regulating endocytosis at the calyx of Held, J. Physiol. 592 (2014) 3495–3510.
[69] R. Eckert, Bioelectric control of ciliary activity, Science 176 (1972) 473–481.
[70] D. Oertel, S.J. Schein, C. Kung, Separation of membrane currents using a Paramecium mutant, Nature 268 (1977) 120–124.
[71] K.P. Hoeflich, M. Ikura, Calmodulin in action: diversity in target recognition and activation mechanisms, Cell 108 (2002) 739–742.
[72] D. Gogendeau, C. Klotz, O. Arnaiz, A. Malinowska, M. Dadlez, N.G. de Loubresse, F. Ruiz, F. Koll, J. Beisson, Functional diversification of centrins and cell morphological complexity, J. Cell Sci. 121 (2008) 65–74.
[73] K. Kim, M. Son, J.B. Peterson, D.L. Nelson, Ca^{2+}-binding proteins of cilia and infraciliary lattice of *Paramecium tetraurelia*: their phosphorylation by purified endogenous Ca^{2+}-dependent protein kinases, J. Cell Sci. 115 (2002) 1973–1984.
[74] E.R. Chapman, Synaptotagmin: a Ca^{2+} sensor that triggers exocytosis? Nat. Rev. Mol. Cell Biol. 3 (2002) 498–508.
[75] M. Momayezi, H. Kersken, U. Gras, J. Vilmart-Seuwen, H. Plattner, Calmodulin in *Paramecium tetraurelia*: localization from the in vivo to the ultrastructural level, J. Histochem. Cytochem. 34 (1986) 1621–1638.
[76] A.K. Fok, M.S. Aihara, M. Ishida, R.D. Allen, Calmodulin localization and its effects on endocytic and phagocytic membrane trafficking in Paramecium multimicronucleatdum, J. Eukaryot. Microbiol. 55 (2008) 481–491.
[77] H. Plattner, The contractile vacuole complex of protists – new cues to function and biogenesis, Crit. Rev. Microbiol. (2013), http://dx.doi.org/10.3109/1040841X. 2013.821650.
[78] K. Gonda, K. Oami, M. Takahashi, Centrin controls the activity of the ciliary reversal-coupled voltage-gated Ca^{2+} channels Ca^{2+}-dependently, Biochem. Biophys. Res. Commun. 362 (2007) 170–176.
[79] J.E. Schultz, S. Klumpp, Cyclic nucleotides and calcium signalling in Paramecium, Adv. Sec. Mess. Phosphoprot. Res. 27 (1993) 25–46.
[80] K.S. Ann, D.L. Nelson, Protein substrates for cGMP-dependent protein phosphorylation in cilia of wild type and atalanta mutants of Paramecium, Cell Motil. Cytoskel. 30 (1995) 252–260.

[81] J.U. Linder, P. Engel, A. Reimer, T. Krüger, H. Plattner, A. Schultz, J.E. Schultz, Guanylyl cyclases with the topology of mammalian adenylyl cyclases and an N-terminal P-type ATPase-like domain in Paramecium, Tetrahymena and Plasmodium, EMBO J. 18 (1999) 4222–4232.

[82] D. Kerboeuf, A. Le Berre, J.C. Dedieu, J. Cohen, Calmodulin is essential for assembling links necessary for exocytotic membrane fusion in Paramecium, EMBO J. 12 (1993) 3385–3390.

[83] S. Quetglas, C. Iborra, N. Sasakawa, L. De Haro, K. Kumakura, K. Sato, C. Leveque, M. Seagar, Calmodulin and lipid binding to synaptobrevin regulates calcium-dependent exocytosis, EMBO J. 21 (2002) 3970–3979.

[84] H.J. Junge, J.S. Rhee, O. Jahn, F. Varoqueaux, J. Spiess, M.N. Waxham, C. Rosenmund, N. Brose, Calmodulin and Munc13 form a Ca^{2+} sensor/effector complex that controls short-term synaptic plasticity, Cell 118 (2004) 389–401.

[85] G.R. Valmonte, K. Arthur, C.M. Higgins, R.M. MacDiarmid, Calcium-dependent protein kinases in plants: evolution, expression and function, Plant Cell Physiol. 55 (2014) 551–569.

[86] K.H. Edel, J. Kudla, Increasing complexity and versatility: how the calcium signalling toolkit was shaped during plant land colonization, Cell Calcium 57 (2015) 231–246.

[87] D. Vetter, R. Kissmehl, T. Treptau, K. Hauser, J. Kellermann, H. Plattner, Molecular identification of a calcium-inhibited catalytic subunit of casein kinase type 2 from *Paramecium tetraurelia*, Eukaryot. Cell 2 (2003) 1220–1233.

[88] R. Kissmehl, T. Treptau, B. Kottwitz, H. Plattner, Occurrence of a para-nitrophenyl phosphate-phosphatase with calcineurin-like characteristics in *Paramecium tetraurelia*, Arch. Biochem. Biophys. 344 (1997) 260–270.

[89] H. Plattner, R. Kissmehl, Dense-core secretory vesicle docking and exocytotic membrane fusion in Paramecium cells, Biochim. Biophys. Acta 1641 (2003) 183–193.

[90] T. Vonderfecht, A.J. Stemm-Wolf, M. Hendershott, T.H. Giddings Jr., J.B. Meehl, M. Winey, The two domains of centrin have distinct basal body functions in Tetrahymena, Mol. Biol. Cell 22 (2011) 2221–2234.

[91] S. Nair, C. Guerra, P. Satir, A Sec7-related protein in Paramecium, FASEB J. 13 (1999) 1249–1257.

[92] J. Laoukili, E. Perret, S. Middendorp, O. Houcine, C. Guennou, F. Marano, M. Bornens, F. Tournier, Differential expression and cellular distribution of centrin isoforms during human ciliated cell differentiation in vitro, J. Cell Sci. 113 (2000) 1355–1364.

[93] S. Martens, H.T. McMahon, Mechanisms of membrane fusion: disparate players and common principles, Nat. Rev. Mol. Cell Biol. 9 (2008) 543–556.

[94] Z.P. Pang, T.C. Südhof, Cell biology of Ca^{2+}-triggered exocytosis, Curr. Opin. Cell Biol. 22 (2010) 496–505.

[95] C.E. Creutz, J.L. Tomsig, S.L. Snyder, M.C. Gautier, F. Skouri, J. Beisson, J. Cohen, The copines, a novel class of C2 domain-containing, calcium-dependent, phospholipid-binding proteins conserved from Paramecium to humans, J. Biol. Chem. 273 (1998) 1393–1402.

[96] V. Gerke, C.E. Creutz, S.E. Moss, Annexins: linking Ca^{2+} signalling to membrane dynamics, Nat. Rev. Mol. Cell Biol. 6 (2005) 449–461.

[97] M. Knochel, R. Kissmehl, J.D. Wissmann, M. Momayezi, J. Hentschel, H. Plattner, R.D. Burgoyne, Annexins in Paramecium cells. Involvement in site-specific positioning of secretory organelles, Histochem. Cell Biol. 105 (1996) 269–281.

[98] J.L. Tomsig, C.E. Creutz, Copines: a ubiquitous family of Ca^{2+}-dependent phospholipid-binding proteins, Cell. Mol. Life Sci. 59 (2002) 1467–1477.

[99] Y. Li, M. Gou, Q. Sun, J. Hua, Requirement of calcium binding, myristoylation, and protein–protein interaction for the copine BON1 function in Arabidopsis, J. Biol. Chem. 285 (2010) 29884–29891.

[100] D. Konopka-Postupolska, G. Clark, A. Hofmann, Structure, function and membrane interactions of plant annexins: an update, Plant Sci. 181 (2011) 230–241.

[101] I.B. Levitan, It is calmodulin after all! Mediator of the calcium modulation of multiple ion channels, Neuron 22 (1999) 645–648.

[102] H. Plattner, A. Habermann, R. Kissmehl, N. Klauke, I. Majoul, H.D. Söling, Differential distribution of calcium stores in Paramecium cells. Occurrence of a subplasmalemmal store with a calsequestrin-like protein, Eur. J. Cell Biol. 72 (1997) 297–306.

[103] J.H. Cho, Y.S. Oh, K.W. Park, J. Yu, K.Y. Choi, J.Y. Shin, D.H. Kim, W.J. Park, T. Hamada, H. Kagawa, E.B. Maryon, J. Bandyopadhyay, J. Ahnn, Calsequestrin, a calcium sequestering protein localized at the sarcoplasmic reticulum, is not essential for body-wall muscle function in *Caenorhabditis elegans*, J. Cell Sci. 113 (2000) 3947–3958.

[104] M. Bilinski, H. Plattner, H. Matt, Secretory protein decondensation as a distinct, Ca^{2+}-mediated event during the final steps of exocytosis in Paramecium cells, J. Cell Biol. 88 (1981) 179–188.

[105] N. Klauke, R. Kissmehl, H. Plattner, N. Haga, T. Watanabe, An exocytotic mutant of *Paramecium caudatum*: membrane fusion without secretory contents release, Cell Calcium 23 (1998) 349–360.

[106] R. Kissmehl, M. Froissard, H. Plattner, M. Momayezi, J. Cohen, NSF regulates membrane traffic along multiple pathways in Paramecium, J. Cell Sci. 115 (2002) 3935–3946.

[107] R.D. Allen, A.K. Fok, Membrane trafficking and processing in Paramecium, Int. Rev. Cytol. 198 (2000) 277–318.

[108] R.D. Allen, Y. Naitoh, Osmoregulation and contractile vacuoles of protozoa, Int. Rev. Cytol. 215 (2002) 379–394.

[109] J.E. Grover, A.F. Rope, E.S. Kaneshiro, The occurrence of biogenic calcian struvite, $(Mg,Ca)NH_4PO_4.6H_2O$, as intracellular crystals in Paramecium, J. Eukaryot. Microbiol. 44 (1997) 366–373.

[110] A.G. Bick, S.E. Calvo, V.K. Mootha, Evolutionary diversity of the mitochondrial calcium uniporter, Science 336 (2012) 886.

[111] Y. Sancak, A.L. Markhard, T. Kitami, E. Kovacs-Bogdan, K.J. Kamer, N.D. Udeshi, S.A. Carr, D. Chaudhuri, D.E. Clapham, A.A. Li, S.E. Calvo, O. Goldberger, V.K. Mootha, EMRE is an essential component of the mitochondrial calcium uniporter complex, Science 342 (2013) 1379–1382.

[112] R.S. Balaban, The role of Ca^{2+} signalling in the coordination of mitochondrial ATP production with cardiac work, Biochim. Biophys. Acta 1787 (2009) 1334–1341.

[113] T. Fenchel, Respiration in heterotrophic unicellular eukaryotic organisms, Protist 165 (2014) 485–492.

[114] H. Machemer, A. Ogura, Ionic conductances of membranes in ciliated and deciliated Paramecium, J. Physiol. 296 (1979) 49–60.

[115] A. Ogura, H. Machemer, Distribution of mechanoreceptor channels in the Paramecium surface-membrane, J. Comp. Physiol. C 135 (1980) 233–242.

[116] R. Eckert, P. Brehm, Ionic mechanisms of excitation in Paramecium, Annu. Rev. Biophys. Bioeng. 8 (1979) 353–383.

[117] P. Brehm, R. Eckert, Calcium entry leads to inactivation of calcium channel in Paramecium, Science 202 (1978) 1203–1206.

[118] R.R. Preston, Y. Saimi, C. Kung, Calcium current activated upon hyperpolarization of *Paramecium tetraurelia*, J. Gen. Physiol. 100 (1992) 233–251.

[119] C. Cárdenas, R.A. Miller, I. Smith, T. Bui, J. Molgó, M. Müller, H. Vais, K.H. Cheung, J. Yang, I. Parker, C.B. Thompson, M.J. Birnbaum, K.R. Hallows, J.K. Foskett, Essential regulation of cell bioenergetics by constitutive $InsP_3$ receptor Ca^{2+}-transfer to mitochondria, Cell 142 (2010) 270–283.

[120] T. Tominaga, R. Allen, Electrophysiology of the in situ contractile vacuole complex of Paramecium reveals its membrane dynamics and electrogenic site during osmoregulatory activity, J. Exp. Biol. 201 (1998) 451–460.

[121] H.K. GrØnlien, C. Stock, M.S. Aihara, R.D. Allen, Y. Naitoh, Relationship between the membrane potential of the contractile vacuole complex and its osmoregulatory activity in *Paramecium multimicronucleatum*, J. Exp. Biol. 205 (2002) 3261–3270.

[122] H. Plattner, R. Stürzl, H. Matt, Synchronous exocytosis in Paramecium cells. IV. Polyamino compounds as potent trigger agents for repeatable trigger-redocking cycles, Eur. J. Cell Biol. 36 (1985) 32–37.

[123] H.C. Lee, Cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP) as messengers for calcium mobilization, J. Biol. Chem. 287 (2012) 31633–31640.

[124] J.D. Fessenden, W. Feng, I.N. Pessah, P.D. Allen, Amino acid residues Gln4020 and Lys4021 of the ryanodine receptor type 1 are required for activation by 4-chloro-m-cresol, J. Biol. Chem. 281 (2006) 21022–21031.

[125] M. Wehner, H. Rueffert, F. Koenig, C.D. Meinecke, D. Olthoff, The Ile2453Thr mutation in the ryanodine receptor gene 1 is associated with facilitated calcium release from sarcoplasmic reticulum by 4-chloro-m-cresol in human myotubes, Cell Calcium 34 (2003) 163–168.

[126] C.W. Taylor, I.C. Marshall, Calcium and inositol 1,4,5-trisphosphate receptors: a complex relationship, Trends Biochem. Sci. 17 (1992) 403–407.

[127] X.L. Zhou, C.W. Chan, Y. Saimi, C. Kung, Functional reconstitution of ion channels from Paramecium cortex into artificial liposomes, J. Membr. Biol. 144 (1995) 199–208.

[128] K. Hamada, K. Mikoshiba, Revisiting channel allostery: a coherent mechanism in IP_3 and ryanodine receptors, Sci. Signal. 5 (2012) pe24.

[129] F.J. Amador, P.B. Stathopulos, M. Enomoto, M. Ikura, Ryanodine receptor calcium release channels: lessons from structure-function studies, FEBS J. 280 (2013) 5456–5470.

[130] S. Ramachandran, A. Chakraborty, L. Xu, Y. Mei, M. Samsó, N.V. Dokholyan, G. Meissner, Structural determinants of skeletal muscle ryanodine receptor gating, J. Biol. Chem. 288 (2013) 6154–6165.

[131] (a) A.C. Gomez, N. Yamaguchi, Two regions of the ryanodine receptor calcium channel are involved in Ca^{2+}-dependent inactivation, Biochemistry 53 (2014) 1373–1379;
(b) R. Zalk, O.B. Clark, A. des Georges, R.A. Grassucci, S. Reiken, F. Mancia, W.A. Hendickson, J. Frank, A.R. Marks, Structure of a mammalian ryanodine receptor, Nature (2014), http://dx.doi.org/10.1038/nature13950.

[132] D.F. Boehning, Molecular architecture of the inositol 1,4,5-trisphosphate receptor pore, Curr. Top. Membr. 66C (2010) 191–207.

[133] H. Plattner, A. Verkhratsky, Ca^{2+} signalling early in evolution – all but primitive, J. Cell Sci. 126 (2013) 2141–2150.

[134] S.R. Collins, T. Meyer, Evolutionary origins of STIM1 and STIM2 within ancient Ca^{2+} signalling systems, Trends Cell Biol. 21 (2011) 202–211.

[135] G. Huang, P.J. Bartlett, A.P. Thomas, S.N. Moreno, R. Docampo, Acidocalcisomes of *Trypanosoma brucei* have an inositol 1,4,5-trisphosphate receptor that is required for growth and infectivity, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 1887–1892.

[136] M. Hashimoto, M. Enomoto, J. Morales, N. Kurebayashi, T. Sakurai, T. Hashimoto, T. Nara, K. Mikoshiba, Inositol 1,4,5-trisphosphate receptor regulates replication, differentiation, infectivity and virulence of the parasitic protist *Trypanosoma cruzi*, Mol. Microbiol. 87 (2013) 1133–1150.

[137] X. Cai, X. Wang, S. Patel, D.E. Clapham, Insights into the early evolution of animal calcium signalling machinery: a unicellular point of view, Cell Calcium 57 (2015) 166–173.

[138] A. Galione, A.M. Evans, J. Ma, J. Parrington, A. Arredouani, X. Cheng, M.X. Zhu, The acid test: the discovery of two-pore channels (TPCs) as NAADP-gated endolysosomal Ca^{2+} release channels, Eur. J. Physiol. 458 (2009) 869–876.
[139] Patel S., Cai X., Evolution of acid Ca^{2+} stores and their resident Ca^{2+}-permeable channels, Cell Calcium 57 (2015) 222–230.
[140] A. Haddad, G.R. Bowman, A.P. Turkewitz, New class of cargo protein in *Tetrahymena thermophila* dense core secretory granules, Eukaryot. Cell 1 (2002) 583–593.
[141] T. Wassmer, I.M. Sehring, R. Kissmehl, H. Plattner, The V-ATPase in Paramecium: functional specialization by multiple gene isoforms, Eur. J. Physiol. 457 (2009) 599–607.
[142] G. Knoll, B. Haacke-Bell, H. Plattner, Local trichocyst exocytosis provides an efficient escape mechanism for Paramecium cells, Eur. J. Protistol. 27 (1991) 381–385.