Calcium signalling in the ciliated protozoan model, *Paramecium*: Strict signal localisation by epigenetically controlled positioning of different Ca^{2+} -channels

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ABSTRACT

The *Paramecium tetraurelia* cell is highly organised, with regularly spaced elements pertinent to Ca^{2+} signalling under epigenetic control. Vesicles serving as stationary Ca^{2+} stores or undergoing trafficking contain Ca^{2+} -release channels (PtCRCs) which, according to sequence and domain comparison, are related either to inositol 1,4,5-trisphosphate (InsP_3) receptors (IP_3R) or to ryanodine receptor-like proteins (RyR-LP) or to both, with intermediate characteristics or deviation from conventional domain structure. Six groups of such PtCRCs have been found. The ryanodine- InsP_3 -receptor homology (RIH) domain is not always recognisable, in contrast to the channel domain with six trans-membrane domains and the pore between transmembrane domain 5 and 6. Two CRC subtypes tested more closely, PtCRC-II and PtCRC-IV, with and without an InsP_3 -binding domain, reacted to InsP_3 and to caffeine, respectively, and hence represent IP_3Rs and RyR-LPs. IP_3Rs occur in the contractile vacuole complex where they allow for stochastic constitutive Ca^{2+} reflux into the cytosol. RyR-LPs are localised to cortical Ca^{2+} stores; they are engaged in dense core-secretory vesicle exocytosis by Ca^{2+} release, superimposed by Ca^{2+} -influx via non-ciliary Ca^{2+} -channels. One or two different types of PtCRCs also occur in other vesicles undergoing trafficking. Since the PtCRCs described combine different features they are considered derivatives of primitive precursors. The highly regular, epigenetically controlled design of a *Paramecium* cell allows it to make Ca^{2+} available very locally, in a most efficient way, along predetermined trafficking pathways, including regulation of exocytosis, endocytosis, phagocytosis and recycling phenomena. The activity of cilia is also regulated by Ca^{2+} , yet independently from any CRCs, by de- and hyperpolarisation of the cell membrane potential.

1. Introduction – *Paramecium*, a highly organised cell under epigenetic control

The ciliated protozoan, *Paramecium tetraurelia*, is a highly organised cell with a regular arrangement of organelles over almost the entire cell surface [1,2], as documented by R.D. Allen at <http://www.5.pbrc.hawaii.edu/allen/> [3] and outlined in Fig. 1. This includes cilia, dense core-secretory organelle (trichocyst) docking sites, cortical Ca^{2+} stores (alveolar sacs), clathrin-coated endocytosis sites (parasomal sacs) located laterally to ciliary bases, and early endosomes (terminal cisternae) located below basal bodies. They constitute repetitive units of the surface area, the kinetids. Each kinetid harbours one cilium (or two in a small area), a parasomal sac, a terminal cisterna, a trichocyst and an alveolar sac (underlying half part of two adjacent kinetids); the size of a kinetid is

~2.0 $\mu\text{m} \times 1.7 \mu\text{m}$ [4], and there are fixed distances for each of the respective substructures.

This regular cortex structure is under epigenetic control in *Paramecium* [5,6] and in *Tetrahymena* [7,8], as became evident from the transgenerational transmission of inverted cortex structure after incomplete separation of conjugants or after microsurgery. In addition, there are some other duplicate or single structures located at invariable sites: two contractile vacuole complexes, serving for the regulation of ion and water homeostasis, are attached to specific sites of the dorsal cell membrane. The site of phagosome formation, i.e. the oral cavity (with cytostome and cytopharynx), as well as the site of release of spent food vacuoles (phago[lyso]somes), the cytoproct, are both located at the ventral side, also at predetermined sites, as is the trafficking route for vesicles recycling from the cytoproct to the cytopharynx [1,9,10].

Clearly the basic principles of Ca^{2+} signalling have been developed already early in the evolution of the eukaryotic cell [11,12] and, thus, also in ciliated protozoa such as *Paramecium* [13]. The evolution of ciliated protozoa started about one billion years ago

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Fig. 1. Epigenetically controlled regular arrangement of surface components in a *Paramecium* cell. Note the regular arrangement of cilia, calcium stores (alveolar sacs), endocytosis sites (parasomal sacs) and early endosomes (terminal cisternae) in the cell cortex. In addition, note the fixed arrangement of trafficking pathways for recycling vesicles from the cytoproct to the oral cavity after release of the contents of spent food vacuoles (phago[lyso]somes). This route is supported by microtubular arrays (postoral fibres). For further explanations, see text.

[14–16], i.e. around the time of radiation of multicellular organisms. Ca^{2+} signalling occurs in protozoa of both groups, unikonts (Amorphea, including myxamoebae such as *Dictyostelium*) and bikonts (Stramenopila, including ciliates such as *Paramecium*). The first line of evolution led to metazoa, the second line of phylogeny culminates in green plants [17,18]. In fact, most data available for Ca^{2+} signalling in protozoa are from *Dictyostelium* and *Paramecium*, respectively.

Epigenetic control, particularly of cell surface pattern formation, is a salient feature of ciliates. Currently particularly this feature makes this group an interesting model for cell biologists [19,20]. On this basis we now envisage two main aspects. First, what is the spectrum of Ca^{2+} signalling mechanisms in ciliated protozoa? Second, to what extent may their regular, epigenetically controlled structural design be of any relevance for efficient Ca^{2+} signalling? In this context we shall briefly address plasmalemmal Ca^{2+} -channels and then focus on intracellular Ca^{2+} -release channels (CRC).

2. Regular surface pattern of the *Paramecium* cell and potential advantages for Ca^{2+} signalling – background information

As seen in an electron micrograph obtained from cell cortex fragments, isolated from *P. tetraurelia* cells and negatively stained (Fig. 2), ciliary basal bodies are arranged in regular intervals, as are docking sites for trichocysts. Alveolar sacs are closely attached, side-by-side, to the cell membrane, also in a regular pattern. These very flat sacs represent cortical Ca^{2+} stores [21] from where Ca^{2+} is mobilised during exocytosis stimulation, but not during any kind of ciliary activity [13,22]. Kinetids are not only distinct structural, but also functional subunits. The ~3000 kinetids per cell [4] are arranged in longitudinal rows, the kineties.

Which consequences does this regular design entail for Ca^{2+} signalling? First, the cell can activate, for instance, exocytosis sites individually or over large areas, thus producing a considerable amplification and synchronisation [23]. Second, the regular arrangement of cilia is prerequisite to their functional coupling during normal beat whereby collectively the beat pattern takes place in metachronal waves [24]. Even normal beat requires a basal $[\text{Ca}^{2+}]$ and its increase accelerates beat frequency [25]. Based on specific Ca^{2+} signals, in response to specific exogenous signals, ciliary activity can be synchronously altered. This includes mechanical or chemical stimuli as well as depolarisation or hyperpolarisation of the membrane potential [26].

Two specific sites serve for the attachment of a pair of contractile vacuole complexes and these contractile complexes themselves are also sites of intense Ca^{2+} signalling [27]. In addition, the cell evidently requires Ca^{2+} signals at the oral cavity (cytostome and cytopharynx) for phagosome/food vacuole formation by vesicle delivery, as well as at the cytoproct for vesicle recycling after the release of undigested materials (Fig. 1), as one may derive from the occurrence of CRCs in the vesicles associated with the cytopharynx and the cytoproct [28]. This is also true for parasomal sacs which, together with terminal cisternae, are at the beginning of an endocytotic vesicle trafficking pathway; as mentioned, both structures are firmly installed under epigenetic control at fixed positions. In metazoan cells endocytosis also depends on Ca^{2+} [29,30].

Until the 19 hundred nineties, analysis of Ca^{2+} signalling in ciliates was restricted to ciliary activity, based mainly on electrophysiology of the *Paramecium* cell. Comparison of deciliated cells with normal control cells enabled the localisation and characterisation of many ion channels in the ciliary and non-ciliary (somatic) cell membrane [26]; see Fig. 3(A and B). In fact, channels determining ciliary activity exhibit a bimodal distribution. As outlined below, those giving the command (receptor potential) for changing beat direction are positioned separate from those executing the command (action potential) [26]. Many of them are sensitive to the intracellular free Ca^{2+} concentration $[\text{Ca}^{2+}]_i$, just adjacent to the cell membrane, or rather to a Ca^{2+} /calmodulin complex [26,31–33], and they may conduct Ca^{2+} themselves. A receptor potential is formed upon de- or hyperpolarisation, depending on the type of mechanosensitive channels activated. These are Ca^{2+} -influx channels, and K^{+} -efflux channels, respectively, and, in consequence, some additional channels are activated [26,31,34]. Each of these channel types is differentially localised; those initiating de- and hyperpolarisation are arranged in the somatic cell membrane in a gradient rising from the mid of the cell to the anterior and posterior apex, respectively (Fig. 3). See Refs. [26,31–33] for detailed reviews on electrophysiology and ref. [35] for partial molecular characterisation of some of these channels.

Thus, the receptor potential generated by the activation of the respective channels in the somatic cell membrane leads to an activation potential and a defined behavioural response. This implies the reversal of the ciliary beat direction (ciliary reversal) and accelerated forward swimming, respectively, depending on whether the cell has been de- or hyperpolarized. Following depolarisation, ciliary reversal is activated by Ca^{2+} -influx into cilia via voltage-dependent Ca^{2+} -channels which are restricted to the

Fig. 2. Electron micrograph of an isolated *Paramecium* cell cortex fragment visualised by negative staining. Note the regular arrangement of unit surface fields (kinetids), arranged in longitudinal lines (kineties) within an egg-case type cell surface relief. It displays cilia (bb = basal bodies) alternating with trichocyst attachment sites (t). Along the bb/t line adjacent alveolar sacs are seen, with borders between arrowheads. Note that cilia and trichocysts have been removed during isolation of cell cortex fragments. Unpublished micrograph from the series published by Bilinski et al. [100]. Scale bar = 1 μ m.

ciliary membrane [36]; see Fig. 3(A and B). This reaction helps the cell to avoid struggling with obstacles and spoiling energy, before it resumes normal forward swimming. Alternatively, enhanced forward swimming enables the cell to follow molecular traces of bacterial food, such as folic acid and biotin in a positive chemore-sponse/chemotaxis [37–39]. Similarly ciliary reversal is also the basis of negative chemoresponses/chemotaxis. Both, positive and negative chemotaxis are regulated by Ca^{2+} , though in different ways, by different ion conductances. Such pile-up to a synchronous response is enabled by the regular arrangement of cilia. Clearly epigenetically determined spacing of cilia is mandatory not only for normal coordinated beat activity but also for coordinated ciliary responses “upon command”. Positioning of cilia in a precise pattern and spacing is of evolutionary advantage as it allows for hydrodynamic coupling of the ciliary response by adhesive forces [24].

In *P. tetraurelia*, Ca^{2+} stores containing Ca^{2+} -release channels (PtCRCs) do not contribute to ciliary beat activity. They are not directly amenable to electrophysiology and they have received attention only quite recently. They are, in part, also arranged in regular patterns to optimise signalling, as to be discussed below.

3. Types of Ca^{2+} -channels in *Paramecium* and their subcellular distribution

Fig. 4(A–C) outlines the types of PtCRCs which are related to IP_3 Rs and RyRs according to overall similarity, domain analysis, subcellular localisation, analysis of the pore domain contained in its transmembrane region etc., as described in detail previously [28]. Eventually this was supported by functional analysis including gene silencing, as well as by biochemical and Ca^{2+} fluorochrome analysis [40,41]. We found a total of 39 of such channels, grouped in 6 subfamilies.

The activation mechanism has been experimentally determined only with PtCRC-II and PtCRC-IV. PtCRC-II binds the correct stereoisomer of InsP_3 , Ins 1,4,5-trisphosphate [28]; modelling of its InsP_3 -binding domain largely coincides with that of mammalian homologues. In situ activation of caged InsP_3 changes pattern of Ca^{2+} signals which constitutively/spontaneously emerge along the contractile vacuole complex. In contrast, PtCRC-IV is activated by the RyR agonists, caffeine and 4-chloro-*meta*-cresol [41]. Because of some deviation from classical RyRs familiar from metazoans, they should better be called RyR-LPs. Any other types of the PtCRCs we found await scrutiny.

Applying up-to-date programmes we determined consistently 6 transmembrane domains in the carboxy-terminal region in all PtCRC sequences and a selectivity filter with the aminoacid sequences Gly-Val-Gly-Asp and Gly-Ile-Gly-Asp [28], as known from IP_3 Rs and RyRs in metazoans, but with some deviations, as will be discussed below.

4. Functional and evolutionary implications and comparison with other systems

During evolution, the arrangement of cilia and their Ca^{2+} dynamics has changed. For instance, in metazoans one finds no comparable regularity of the surface pattern, including the arrangement of cilia. Hypothetically, during evolution of ciliated protozoa, the regular arrangement of cilia may have served as a scaffold for the coassembly of additional elements that now form a kinetid. In one way or another all structures composing a kinetid are involved in Ca^{2+} signalling; for instance, as documented in Fig. 5, parasomal sacs contain an unexpectedly high number of CRCs, type PtCRC-V-4 with an InsP_3 -binding domain [28]. The functional significance of these very abundant channels is as uncertain as that of the extremely small numbers, less than three, of IP_3 Rs detected in the cell membrane of some mammalian cells [42]. In *Paramecium*, these channels could account for the ill defined non-selective, high conductance channels responsible for leaking conductances registered by electrophysiologists [26]. Such channels may provide a *Paramecium* cell with sufficient Ca^{2+} under natural conditions, although under laboratory conditions the opposite, i.e. elimination of an excess of Ca^{2+} , appears more significant.

The highly organised cell surface pattern can mediate a clear local response to a local stimulus, e.g. a secretagogue. Stimulation of larger areas causes a pile-up of synchronous responses [23,43]. Nevertheless, differential Ca^{2+} responses can activate selectively one or another activity in the *Paramecium* cell cortex. An example is the Ca^{2+} signal that triggers a ciliary reversal response without triggering trichocyst exocytosis at the same time.

In each case it is not only functionally, but also energetically favourable to have the source and the respective target of Ca^{2+} in close proximity, particularly also because the signal and its efficiency decay very rapidly with distance [44,45]. Possible epigenetic mechanisms mediating such strict confinement of signalling have been discussed somewhere else [20]. There are good reasons to speculate that Ca^{2+} may even be part of the epigenetic signals leading to the formation of the regular cell surface pattern [27].

Fig. 3. Cation-channels relevant for ciliary activity in *Paramecium*. (A) Channels pertinent to the regulation of ciliary activity. Note anterior enrichment of mechanosensitive depolarisation channels and posterior enrichment of hyperpolarisation channels (see text). They are both restricted to the somatic (non-ciliary) cell membrane, each type with a gradient evanescent towards the middle of the cell. Also note restriction of voltage-dependent Ca^{2+} -influx channels to ciliary membranes. Cortical localisation of centrin, including basal regions of cilia, is also important for Ca^{2+} signalling (for details, see text). The occurrence of a PMCA-type Ca^{2+} -ATPase/pump in the somatic cell membrane is certain, but unsettled for the ciliary membrane. (B) Scheme of a cilium summarising Ca^{2+} fluxes pertinent to ciliary reversal. Note distribution of voltage-dependent Ca^{2+} -channels over the entire cilium and “trapping” of Ca^{2+} by centrin located at and near the ciliary basis. Again the occurrence of a PMCA-type Ca^{2+} -ATPase/pump for the somatic cell membrane has been ascertained, but is unsettled for the ciliary membrane. Note the absence of Ca^{2+} spill-over into the soma and the fact that alveolar sacs do not normally contribute to the regulation of ciliary activity during ciliary reversal (for exceptions, see text).

4.1. Cilia

For enduring swimmers, such as ciliates, it is important that spacing of cilia is appropriate to guarantee hydrodynamic coupling in metachronal waves [24]. It is also important to draw consequences when running against an obstacle and ciliary reversal serves just for this purpose. How has this mechanism been optimised?

Voltage-dependent Ca^{2+} -channels also regulate the activity of cilia in ciliates other than *Paramecium* [26] and in ctenophores [46], but not in higher metazoans. For instance, Ca^{2+} -channels

responsible for the activity of the primary cilium in mammalian cells are functionally different and not restricted to the cilium [47].

As mentioned, in *Paramecium* voltage-dependent Ca^{2+} -influx channels are restricted to the ciliary membrane [36]; see Fig. 3(A and B). Restriction of the Ca^{2+} signal guarantees a maximal effect for signalling specifically within the cilium and, therefore, is economic. The number of Ca^{2+} ions required to mediate ciliary reversal has been estimated as ~ 700 by energy dispersive X-ray microanalysis [48], whereas somewhat higher or lower values have been derived from electrophysiology, depending on the conditions [49,50]. Spillover deeper into the cell soma is avoided by binding

Fig. 4. Distribution of PtCRCs in the *Paramecium* cell. In figs. A–C, roman numbers indicate the PtCRC subfamily, arabic numbers added to the PtCRC channel designation indicate subtypes experimentally localised by antibodies and/or GFP fusion. Note that, beyond the channel domain, the occurrence of some other domains, such as the RIH domain and the InsP₃-binding domain, is quite variable. Vesicle transport is outlined by arrows. (A) Distribution of PtCRCs along the secretory pathway. Note the occurrence of two different channel types, PtCRC-I and PtCRC-IV, in the endoplasmic reticulum, whereas no PtCRCs have been recognised so far in the Golgi apparatus and in trichocysts. One of these channels, PtCRC-IV-1, also occurs in alveolar sacs where it has been identified as a RyR-LP and as an initiator of Ca^{2+} signalling, during exocytosis stimulation, in a SOC-type mechanism. Alveolar sacs also contain a channel of type PtCRC-V-4. Its function, specifically the significance of its InsP₃-binding domain, is unknown. (B) Distribution of PtCRCs along the endocytotic and phagocytotic pathway. All stages of food vacuoles/phago(lyso)somes are equipped with PtCRC-III, as are different sorts of recycling vesicles. These originate in part from the cytoproct and in part from advanced stages of food vacuoles during their migration through the cell (cyclosis). Remarkably parasomal sacs (coated pits) contain a PtCRC of a different type (PtCRC-V) and remarkably this is different from that seen in terminal cisternae (early endosomes), i.e. PtCRC-VI which is a rather short molecule. For details, see text. (C) PtCRCs in the contractile vacuole complex. In peripheral parts its smooth spongione contains PtCRC-II and PtCRC-V, whereas PtCRC-VI-2 and PtCRC-VI-3 are restricted to the small region at the cell surface containing the exocytosis site (“pore”) for the periodic release of vacuole fluid. This may be facilitated by the nearby PtCRC-VI channels, whereas PtCRC-II has been specified as a genuine IP₃R serving for reflux of some of the Ca^{2+} that has been sequestered and which is predominantly released by the organelle. The occurrence of a PMCA in the organelle is uncertain.

to calmodulin and centrin. This occurs still in the cilium where centrin activates the voltage-dependent Ca^{2+} -influx channels [51]. Also in the cilia of ciliates, Ca^{2+} /calmodulin exerts a negative feedback on Ca^{2+} -influx channels and, thus, terminates Ca^{2+} -influx [52]. Not only the depolarisation [32,33], but also the hyperpolarisation response is based on Ca^{2+} and calmodulin by regulating channel conductances [38,39].

It was the *Paramecium* cell where termination of the depolarisation response by formation of a Ca^{2+} /calmodulin complex has been detected [52]. Remarkably, the on/off switch of voltage dependent Ca^{2+} -channels by a Ca^{2+} /calmodulin complex in the ciliary membrane is conserved up to pyramidal cells of the mammalian brain [53] where they serve for long-term potentiation (learning). In ciliates, the intraciliary Ca^{2+} signal does normally not spill into the cell soma [48] but vanishes by binding to centrin contained in the cell cortex [54]; here, centrin mainly contributes to the formation of a regularly arranged filamentous cortical network, the infraciliary lattice [55]. Centrin is endowed, also in ciliates, not only with EF-hand high affinity Ca^{2+} binding loops [56,57], but also with acidic aminoacid clusters for high capacity Ca^{2+} binding [56]. Presence of a plasmamembrane Ca^{2+} -ATPase (PMCA, Ca^{2+} pump) is ascertained for the somatic (non-ciliary) cell membrane [58], its occurrence in cilia, though likely, is still under debate [59]. Its activity, like that of the SERCA pump of alveolar sacs [60], may be much less efficient than the secondary active Ca^{2+} transport coupled to H^{+} -ATPase activity in other parts of the cell [27,40].

A K^{+} current carries the hyperpolarization-induced receptor potential [61] and then highly Ca^{2+} -selective hyperpolarization-activated channels in the somatic membrane are activated. They peak within one ciliary stroke, i.e. within 20–80 ms [62]. In total it looks as if ciliary reversal, thanks to the more distinct localisation of the underlying Ca^{2+} -channels, is more precisely regulated at the organelle level than enhanced forward swimming – escape from an attack being more important than feeding.

4.2. Exocytosis sites for trichocysts

These sites, characterised in Fig. 6, are also regularly arranged (Figs. 1 and 2). In freeze-fractures they are highly organised, with particle pattern [63] whose molecular identity has not been clearly elucidated as yet. According to gene silencing experiments they must dispose of SNARE proteins for membrane fusion to occur [64,65], as well as of a sensor responding to the $[\text{Ca}^{2+}]_i$ increase that is also mandatory for exocytotic membrane fusion [22]. However, the Ca^{2+} sensor still requires identification. Low capacity/high sensitivity sensors of the type known from metazoans, i.e. synaptotagmin-type molecules with two C2 domains, are not found in the *Paramecium* database [65]. Here, proteins with eight, rather than two C2-domains are found. They thus resemble another type of Ca^{2+} sensors, the “extended synaptotagmin-LPs” (E-Syts) [66,67] that substitute for synaptotagmin in some mammalian cells.

Fig. 5. Immuno-localisation of *PtCRC-V-4* to coated pits (parasomal sacs). From Ladenburger and Plattner [28]. (A–D) Confocal laser scanning microscopy of immune-fluorescence localisation of *PtCRC-V-4* channels using monospecific antibodies (green) together with antibodies against α -tubulin (red). To be compared with Figs. 1 and 4B for gross orientation. (A and C) Surface view with red and green signal in close association because parasomal sacs are closely associated with ciliary basal bodies (bb). Note absence of green label from the pore (p) of the contractile vacuole complex. (B and D) median view, again with close association of red and green signal. Moreover, the oral apparatus (oa) is flanked by green label indicating presence of *PtCRC-V-4*. Bars = 10 μ m. (E) Electron microscopic immuno-gold (arrows) localisation of *PtCRC-V-4* at parasomal sacs in cross-section (left), oblique section (middle) and longitudinal/median section (right); pm = plasmamembrane. Bar = 0.1 μ m.

In *Paramecium*, exocytosis is paralleled by a cortical $[\text{Ca}^{2+}]_i$ increase, as has been analysed by fluorochrome analysis [68], energy-dispersive X-ray microanalysis [69] and by electrophysiology [4]. In this context, none of the ciliary membrane channels play a role. During exocytosis stimulation Ca^{2+} /calmodulin activated currents at the somatic cell membrane have been recorded to monitor Ca^{2+} signals as close to the cell membrane as possible. The duration of one single current event accompanying one exocytosis event was ~ 50 ms, $t_{1/2} = 21$ ms. This corresponds to the analysis time which was available also for fast confocal fluorochrome analysis and for quenched-flow/X-ray microanalysis. With $[\text{Ca}^{2+}]_o$ reduced to slightly below $[\text{Ca}^{2+}]_i$ at rest, the signal is weak and causes only a small extent of trichocyst release. All three methods converged in the conclusion that the activation mechanism underlying Ca^{2+}

signalling during trichocyst exocytosis involves a store-operated Ca^{2+} -influx, SOC [22,23]. A full exocytotic response requires both, Ca^{2+} release in a first step, influx as a second step and another release as the third step (Fig. 6), all steps being very tightly coupled.

Since the explosive discharge of trichocyst contents by decondensation from a carrot- to a needle-shape, also depends on extracellular Ca^{2+} [70], experiments were repeated with mutants devoid of any Ca^{2+} -influx in presence of normal $[\text{Ca}^{2+}]_o$ [71]. A Ca^{2+} -induced Ca^{2+} release, CICR, could thus be excluded. Evidently SOC may be older than CICR. This is supported by the following experiments.

Ca^{2+} release from alveolar sacs as a first step, before additional Ca^{2+} entry, can be seen also in energy-dispersive X-ray microanalysis, but only when $[\text{Ca}^{2+}]_o$ is reduced to near resting $[\text{Ca}^{2+}]_i$ levels

Fig. 6. Trichocyst docking site flanked by alveolar sacs tightly attached to the cell membrane. Alveolar sacs contain PtCRC-IV channels which have been identified as RyR-LPs. Upon exocytosis stimulation, Ca²⁺ is first released from alveolar sacs by the PtCRC-IV channels (step 1), followed by a store-operated Ca²⁺-influx (SOC) into the sacs (step 2), and a second wave of Ca²⁺ release from alveolar sacs (step 3). Sequences 1–3 of Ca²⁺ fluxes are tightly coupled in time and, thus, can be ascertained only with special techniques indicated in the text. Channels type PtCRC-V-4 in alveolar sacs, as indicated in Fig. 4A, are of unknown function and, therefore, omitted here. Note the occurrence of SERCA in domains facing the cell centre, opposite to RyR-LPs/PtCRC-IV channels whose arrangement guarantees a Ca²⁺ flux directed to exocytosis sites.

and when Ca²⁺ in the medium is rapidly exchanged for Sr²⁺ during stimulation in quenched flow/cryofixation/X-ray microanalysis experiments [69]. The latter experiments show Sr²⁺ in alveolar sacs, whereas the Ca²⁺ signal in the sacs has vanished. How such rapid entry of Ca²⁺ (or of Sr²⁺ in the substitution experiments) into alveolar sacs is possible, to make Ca²⁺ depletion unrecognisable under standard conditions, is not known, particularly since reuptake by the SERCA pump is unexpectedly slow [72]. Later on a similar phenomenon, called “direct mass-action mechanism” has also been reported for a chicken lymphocyte cell line [73].

In *Paramecium*, both, endogenous and exogenous Ca²⁺ is spilled over exocytosis sites (Fig. 6) and, thus, maximal stimulation of exocytosis is achieved. In addition, increased [Ca²⁺]_o during stimulation also accelerates resealing of exocytosis sites (exocytosis-coupled endocytosis) and internalisation of empty “ghosts” [63].

The CRCs involved in Ca²⁺ release from alveolar sacs during exocytosis are of type PtCRC-IV. They have structural characteristics of RyR-LPs and are activated not only by the RyR agonists, caffeine and 4-chloro-*meta*-cresol, but, in addition, also by the polyamine secretagogue, aminoethyl dextrane, AED [41]. Ca²⁺ is spilled over exocytosis sites, where it has to reach a local concentration of ~5 μM, as determined by Ca²⁺ chelators of different affinity [68]. This construction principle for a site-directed Ca²⁺ flow within the narrow space between the plasmamembrane and a cortical Ca²⁺ store (alveolar sacs in ciliates) is found not only in ciliates, but also in some metazoan cells up to mammals. However, molecules communicating store emptying and mediating Ca²⁺-influx in a SOC-type mechanism, such as Stim and Orai [74], have not been found in ciliates, neither by us [75] nor by others [76]. Thus, the molecular basis of this part of Ca²⁺ signalling in ciliates remains open for the time being. Currently several alternative molecules are discussed for linking cortical compartments to the cell membrane, as outlined previously [75]. In addition Giordano et al. [77] have assigned such a role to synaptotagmin-LPs whose presence in *Paramecium* has been mentioned above. It may well be that Orai/Stim are used for dynamically rearranged cortical Ca²⁺ stores, in contrast to stationary cortical stores, such as alveolar sacs.

In essence, a site-directed Ca²⁺ flow is required to achieve trichocyst exocytosis. The regular arrangement of relevant components (Figs. 1, 2 and 6) appears mandatory. Trichocyst exocytosis sites somehow recall a skeletal muscle cell because of the tight linkage of a subplasmalemmal Ca²⁺ store (terminal cisternae of the

sarcoplasmic reticulum) to the cell membrane. It also recalls the end plate of a motor neuron, considering the regular arrangement of intramembranous particles representing Ca²⁺-influx channels in close association with exocytosis sites [78]. With this regard it would be desirable to know the signification of the regular particle arrays seen in freeze-fractures at trichocyst exocytosis sites as well as of their dynamic rearrangement during exocytotic membrane fusion [23,65]. What is the relation between the structural organisation and Ca²⁺ signalling?

The flood of Ca²⁺ over trichocyst exocytosis sites can be calculated from the values determined by X-ray microanalysis. If it were restricted to the subplasmalemmal space it would exceed by several orders of magnitude the [Ca²⁺]_i actually needed (when available in free form) at exocytosis sites [22]. This situation, i.e. the requirement of an unexpectedly strong Ca²⁺-flux, strikingly resembles that in endocrine cells [79]. In chromaffin cells the actual distance of Ca²⁺-influx channels from the potential fusion site of a docked dense core-vesicle determines actual fusion capacity [45] and only a fraction of chromaffin granules attached to the cell membrane is released upon depolarisation [80]. Just like in motor end plates and in nerve terminals [81], ciliated protozoan cells have optimised this principle already early on by epigenetically determined coassembly of both, Ca²⁺ signal generation and membrane fusion sites as targets. Consider that strict coordination between local Ca²⁺ signalling and local trichocyst exocytosis is vital, as it is mandatory for warding off with maximal efficiency the deadly attacks by predatory cells [82,83].

So far unknown is the function of a second CRC type, PtCRC-V-4, also in alveolar sacs, where these channels are enriched near contact sites between adjacent sacs [28]. Unexpectedly PtCRC-V-4 contains an InsP₃-binding domain. Particularly intriguing is also the occurrence of these CRCs in parasomal sacs (see below).

Even though the calcium leakage rate determined with ⁴⁵Ca²⁺ is rather high in unstimulated *Paramecium* cells [84,85] spontaneous trichocyst release is relatively scarce [43]. No trichocyst exocytosis takes place when [Ca²⁺]_i in the medium is suddenly increased in the medium, even though this yields a strong overall fluorochrome signal [4]. The same is true of Ca²⁺ injection [22,68]. All this supports the concept of strictly localised microdomain Ca²⁺ signalling, based on co-assembly of components, including Ca²⁺-channels.

4.3. Endoplasmic reticulum

The endoplasmic reticulum contains not only PtCRC-I-1, but also PtCRC-IV-1 type channels [28]. The first type contains an InsP₃-binding domain, in contrast to the second type (Fig. 4A). Since the latter are also present in alveolar sacs, where they represent the main type of CRCs (see above), this supports the hypothesis that alveolar sacs are formed by vesicle transport from the endoplasmic reticulum. This pathway has been found by in vivo-tracing of the transport of green fluorescent protein-labelled SERCA molecules [86]. The same can be derived from studies where vesicle delivery has been suppressed by silencing of the SNARE-specific chaperone, N-ethylmaleimide sensitive factor, NSF [87]. This transport must encompass a selective step since the endoplasmic reticulum contains a calreticulin-LP, whereas the alveolar sacs are endowed with a calsequestrin-LP [88]. This distinction was achieved by immunocytochemistry using distinct antibodies selective for either one of these luminal high capacity/low affinity Ca²⁺-binding proteins. This finding also underscores the similarity of the alveolar sacs with the sarcoplasmic reticulum and, moreover, indicates selective transport for coassembly of proper components in specific organelles that are relevant for Ca²⁺ signalling.

Fig. 7. Confocal laser scanning microscopy of immune-fluorescence localisation of *PtCRC-III-4* channels using monospecific antibodies (green) together with antibodies against α -tubulin (red). Phagosomes are black due to filling with Indian ink. To be compared with Fig. 4B. From Ladenburger and Plattner [28]. Bars = 10 μ m. (A–A' and B) Tubulin staining outlines the trafficking avenue along postoral fibres (pof), between the cytoproct and the oral apparatus (oa) whose cilia are also stained, as are cilia on the cell surface and the outlines of the contractile vacuole complex (cvc). Confocal planes (z) and pile-up (z1–z24) as indicated. (C) Brightfield image with Indian ink-filled food vacuole with superimposed immunostaining for *PtCRC-III-4* contained in aggregates of recycling vesicles (as confirmed by electron microscopy [not shown]). Note intense interaction between recycling vesicles and the food vacuole.

4.4. Endo- and phagocytotic pathways

Epigenetic programming is also evident for the precise positioning of the oral cavity and the cytoproct, though less directly for the mobile, small and large vesicular components of the endocytotic and phagocytotic cycle. They all contain *PtCRC-III* channels (Fig. 4B). This is documented by confocal immune-fluorescence microscopy in Fig. 7. It may provide Ca^{2+} for the multiple interactions and fusions which the many types of vesicles can undergo [10]. As mentioned, other types of CRCs are contained in coated pits (parasomal sacs) and in early endosomes that are located near ciliary basal bodies (Figs. 1, 4B and 5) and contain *PtCRC-V-4* and *PtCRC-VI-3*, respectively [28]. One can think of several mechanisms to explain that CRCs (just like SNARE proteins) cannot in all cases be traced throughout a specific trafficking pathway; for instance, this can depend on the actual retention rate of a specific membrane

protein. These two channel types also occur along the oral cavity [28]; in this part of the cell one also has to take into account the occurrence of endocytotic structures and of small alveoli (see R.D. Allen at <http://www.5.pbrc.hawaii.edu/allen/> [3]).

4.5. Contractile vacuole complex

The pores of the two contractile vacuoles, whose location is also epigenetically predetermined, undergo periodic constitutive exocytosis of the kiss-and-run type in ~ 10 s intervals. The filling state may be sensed by mechanosensitive channels [27], but CRCs of type *PtCRC-VI-2* and *PtCRC-VI-3*, both restricted to the pore area [28] as indicated in Fig. 4C, could also provide Ca^{2+} locally. (Note, however, that the expression of *PtCRC-VI-3* is variable, eventually in fragmented or abortive form.) *PtCRC-V-4* is another candidate, as it is also present in this region of the contractile vacuole complex. Its

InsP₃-binding domain is shorter than in the most abundant form, *PtCRC-II*, occurring over large parts of the contractile vacuole complex [28]. No details are known about the function of *PtCRC*-type channels in the contractile vacuole complex except *PtCRC-II*, as to be discussed now.

In the following we have to consider the most elaborate labyrinth of a 3D-meshwork of membrane-enveloped narrow tubules, the so-called spongiome. This part sequesters H⁺ ions into the lumen, enabled by its endowment with a V-type H⁺-ATPase [89,90]; in the electron microscope these molecules mediate a decorated appearance and this part is, therefore, called the “decorated spongiome”. The sequestration of Ca²⁺ is coupled to the activity of the H⁺-ATPase [75,91,92]. In contrast, the smooth spongiome, which on its peripheral part is continuous with the decorated spongiome and on the proximal part directly attached to the collecting canals (feeding into the contractile vacuole), contains IP₃Rs, type *PtCRC-II* [40]. Here, *PtCRC-II* type CRCs mediate some spontaneous reflux of Ca²⁺ from the lumen into the cytosol, be it for fine-tuning of local [Ca²⁺]_i and/or to enable internal restructuring of the 3D network of the spongiome [27]. This finding was unexpected insofar as this system continuously and efficiently extrudes not only an excess of water, but also of Ca²⁺ [91]. A comparable capability of spontaneous, constitutive IP₃R activation has then also been described for a chicken lymphocyte cell line [93].

5. The pore region of the *PtCRCs* molecules in comparison to mammalian IP₃Rs and RyRs

One aspect concerning *PtCRCs*, types IP₃Rs and RyR-LPs, emerging from our work with *Paramecium* is the formation of the pore region by six transmembrane domains [28]. It thus agrees with the general assumption of 6 transmembrane domains for the IP₃R, but deviates from the tentative assumption of only 4 [94], 6–8 [95] or 4–10 [96] for RyRs, as summarised in the reviews cited. More recently another type of bioinformatic analysis led to the same conclusion, i.e. 6 transmembrane domains, for the skeletal muscle RyR [97] and this now seems to be tacitly accepted also by others [98]. Furthermore, the general assignment of a sequence Gly-Val-Gly-Asp to the selectivity filter of IP₃Rs and Gly-Ile-Gly-Asp to that of RyRs [99] is not applicable to lower eukaryotes; not only CRCs of *Paramecium* [28], but also those in lower metazoans show these two motives alternatively [12].

There is also evidence for the occurrence of receptors for cADPR and NAADP in *Paramecium*, founded on microinjection and on NAADP binding studies [75], but neither any molecular identity nor any specific localisation can be assigned to the respective CRCs as yet.

6. Conclusions

In *Paramecium*, Ca²⁺-influx from the outside medium and/or mobilised from internal stores can contribute to signalling. Any Ca²⁺ effect on ciliary activity is regulated exclusively by Ca²⁺-influx; specifically voltage-dependent Ca²⁺-channels, restricted to the ciliary membrane, serve for the reversal of the ciliary beat. Hyperpolarisation-induced accelerated forward swimming also involves Ca²⁺ signals, though not primarily in the cilium. Many different types of *PtCRCs*, i.e. release channels related to IP₃Rs and/or RyRs, are scattered over a multitude of vesicular compartments. For instance, Ca²⁺ required for trichocyst exocytosis is provided by a SOC mechanism, starting with Ca²⁺ release from alveolar sacs which is superimposed by Ca²⁺-influx without any recognisable delay. The underlying *PtCRC*, type *PtCRC-IV*, is a RyR-related channel restricted to the endoplasmic reticulum and alveolar sacs. Since trichocyst exocytosis sites and cilia are only ~1.7–2.0 μm apart, spillover

phenomena have to be restricted by properly positioned Ca²⁺-binding proteins, such as centrin. Therefore, normally no Ca²⁺ flux from the cilium into the cell soma occurs. However, a remarkable Ca²⁺ spillover into cilia does occur during massive synchronous exocytosis stimulation, thus enforcing escape from predator attacks [83]. Hereby depolarisation as a trigger is bypassed. Not only cilia and alveolar sacs, but also parasomal sacs (coated pits) and early endosomes are endowed with specific *PtCRCs*. The localisation of all these compartments is also under strict epigenetic control, though with unknown functional implications. Rather, such coassembly may represent not only a prerequisite to, but also the result of epigenetically controlled Ca²⁺ signalling. *PtCRC-II*-type CRCs, localised to the pair of contractile vacuole complexes are IP₃Rs. These serve for the extrusion of an excess of water and Ca²⁺. Their *PtCRC-II* can serve for fine-tuning of cytosolic Ca²⁺ by partial reflux and probably also for reversible restructuring processes within the spongiome. Finally compartments of the phagocytotic system and vesicles serving for membrane recycling also possess distinct CRCs, type *PtCRC-III*. The pathway of some of the vesicles (those shuttling from the cytoproct to the cytopharynx) is also epigenetically fixed within the *Paramecium* cell. All this differential distribution of *PtCRCs* has its counterpart in a comparable distribution pattern of SNARE proteins [65]. Together with other components, whose localisation is also under epigenetic control, the regular design of the *Paramecium* cell makes it highly efficient in vesicle trafficking and Ca²⁺ signalling already at a low level of evolution.

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References

- [1] R.D. Allen, Cytology, in: H.-D. Görtz (Ed.), *Paramecium*, Springer-Verlag, Berlin, Heidelberg, 1988, pp. 4–40.
- [2] H. Plattner, My favorite cell – *Paramecium*, *Bioessays* 24 (2002) 649–658.
- [3] R.D. Allen, Available at: <http://www.5.pbrc.hawaii.edu/allen/>
- [4] C. Erxleben, N. Klauke, M. Flötenmeyer, M.-P. Blanchard, C. Braun, H. Plattner, Microdomain Ca²⁺ activation during exocytosis in *Paramecium* cells. Superposition of local subplasmalemmal calcium store activation by local Ca²⁺ influx, *J. Cell Biol.* 136 (1997) 597–607.
- [5] J. Beisson, T.M. Sonneborn, Cytoplasmic inheritance of the organization of the cell cortex in *Paramecium aurelia*, *Proc. Natl. Acad. Sci. U. S. A.* 53 (1965) 275–282.
- [6] J. Beisson, Preformed cell structure and cell heredity, *Prion* 2 (2008) 1–8.
- [7] J. Frankel, Dimensions of control of cortical patterns in *Euplotes* – role of pre-existing structure, clonal life-cycle, and genotype, *J. Exp. Zool.* 183 (1973) 71–94.
- [8] D. Wloga, J. Frankel, From molecules to morphology: cellular organization of *Tetrahymena thermophila*, *Methods Cell Biol.* 109 (2012) 83–140.
- [9] A.K. Fok, R.D. Allen, Membrane flow in the digestive cycle of *Paramecium*, in: H. Plattner (Ed.), *Membrane Traffic in Protozoa*, JAI Press, Greenwich (CONN), London (GB), 1993, pp. 311–337.
- [10] R.D. Allen, A.K. Fok, Membrane trafficking and processing in *Paramecium*, *Int. Rev. Cytol.* 198 (2000) 277–318.
- [11] X. Cai, Unicellular Ca²⁺ signaling ‘toolkit’ at the origin of metazoa, *Mol. Biol. Evol.* 25 (2008) 1357–1361.
- [12] H. Plattner, A. Verkhatsky, Ca²⁺ signalling early in evolution – all but primitive, *J. Cell Sci.* 126 (2013) 2141–2150.
- [13] H. Plattner, Calcium regulation in the protozoan model, *Paramecium tetraurelia*, *J. Eukaryot. Microbiol.* 61 (2014) 95–114.
- [14] 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. Protistenk.* 148 (1997) 329–341.
- [15] E.J. Douzery, E.A. Snell, E. Baptiste, 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.
- [16] C. Berney, J. Pawłowski, A molecular time-scale for eukaryote evolution recalibrated with the continuous microfossil record, *Proc. R. Soc. B Biol. Sci.* 273 (2006) 1867–1872.
- [17] S.M. Adl, A.G. Simpson, C.E. Lane, J. Lukes, D. Bass, S.S. Bowser, M.W. Brown, F. Burki, M. Dunthorn, V. Hampl, A. Heiss, M. Hoppenrath, E. Lara, L. Le Gall, D.H. Lynn, H. McManus, E.A. Mitchell, S.E. Mozley-Standridge, L.W. Parfrey, J. Pawłowski, S. Rueckert, R.S. Shadwick, C.L. Schoch, A. Smirnov, F.W. Spiegel,

- The revised classification of eukaryotes, *J. Eukaryot. Microbiol.* 59 (2012) 429–493.
- [18] D. He, O. Fiz-Palacios, C.-J. Fu, J. Fehling, C.-C. Tsai, S.L. Baldauf, An alternative root for the eukaryote tree of life, *Curr. Biol.* 24 (2014) 465–470.
 - [19] B. Alberts, Impact factor distortions, *Science* 340 (2013) 787.
 - [20] M. Simon, H. Plattner, Unicellular eukaryotes as models in cell and molecular biology: a critical appraisal of their past and future value, *Int. Rev. Cell Mol. Biol.* 309 (2014) 141–198.
 - [21] N. Stelly, J.P. Mauger, M. Claret, A. Adoutte, Cortical alveoli of *Paramecium*: a vast submembranous calcium storage compartment, *J. Cell Biol.* 113 (1991) 103–112.
 - [22] H. Plattner, N. Klauke, Calcium in ciliated protozoa: sources, regulation, and calcium-regulated cell functions, *Int. Rev. Cytol.* 201 (2001) 115–208.
 - [23] H. Plattner, J. Hentschel, Sub-second cellular dynamics: time-resolved electron microscopy and functional correlation, *Int. Rev. Cytol.* 255 (2006) 133–176.
 - [24] M.A. Sleight, Encounters with cilia, in: K. Hausmann, R. Radek (Eds.), *Cilia and Flagella, Ciliates and Flagellates*, Schweizerbart Science Publishers, Stuttgart, Germany, 2014, pp. 121–131.
 - [25] Y. Nakaoka, H. Tanaka, F. Oosawa, Ca^{2+} -dependent regulation of beat frequency of cilia in *Paramecium*, *J. Cell Sci.* 65 (1984) 223–231.
 - [26] H. Machemer, Electrophysiology, in: H.-D. Götz (Ed.), *Paramecium*, Springer-Verlag, Berlin, Heidelberg, 1988, pp. 185–215.
 - [27] 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>.
 - [28] E.-M. Ladenburger, H. Plattner, Calcium-release channels in *Paramecium*. Genomic expansion, differential positioning and partial transcriptional elimination, *PLoS ONE* 6 (11) (2011) e27111.
 - [29] C.R. Artalejo, J.R. Henley, M.A. McNiven, H.C. Palfrey, Rapid endocytosis coupled to exocytosis in adrenal chromaffin cells involves Ca^{2+} , GTP, and dynamin but not clathrin, *Proc. Natl. Acad. Sci. U. S. A.* 92 (1995) 8328–8332.
 - [30] M.M. Lai, J.J. Hong, A.M. Ruggiero, P.E. Burnett, V.I. Slepnev, P. De Camilli, S.H. Snyder, The calcineurin-dynamin 1 complex as a calcium sensor for synaptic vesicle endocytosis, *J. Biol. Chem.* 274 (1999) 25963–25966.
 - [31] R.R. Preston, Genetic dissection of Ca^{2+} -dependent ion channel function in *Paramecium*, *Bioessays* 12 (1990) 273–281.
 - [32] C. Kung, R.R. Preston, M.E. Maley, K.Y. Ling, J.A. Kanabrocki, B.R. Seavey, Y. Saimi, In vivo *Paramecium* mutants show that calmodulin orchestrates membrane responses to stimuli, *Cell Calcium* 13 (1992) 413–425.
 - [33] Y. Saimi, C. Kung, Calmodulin as an ion channel subunit, *Annu. Rev. Physiol.* 64 (2002) 289–311.
 - [34] Y. Saimi, C. Kung, Behavioral genetics of *Paramecium*, *Annu. Rev. Genet.* 21 (1987) 47–65.
 - [35] D.L. Prole, C.W. Taylor, Identification and analysis of putative homologues of mechanosensitive channels in pathogenic protozoa, *PLOS ONE* 8 (6) (2013) e66068.
 - [36] H. Machemer, A. Ogura, Ionic conductances of membranes in ciliated and deciliated *Paramecium*, *J. Physiol.* 296 (1979) 49–60.
 - [37] H. Machemer, P.F.M. Teunis, Sensory motor coupling and motor responses, in: K. Hausmann, P.C. Bradbury (Eds.), *Ciliates: Cells and Organisms*, Gustav Fischer Verlag, Stuttgart, 1996, pp. 379–402.
 - [38] J.L. Van Houten, Chemosensory transduction in *Paramecium*, *Eur. J. Protistol.* 34 (1998) 301–307.
 - [39] W.E. Bell, R.R. Preston, J. Yano, J.L. Van Houten, Genetic dissection of attractant-induced conductances in *Paramecium*, *J. Exp. Biol.* 210 (2007) 357–365.
 - [40] E.-M. Ladenburger, I. Korn, N. Kasielke, T. Wassmer, H. Plattner, An $\text{Ins}(1,4,5)\text{P}_3$ receptor in *Paramecium* is associated with the osmoregulatory system, *J. Cell Sci.* 119 (2006) 3705–3717.
 - [41] 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.
 - [42] O. Dellis, A.M. Rossi, S.G. Dedos, C.W. Taylor, Counting functional inositol 1,4,5-trisphosphate receptors into the plasma membrane, *J. Biol. Chem.* 283 (2008) 751–755.
 - [43] H. Plattner, H. Matt, H. Kersken, B. Haacke, R. Stürzl, Synchronous exocytosis in *Paramecium* cells. I. A novel approach, *Exp. Cell Res.* 151 (1984) 6–13.
 - [44] E. Neher, Vesicle pools and Ca^{2+} microdomains: new tools for understanding their roles in neurotransmitter release, *Neuron* 20 (1998) 389–399.
 - [45] U. Becherer, T. Moser, W. Stühmer, M. Oheim, Calcium regulates exocytosis at the level of single vesicles, *Nat. Neurosci.* 6 (2003) 846–853.
 - [46] S.L. Tamm, Ctenophores and termites – systems for motility, in: K. Hausmann, R. Radek (Eds.), *Cilia and Flagella, Ciliates and Flagellates*, Schweizerbart Science Publishers, Stuttgart, Germany, 2014, pp. 147–171.
 - [47] H.A. Praetorius, K.R. Spring, Removal of the MDCK cell primary cilium abolishes flow sensing, *J. Membr. Biol.* 191 (2003) 69–76.
 - [48] 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.
 - [49] R. Eckert, Bioelectric control of ciliary activity, *Science* 176 (1972) 473–481.
 - [50] D. Oertel, S.J. Schein, C. Kung, Separation of membrane currents using a *Paramecium* mutant, *Nature* 268 (1977) 120–124.
 - [51] 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.
 - [52] P. Brehm, R. Eckert, Calcium entry leads to inactivation of calcium channel in *Paramecium*, *Science* 202 (1978) 1203–1206.
 - [53] I.B. Levitan, It is calmodulin after all! Mediator of the calcium modulation of multiple ion channels, *Neuron* 22 (1999) 645–648.
 - [54] 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.
 - [55] C. Klotz, N. Garreau de Loubresse, F. Ruiz, J. Beisson, Genetic evidence for a role of centrin-associated proteins in the organization and dynamics of the infraciliary lattice in *Paramecium*, *Cell Motil. Cytoskel.* 38 (1997) 172–186.
 - [56] 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.
 - [57] D. Gogendeau, C. Klotz, O. Arnaiz, A. Malinowska, M. Dadlez, N. Garreau de Loubresse, F. Ruiz, F. Koll, J. Beisson, Functional diversification of centrins and cell morphological complexity, *J. Cell Sci.* 121 (2008) 65–74.
 - [58] N.L. Elwess, J.L. Van Houten, Cloning and molecular analysis of the plasma membrane Ca^{2+} -ATPase gene in *Paramecium tetraurelia*, *J. Eukaryot. Microbiol.* 44 (1997) 250–257.
 - [59] J. Yano, A. Rajendran, M.S. Valentine, M. Saha, B.A. Ballif, J.L. Van Houten, Proteomic analysis of the cilia membrane of *Paramecium tetraurelia*, *J. Proteomics* 78 (2013) 113–122.
 - [60] 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.
 - [61] Y. Naitoh, R. Eckert, Ionic mechanisms controlling behavioral responses of *Paramecium* to mechanical stimulation, *Science* 164 (1969) 963–965.
 - [62] R.R. Preston, Y. Saimi, C. Kung, Calcium current activated upon hyperpolarization of *Paramecium tetraurelia*, *J. Gen. Physiol.* 100 (1992) 233–251.
 - [63] 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.
 - [64] R. Kissmehl, C. Schilde, T. Wassmer, C. Danzer, K. Nuehse, K. Lutter, H. Plattner, Molecular identification of 26 syntaxin genes and their assignment to the different trafficking pathways in *Paramecium*, *Traffic* 8 (2007) 523–542.
 - [65] 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.
 - [66] S.W. Min, W.P. Chang, T.C. Südhof, E.-Syts, a family of membranous Ca^{2+} -sensor proteins with multiple C2 domains, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 3823–3828.
 - [67] S. Martens, H.T. McMahon, Mechanisms of membrane fusion: disparate players and common principles, *Nat. Rev. Mol. Cell Biol.* 9 (2008) 543–556.
 - [68] N. Klauke, H. Plattner, Imaging of Ca^{2+} transients induced in *Paramecium* cells by a polyamine secretagogue, *J. Cell Sci.* 110 (1997) 975–983.
 - [69] 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.
 - [70] 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.
 - [71] 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.
 - [72] 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.
 - [73] B. Narayanan, M.N. Islam, D. Bartelt, R.S. Ochs, A direct mass-action mechanism explains capacitative calcium entry in Jurkat and skeletal L6 muscle cells, *J. Biol. Chem.* 278 (2003) 44188–44196.
 - [74] M.M. Wu, R.M. Luik, R.S. Lewis, Some assembly required: constructing the elementary units of store-operated Ca^{2+} entry, *Cell Calcium* 42 (2007) 163–172.
 - [75] H. Plattner, I.M. Sehring, I.K. Mohamed, K. Miranda, W. De Souza, R. Billington, A. Genazzani, E.-M. Ladenburger, Calcium signaling in closely related protozoan groups (Alveolata): non-parasitic ciliates (*Paramecium*, *Tetrahymena*) vs. parasitic Apicomplexa (*Plasmodium*, *Toxoplasma*), *Cell Calcium* 51 (2012) 351–382.
 - [76] D.L. Prole, C.W. Taylor, Identification of intracellular and plasma membrane calcium channel homologues in pathogenic parasites, *PLoS ONE* 6 (10) (2011) e26218.
 - [77] F. Giordano, Y. Saheki, O. Idevall-Hagren, S.F. Colombo, M. Pirruccello, I. Milosevic, E.O. Gracheva, S.N. Bagriantsev, N. Borgese, P. De Camilli, $\text{PI}(4,5)\text{P}_2$ -dependent and Ca^{2+} -regulated ER-PM interactions mediated by the extended synaptotagmins, *Cell* 153 (2013) 1494–1509.
 - [78] J.E. Heuser, T.S. Reese, M.J. Dennis, Y. Jan, L. Jan, L. Evans, Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release, *J. Cell Biol.* 81 (1979) 275–300.

- [79] 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.
- [80] H. Plattner, A.R. Artalejo, E. Neher, Ultrastructural organization of bovine chromaffin cell cortex – analysis by cryofixation and morphometry of aspects pertinent to exocytosis, *J. Cell Biol.* 139 (1997) 1709–1717.
- [81] R.G. Zhai, H.J. Bellen, The architecture of the active zone in the presynaptic nerve terminal, *Physiology* 19 (2004) 262–270.
- [82] T. Harumoto, A. Miyake, Defensive function of trichocysts in *Paramecium*, *J. Exp. Biol.* 260 (1991) 84–92.
- [83] 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.
- [84] J.L. Browning, D.L. Nelson, Biochemical studies of the excitable membrane of *Paramecium aurelia*. I. $^{45}\text{Ca}^{2+}$ fluxes across resting and excited membrane, *Biochim. Biophys. Acta* 448 (1976) 338–351.
- [85] D. Kerboeuf, J. Cohen, A Ca^{2+} influx associated with exocytosis is specifically abolished in a *Paramecium* exocytotic mutant, *J. Cell Biol.* 111 (1990) 2527–2536.
- [86] 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.
- [87] 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.
- [88] 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.
- [89] A.K. Fok, K. Yamauchi, A. Ishihara, M.S. Aihara, M. Ishida, R.D. Allen, The vacuolar-ATPase of *Paramecium multimicronucleatum*: gene structure of the B subunit and the dynamics of the V-ATPase-rich osmoregulatory membranes, *J. Eukaryot. Microbiol.* 49 (2002) 185–196.
- [90] T. Wassmer, I.M. Sehring, R. Kissmehl, H. Plattner, The V-ATPase in *Paramecium*: functional specialization by multiple gene isoforms, *Pflügers Arch. Eur. J. Physiol.* 457 (2009) 599–607.
- [91] 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.
- [92] 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.
- [93] 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.
- [94] A.J. Williams, D.J. West, R. Sitsapesan, Light at the end of the Ca^{2+} -release channel tunnel: structures and mechanisms involved in ion translocation in ryanodine receptor channels, *Q. Rev. Biophys.* 34 (2001) 61–104.
- [95] R. Zalk, S.E. Lehnart, A.R. Marks, Modulation of the ryanodine receptor and intracellular calcium, *Annu. Rev. Biochem.* 76 (2007) 367–385.
- [96] S.L. Hamilton, Ryanodine receptors, *Cell Calcium* 38 (2005) 253–260.
- [97] 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.
- [98] 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.
- [99] D.F. Boehning, Molecular architecture of the inositol 1,4,5-trisphosphate receptor pore, *Curr. Top. Membr.* 66C (2010) 191–207.
- [100] M. Bilinski, H. Plattner, R. Tiggemann, Isolation of surface membranes from normal and exocytotic mutant strains of *Paramecium tetraurelia*. Ultrastructural and biochemical characterization, *Eur. J. Cell Biol.* 24 (1981) 108–115.