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Intracellular calcium channels in protozoa

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ABSTRACT

Ca^{2+} signaling pathways and intracellular Ca^{2+} channels are present in protozoa. Ancient origin of inositol 1,4,5 trisphosphate receptors (IP_3Rs) and other intracellular channels predates the divergence of animals and fungi as evidenced by their presence in the choanoflagellate *Monosiga brevicollis*, the closest known relative to metazoans. The first protozoan IP_3R cloned, from the ciliate *Paramecium*, displays strong sequence similarity to the rat type 3 IP_3R. This ciliate has a large number of IP_3 and ryanodine (Ry) like receptors in six subfamilies suggesting the evolutionary adaptation to local requirements for an expanding diversification of vesicle trafficking. IP_3Rs have also been functionally characterized in trypanosomatids, where they are essential for growth, differentiation, and establishment of infection. The presence of the mitochondrial calcium uniporter (MCU) in a number of protozoa indicates that mitochondrial regulation of Ca^{2+} signaling is also an early appearance in evolution, and contributed to the discovery of the molecular nature of this channel in mammalian cells. There is only sequence evidence for the occurrence of two pore channels (TPCs), transient receptor potential Ca^{2+} channels (TRPCs) and intracellular mechanosensitive Ca^{2+} channels in *Paramecium* and in parasitic protozoa.

Contents

1. Introduction

Calcium ion (Ca^{2+}) controls a variety of cellular functions in protozoa. As occurs with mammalian cells, the cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ of protozoa is maintained at very low levels (of the order of 10^{-7} M). The cytosolic Ca^{2+} level is responsible for

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the regulation of Ca^{2+} dependent and Ca^{2+} controlled proteins. Although the total calcium inside protozoan cells is much higher than 10 ^{7} M, the bulk of this calcium is either bound to proteins, polyphosphate, membranes or other cellular constituents, or is sequestered inside intracellular organelles through the activity of pumps, channels, and exchangers, and released when needed by a variety or intracellular Ca^{2+} channels.

Recent genomic studies (King et al., 2008) have revealed that many ion channels including Ca^{2+} channels previously thought to be restricted to animals, can be traced back to one of the unicellular ancestors of animals, *Monosiga brevicollis*, a choanoflagellate proto zoan belonging the supergroup Opisthokonta, which also includes animals, and fungi. Genes encoding homologs to various types of plasma membrane Ca^{2+} channels are present: store operated channel (Orai) and the endoplasmic reticulum sensor protein stromal interaction molecule (Stim); voltage operated channel (similar to dihydropyridine sensitive L type Ca^{2+} channel); ligand operated channels (nicotinic acetylcholine receptor and P2X purinergic receptor); transient receptor potential (TRP) channels; and second messenger operated channel (cyclic nucleotide gated channel) (Cai, 2008). This protozoan appears to possess all five modes of regulated Ca^{2+} entry across the plasma membrane identified in animals (Parekh and Putney, 2005), although their physiological validation is needed (Cai, 2008). *Monosiga brevicollis* has also four homologs of the inositol 1,4,5 trisphosphate receptor (IP_3R), and a homolog to the mitochondrial calcium uniporter (XP_001749044), but no homologs to ryanodine receptors (RyR) (Cai, 2008). However, no functional studies have been reported with any of these channels.

Evidently the evolution of eukaryotic cells is characterized by increasing genomic information that allows for increasing complexity of intracellular structure, dynamics and signaling mechanisms. Target oriented vesicle trafficking requires not only an inventory of membrane specific proteins, such as SNAREs (Malsam et al., 2008) and small GTPases (Zerial and McBride, 2001), but also provisions for Ca^{2+} signaling in a very local area where membranes have to interact (Neher, 1998). Ca^{2+} may come from the external medium or be locally released from stores via Ca^{2+} release channels (CRC) so that Ca^{2+} can locally drive docking, priming and eventual fusion of membranes (Rizo et al., 2006). Cell contraction is another example. Ca^{2+} is most appropriate for such functions because of its specific, reversible binding to Ca^{2+} binding proteins, CaBP, which in the end transmit the signal by a conformational change in effector protein molecules (Klee et al., 1980; Rizo et al., 2006). On the one hand global regulation of intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, is mandatory to avoid the overall toxic effect of Ca^{2+} (Case et al., 2007). On the other hand, local $[Ca^{2+}]_i$ regulation also has to account for diffusional spread by a square function, whereas most molecular effects of Ca^{2+} depend on a higher power function of $[Ca^{2+}]_i$ (Neher, 1998). Binding to CaBPs, sequestration into organelles and extrusion from the cell antagonize the occurrence of too high and diffuse $[Ca^{2+}]_i$ values after stimulation. Remarkably, the phenomena described in this review, as well as the CRC types mentioned, are all found already in protozoa. Nevertheless, with these cells stringent analyses of Ca^{2+} signaling and the subsiding intracellular CRCs have remained elusive until quite recently.

The protozoan organisms whose Ca^{2+} signaling and subsiding CRCs are currently investigated in our labs include ciliates (*Para mecium*), their close relatives, Apicomplexa (including pathogenic species of *Plasmodium* [malaria causing agent] and *Toxoplasma*) as well as some pathogenic flagellates (trypanosomatids). With these organisms, CRCs have been characterized at a molecular level, in conjunction with functional studies. There is a wide gap between evolutionary levels: ciliates close to recent forms have emerged ~800 to 850 million years ago, non parasitic Apicomplexa ~500 million years (Douzery et al., 2004) and mammalian apicomplexan parasites ~13 million years ago (Ricklefs and Outlaw, 2010). There is also some information available on the Ca^{2+} dynamics in social ameba of the myxomycete *Dictyostelium*, which clearly possesses Ca^{2+} signaling pathways (Allan and Fisher, 2009), but information about CRCs in these cells is scant.

A *Paramecium tetraurelia* cell is up to ~100 μm in size and exhibits distinct intracellular vesicle trafficking pathways (Allen and Fok, 2000), essentially including all those known from metazoan cells. The pathogenic forms discussed are ~10 times smaller, but also contain specific vesicle trafficking pathways, such as endocytosis vesicles and organelles for intracellular digestion (trypanosomatids, Apicomplexa). Apicomplexa also possess secre tory organelles for exocytosis. Due to their small size and their complicated lifestyle the parasites are much more difficult to study than their free living relatives. Using fluorescent dyes in both ciliates and Apicomplexa, a considerable Ca^{2+} signal could be recorded during exocytosis of secretory organelles, such as tricho cysts (Klauke and Plattner, 1997) and during motility (Lovett and Sibley, 2003), respectively.

Values for steady state $[Ca^{2+}]_i$ in widely different cells, from protozoa to mammals, are of the order of 50 100 nM at rest and stimulation generally causes an increase by a factor of 10 100 (Bootman and Berridge, 1995). This frame also applies to ciliates (Klauke and Plattner, 1997) and to parasitic protozoa (Vieira and Moreno, 2000; Moreno et al., 1994). $[Ca^{2+}]_i$ determined in *Para mecium* under steady state conditions yields values between 60 and 100 nM. It has to be stressed that measurements performed with fluorescent dyes, even when calibrated, systematically under estimate the real local $[Ca^{2+}]_i$ increase during activation because of its considerable local restriction. More realistic local, function ally relevant values are obtained by probing the threshold inhibi tory effect of Ca^{2+} chelators with appropriate binding properties (Neher, 1995). For instance, during exocytosis stimulation $[Ca^{2+}]_i$ in the cell cortex peaked at ~400 nM with fluorescent dyes measurements, whereas chelator application during stimulation indicated the increase in $[Ca^{2+}]_i$ to the micromolar range (Klauke and Plattner, 1997).

2. Calcium stores

The paradigm of a Ca^{2+} store in all eukaryotic cells is the endoplasmic reticulum (ER), together with the sarcoplasmic reti culum (SR) in muscle cells (Berridge et al., 2000, 2003; Clapham, 2007; Cai, 2008). Since Ca^{2+} is stored in many more organelles such stores and their CRCs deserve special attention also in protozoa, including ciliates and parasitic protozoa (Plattner et al., 2012).

Subsequent to stimulation Ca^{2+} is sequestered into different organelles and then may be available later on for release via CRCs in a constitutive manner or in the context of signaling processes. Ca^{2+} can, thus, regulate exocytosis, endocytosis, phagocytosis, fusion of endosomes of different stages with phagosomes, phago some formation, membrane recycling, phago lysosome fusion etc. (Hay, 2007; Zampese and Pizzo, 2012). In mammalian cells, many of these organelles, specifically early endosomes (Luzio et al., 2010) and lysosomes (Christensen et al., 2002), are known to store Ca^{2+} (Hay, 2007; Sherwood et al., 2007) and the membranes of many of them contain CRCs (Zampese and Pizzo, 2012). The main types of CRCs found in metazoans up to mammalian cells are IP_3R (Taylor et al., 2004; Bezprozvanny, 2005), RyR (Hamilton, 2005; Mackrill, 2012), transient receptor potential Ca^{2+} channels, TRPC (Patel and Docampo, 2009), and two pore channels, TPC, occurring mainly in acidic compartments (Galione et al., 2009; Galione et al., 2010; Patel and Docampo, 2010). All these channel types also occur in protozoa.

Work with *P. tetraurelia* was started with *Paramecium* database (DB) analysis and further evaluation by expression, localization and functional studies. Thus, a plethora of CRCs related to RyRs and to IP_3Rs, or to both, were identified (Ladenburger et al., 2006; Ladenburger et al., 2009; Ladenburger and Plattner, 2011). The different CRC types are scattered over the many sites of specific membrane interactions. The functions of IP_3Rs (Ladenburger et al., 2006) and of RyRs (more safely to be addressed as RyR like proteins, RyR LP) (Ladenburger et al., 2009) were investigated in more detail. Further on the analysis concentrated on cortical stores (alveolar sacs) and dense core secretory organelle (trichocyst) exocytosis as well as on the contractile vacuole complex that serves for osmoregulation and maintaining the internal ionic balance, particularly of Ca^{2+}.

In Apicomplexa secretory organelles include rhoptries, micro nemes, and dense granules whose exocytosis is mandatory for attachment to a host cell, invasion and establishment of the parasitophorus vacuole. Their secretion requires Ca^{2+} signals, presumably based on IP_3 (Lovett et al., 2002; Lovett and Sibley, 2003) or cADP ribose (cADPR) (Chini et al., 2005; Nagamune et al., 2008) signaling. Database search by different groups did not allow for the identification of either IP_3Rs or of RyRs (or RyR LPs) in Apicomplexa (Nagamune and Sibley, 2006; Plattner et al., 2012) although intracellular application of IP_3 has facilitated host cell infection (Lovett et al., 2002). In trypanosomatids IP_3Rs have recently been identified on a molecular level, and probed func tionally, in two species, *Trypanosoma brucei* (Huang et al., 2013) and *T. cruzi* (Hashimoto et al., 2013). In *Paramecium*, the dense core secretory organelles called trichocysts can explosively be released by exocytosis within fractions of a second, thus making this system amenable to sub second analysis (Plattner and Hentschel, 2006). The reaction serves for warding off predators very efficiently (Harumoto and Miyake, 1991).

In summary, CRCs must have evolved early in evolution, i.e. already at the level of protozoa. These CRCs include not only IP_3Rs and RyR LPs (Plattner and Verkhratsky, 2013) but also TRPCs and TPCs (Patel and Docampo, 2010; Plattner et al., 2012) as well as the mitochondrial calcium uniporter (Docampo and Lukes, 2012) that will be discussed later. On a speculative basis one may envisage also intracellular mechano sensitive Ca^{2+} channels as part of an ancient CRC inventory. This expectation is based on the finding of stomatin in the membranes of the contractile vacuole complex and of food vacuoles of *Paramecium* (Reuter et al., 2013), considering that generally the scaffolding protein stomatin is structurally and functionally associated with mechano sensitive Ca^{2+} channels in metazoans (Lapatsina et al., 2012). By sensing the internal tension in these organelles such channels may initiate a Ca^{2+} signal for the release of contractile vacuole contents by exocytosis or for fusion processes along the food vacuole pathway (Reuter et al., 2013).

Scrutiny at a molecular level, including domain analysis, intra cellular localization and functional analysis, including also gene silencing, will provide us with important new insight into Ca^{2+} signaling mechanisms not only in free living, but also in patho genic protozoan species. Also the precise function of many of the CRCs recently identified remains to be elucidated.

3. Intracellular calcium channels in ciliates

3.1. IP_3R and RyR type CRCs in Paramecium identification and localization

Only quite recently could such channels be identified, based on genomic sequences indicative of characteristic domain structures. Six subfamilies of putative CRCs, *Pt*CRC I to *Pt*CRC VI, which all encompass several paralogs, were found and cloned. These are identified by addition of arabic numbers, e.g. *Pt*CRC IV 2 (some with further subforms, such as *Pt*CRC III 1a and 1b). For detailed terminology, see (Ladenburger and Plattner, 2011). In part they show characteristics of IP_3Rs and in part of RyRs and they were the first of these types unambiguously identified in protozoa at a molecular level. Some other CRCs of this family show one or the other, but not all of the characteristic features of either IP_3Rs or RyRs. Domains under consideration include the IP_3 binding domain (IP_3 BD), the RyR/IP_3R homology (RIH) domain, the pore domain with its transmembrane domains and the selectivity filter as well as regions with variable similarity to mammalian IP_3Rs or

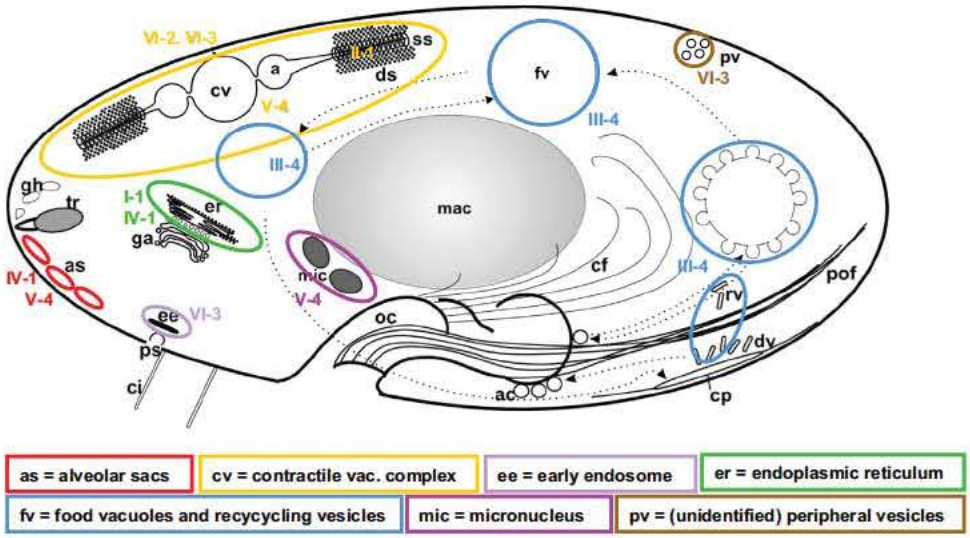

Fig. 1. Distribution of CRCs in the *P. tetraurelia* cell (*Pt*CRC), subfamilies I to VI (and subtypes analyzed in some detail given in arabic numbers), as localized by immuno-fluorescence. Additional sites of *Pt*CRC localization are not included in the scheme. These are *Pt*CRC-V-4, occurring along the oral cavity (together with *Pt*CRC-VI-3) and along the cleavage furrow; remarkably *Pt*CRC-V-4 also occurs in the parasomal sacs (clathrin coated pits at the cilairy basis) membranes. Beyond structures identified below the scheme, the following abbreviations are used: in the contractile vacuole (*cv*) system, *a* marks ampullae (with extending radial/collecting canals), *ds* decorated spongiome and *ss* smooth spongiome. Other structures are *ac* acidosomes (vesicles of endosomal origin contributing to phagosome/food vacuole formation), *cf* cytopharyngeal fibers; *ci* cilia; *cp* cytoproct, *dv* discoidal vesicles, *rv* recycling vesicles, *mac* macronucleus, *oc* oral cavity, *pof* postoral fibers, *ps* parasomal sacs (clathrin-coated pits), *tr* trichocysts and their "ghosts" (*gh*) occurring after contents release. Compiled according to Ladenburger and Plattner (2011).

RyRs. It was thus identified an unexpected total of 34 IP_3R and RyR like channels in the *P. tetraurelia* cell (Ladenburger and Plattner, 2011). Generally only a selected paralog of one subfamily has been analyzed in more detail. This high number of *Pt*CRCs clearly is the result of several whole genome duplications (Aury et al., 2006) as one can derive from the high similarity of many though not all paralogs contained in each subfamily. Remarkably all sequences are expressed, except one from subfamily VI, which may be on the way to pseudogene formation (Ladenburger and Plattner, 2011).

These attempts of identification were complemented by localiza tion of *Pt*CRCs at the light and electron microscope level and by functional analyses: IP_3 binding and activation of injected caged IP_3 on the one hand (Ladenburger et al., 2006) and on the other hand by activation by RyR agonists (Ladenburger et al., 2009) whose secre tagogue effect had been probed before (Klauke and Plattner, 1998; Klauke et al., 2000; Plattner and Klauke, 2001). Also discussed in more detail below are fluorescent imaging experiments.

The members of the six subfamilies are all distinctly placed in the cell (Fig. 1) and, according to gene silencing experiments, they can account for the regulation of widely different functions in different regions of the highly complex *Paramecium* cell. In detail, subfamily I channels (in our designation *Pt*CRC I) are associated with the ER (Ladenburger and Plattner, 2011). *Pt*CRC II/IP_3Rs are restricted to the contractile vacuole complex (serving in fresh water organisms for the expulsion of water and of some ions, including an excess of Ca^{2+}) and, therefore, may fine tune Ca^{2+} homeostasis by partial reflux of Ca^{2+} (Ladenburger et al., 2006). Spontaneous Ca^{2+} puffs are seen along the tubular extensions of this organelle, indicating constitutively active IP_3R type channels a phenomenon reported later on also for a chicken lymphocyte cell line (Cardenas et al., 2010) and mammalian atrial myocytes

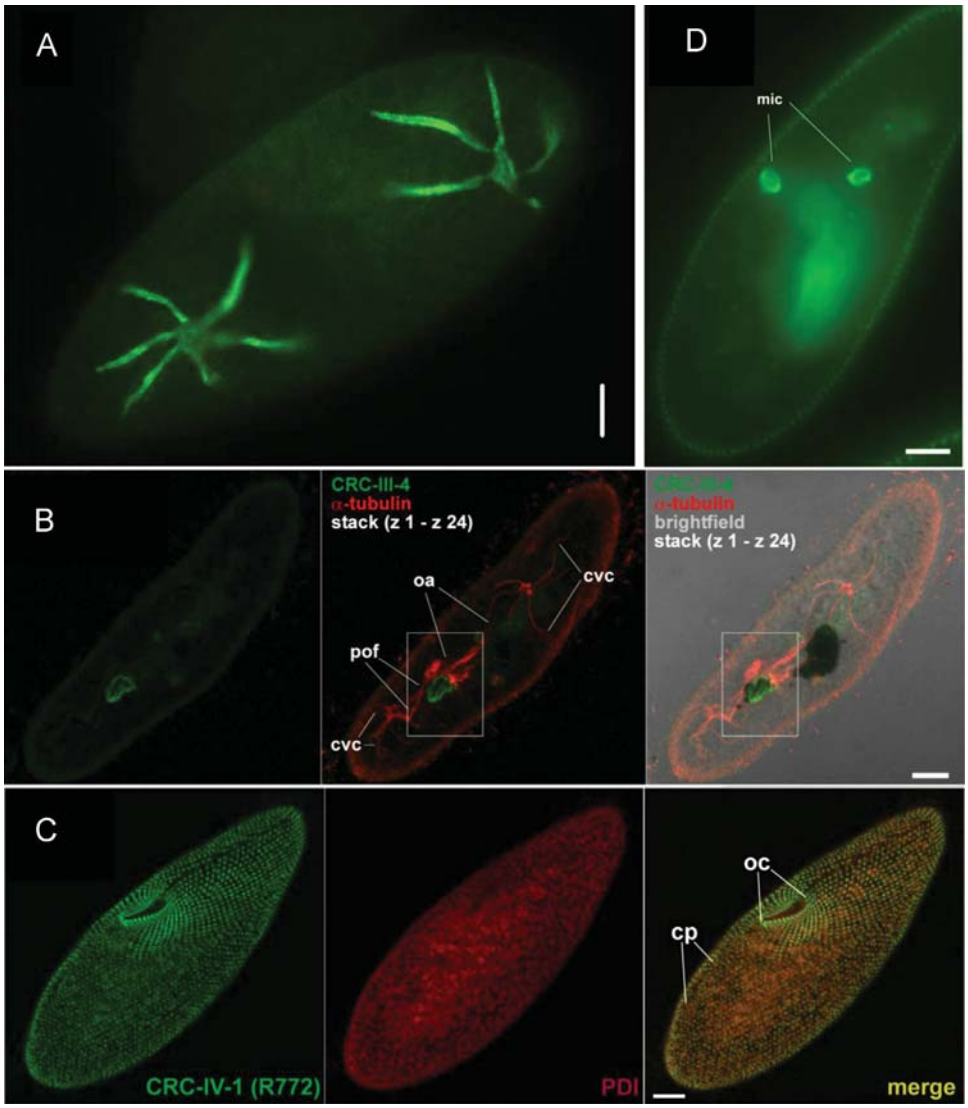

Fig. 2. Examples of immuno-localization of different *Pt*CRCs. (A) *Pt*CRC-II (IP_3R) is localized to the star-shaped contractile vacuole complex. (B) *Pt*CRC-III-4 is localized to the phagocytic pathway, including recycling vesicles. Here, phagosomes (food vacuoles) show up in dark due to the addition of Indian ink in the medium. For better identification of structures labeled with antibodies against *Pt*CRC-III-4 (*green*) *red* antibodies have been applied to stain microtubules. Here, *Pt*CRC-III-4 traffic with recycling vesicles along microtubules that are associated with the postoral fibers (*pof*) which originate from the oral apparatus (*oa*). By contrast, the contractile vacuole complex (*cvc*) is stained only for tubulin, but not for *Pt*CRC-III-4. Also indicated is the number of z-stacks used for a pile-up image in (B). See Fig. 1 for further details. (C) Immunolocalization of *Pt*CRC-IV-1 (RyR-LP) by *green* fluorescence. *Red* staining comes from labeling of protein disulfide isomerase (PDI), an ER-specific marker. Note the localization of *Pt*CRC-IV-1 preferably to the alveolar sacs (Ca^{2+}-stores, represented by the green patches in the cell cortex) and less to the ER. Abbreviations are: *oc* oral cavity, *cp* cytoproct. (D) *Pt*CRC-V-4 localizes to micronuclei (*mic*), i.e. their envelopes, as well as to cell surface components (see Fig. 1 for details). B-D are from (Ladenburger et al., 2009). Scale bars 10 μm.

(Horn et al., 2013). *Pt*CRC III molecules are associated with recycling vesicles engaged in phagosome formation (Ladenburger and Plattner, 2011). A sequence indicating an IP_3 BD occurs in the members of these three subfamilies, I to III, but has been experimentally verified only with *Pt*CRC II type channels. *Pt*CRC IV channels display structural and functional characteristics of RyRs (Ladenburger et al., 2009). They are localized to the estab lished subplasmalemmal Ca^{2+} stores, the alveolar sacs. Note that in apicomplexan parasites, the structural equivalent is the "inner membrane complex", whose relevance for Ca^{2+} signaling is not known (Plattner et al., 2012). Silencing reduces stimulated exocy tosis in response to RyR agonists. The remaining *Pt*CRC subfamily types, *Pt*CRC V and *Pt*CRC VI, have a more complex distribution (Ladenburger and Plattner, 2011), as shown in Fig. 1.

Altogether, immunolocalization studies with antibodies against subfamily *Pt*CRC members revealed widely different, but distinct localization and functional engagement of the different Ca^{2+} release channel types also turned out to be different. Some *Pt*CRCs occur in different organelles and some organelles possess different CRC types, as summarized in Fig. 1, with some selected examples presented in Figs. 2 and 3. In alveolar sacs *Pt*CRC IV molecules are scattered over the entire peripheral part where they face the cell membrane (Ladenburger et al., 2009), thus allowing spilling of Ca^{2+} over exocytosis sites. An additional type of CRCs is found in this organelle, i.e. *Pt*CRC V 4, with characteristics of an IP_3R; it is positioned laterally, i.e. where adjacent sacs approach each other (Ladenburger and Plattner, 2011). Other *Pt*CRCs are generally associated with vesicles participating in trafficking, as known from in vivo and ultrastructural analyses, as well as from the topology of specific SNARE proteins (Plattner, 2010) that mediate specific membrane interactions.

3.2. *Functional aspects of IP_3R and RyR type CRCs in Paramecium*

Only *Pt*CRC II (IP_3Rs) and *Pt*CRC IV (RyR LP) channels have been analyzed in some more detail by post transcriptional gene silencing, i.e. by applying the method described by (Galvani and Sperling, 2002). Genes diverging by $>15\%$ can thus be differen tially silenced at a post transcriptional level. This means that not all paralogs of a subfamily can be safely addressed and a similar uncertainty holds for antibodies used for immunolocalization.

3.2.1. *IP_3 receptors*

The *Pt*CRC II/IP_3R localized selectively to the contractile vacuole system, from the contractile vacuole bladder, over emanating radial (connecting) arms to the entire "smooth spongiome", a network of anastomosing membrane bounded tubules. Here, a H^+ gradient generated by a vacuolar H^+ ATPase pump in the adjacent "decorated

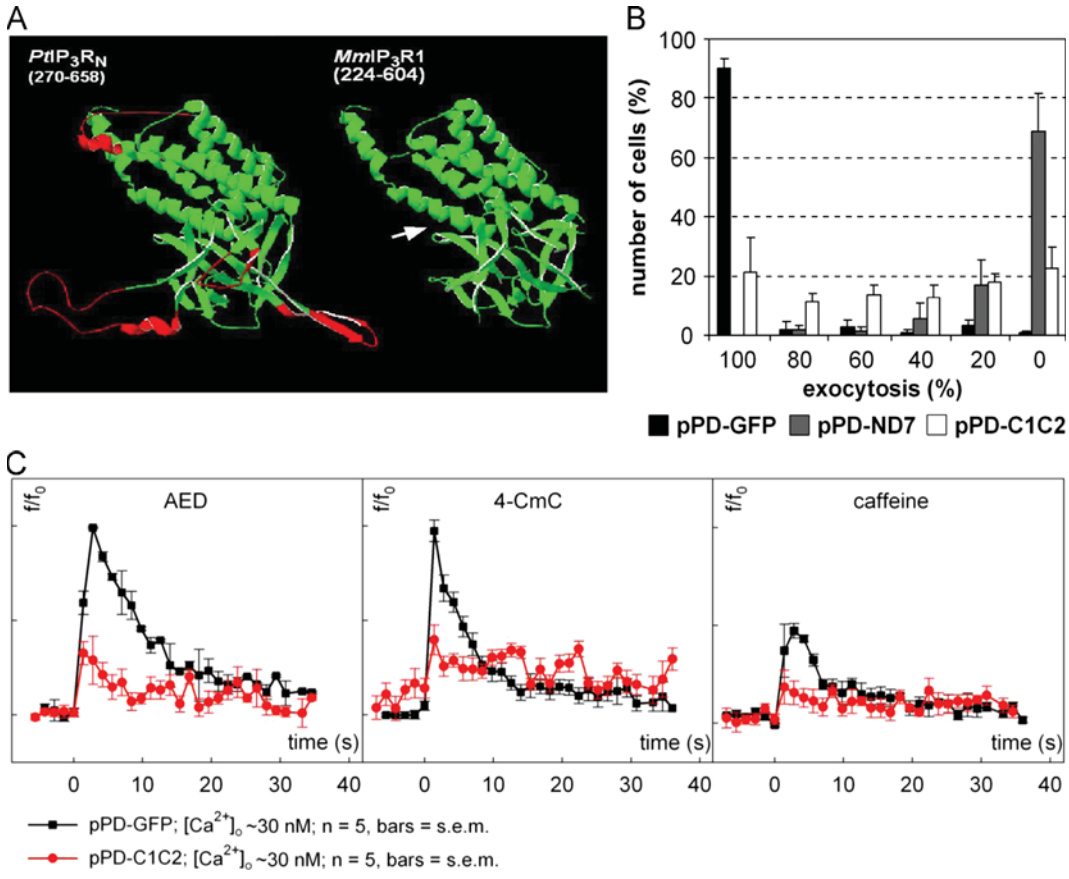

Fig. 3. (A) Molecular modeling of the inositol 1,4,5-trisphosphate-BD of *Pt*CRC-II (designated *Pt*IP_3R_N) in comparison to type 1 IP_3R of the mouse (*Mm*IP_3R1). The IP_3-BD of the *Paramecium* molecule has been modeled by comparison with that of *Mm*IP_3R1. Numbers in parenthesis indicate the amino acid sequence included in the modeling procedure. Note considerable coincidence of the motifs in both IP_3Rs, with some additional loops (of yet unexplained significance) in the IP_3-BD of *Paramecium*. From (Ladenburger et al., 2006). (B) Exocytosis performance (percent of cells performing exocytosis [ordinate] to a different extent indicated in the abscissa) and the effect of *Pt*CRC-IV-1 (RyR-LP) silencing. Two types of control cells have been evaluated: (i) Cells mock-silenced with the vector (pPD) containing only a GFP sequence served as a negative control; (ii) cells silenced in the exocytosis-relevant *ND7* gene served as a positive control. The relevant experiment was carried out with a vector containing a sequence appropriate to silence *Pt*CRC-IV. Note considerable depression of AED-stimulated exocytosis after ND7 and *Pt*CRC-IV silencing, respectively. From (Ladenburger et al., 2009). (C) Effect of silencing of *Pt*CRC-IV-1 (RyR-LP) on Ca^{2+}-signaling. Cells were exceptionally contained in a medium with $[Ca^{2+}]_o$ reduced to a calculated value of ~30 nM, i.e. slightly below internal resting level ($[Ca^{2+}]_i \sim 50$ nM), thus excluding signals from Ca^{2+}-influx. The Ca^{2+} signal generated during exocytosis stimulation was evaluated by ratio imaging (f/f_o). *Black lines*: non-silenced cells, *red lines*: silenced cells. Exocytosis was stimulated by aminoethyldextrane (AED), 4-chloro-m-cresol (4CmC) and caffeine, respectively. For further details, see text. From (Ladenburger et al., 2009).

spongiome" drives chemiosmotically the uptake of Ca^{2+} and water (Fok et al., 1995; Wassmer et al., 2009) for collection by the smooth spongiome, delivery to the vacuole bladder and periodic extrusion by exocytosis (Stock et al., 2002). What may the function of the organelle specific CRCs then be? In fact, using a Ca^{2+} sensitive fluorescent dye, irregular, spontaneous Ca^{2+} puffs along the radial arms/smooth spongiome part of the organelle were observed (Ladenburger et al., 2006). Ca^{2+} signals have been altered by UV activation of caged Ca^{2+} and Ca^{2+} dependent biosynthetic pathways (organelle biogenesis) have been inhibited by silencing the *Pt*CRC II/IP_3R; this, together with the unambiguous identification of an IP_3 BD (with characteristics of a low affinity type of mammalian IP_3R), shown in Fig. 3A, led the authors (Ladenburger et al., 2006) to conclude that these channels allow for some Ca^{2+} reflux from the organelle. However, the function may be dual. First, they may serve for fine tuning of Ca^{2+} secretion (Ladenburger et al., 2006) similar to systemic functions in kidney. Second, they may provide Ca^{2+} for local restructuring of the spongiome by reversible fusion and fission processes by the numerous organelle specific SNARE proteins (Schönemann et al., 2013). This may enable the adjustment of organelle structure and function to the actual physiological requirements (Plattner, 2013).

3.2.2. *Ryanodine receptor like proteins*

The *Pt*CRC IV/RyR LP are localized to the alveolar sacs (Ladenburger et al., 2009) that had been previously identified (Stelly et al., 1991) and further characterized (Länge et al., 1995; Hardt and Plattner, 2000) as cortical Ca^{2+} stores. Alveolar sacs are tightly attached to the cell membrane. By immunogold EM analysis these channels have been localized to the outer part of alveolar sacs facing the cell membrane (Ladenburger et al., 2009). Aspects of functional characterization are contained in Fig. 3B and C. These were identified by activation with the secretagogue aminoethyldextran (AED), caffeine, or with the ryanodine substitute 4 chloro *meta* cresol (4CmC) in conjunction with Ca^{2+} imaging, paralleled by gene silencing (Fig. 3C). Since gene silencing inhibited secretagogue induced Ca^{2+} signals (Fig. 3C), as well as trichocyst exocytosis (Fig. 3B), these channels were concluded to transport Ca^{2+}. This function has been identified as the first step of signal transduction, which induces, as a second step, a superimposed Ca^{2+} influx from the medium (store operated Ca^{2+} entry, SOCE). The activity of both components in concert had been envisioned previously by whole cell patch electrophysiology (Erxleben and Plattner, 1994; Erxleben et al., 1997), by fluorochrome analysis under selective conditions (Klauke et al., 2000) and by elemental analysis at the EM level using energy dispersive x ray microanalysis (Hardt and Plattner, 2000). The situation clearly recalls that of the junction between the SR and the plasma membrane in striated muscle, although SOCE is much more widely distributed.

The simultaneous occurrence of *Pt*CRC IV channels (RyR type) and additionally of *Pt*CRC V 4 (IP_3R type) in alveolar sacs is also not without precedent. It also occurs in the ER of rat sensory neurons (Solovyova and Verkhratsky, 2003) and in mammalian skeletal muscle RyRs and IP_3Rs cooperate to activate Ca^{2+} signaling via the SR (Tjondrokoesoemo et al., 2013). A similar cooperativity has been detected in atrial cells of the heart (Horn et al., 2013).

There are several unexplored properties of *Pt*CRCs including the occurrence of mixed features in some subfamilies. For instance, the molecular size of *Pt*CRCs of the RyR type (e.g. *Pt*CRC IV) is unusually small. According to their amino acid sequence all *Pt*CRCs are around 300 kDa in size (Ladenburger and Plattner, 2011) and thus resemble IP_3Rs in metazoans. Functional implications of this peculiarity remain to be explored. This may be one of the criteria indicating the occurrence of a common ancestral form of IP_3Rs and RyR LPs in protozoa (Plattner and Verkhratsky, 2013), whereas in metazoa RyRs are much larger than IP_3Rs (Taylor et al., 2009). Also unusual is the occurrence of two large loops in the *Pt*CRC II 1 (IP_3R) molecule (Ladenburger et al., 2006) (Fig. 3A).

Evaluation by very recently developed data based algorithms for the determination of transmembrane domains as specified (Ladenburger and Plattner, 2011), has indicated that *Pt*CRCs, including RyR LPs, possess six transmembrane domains (TMD) previously a matter of debate. Thus, *Pt*RyR LPs differ from the most widely maintained assumption of only four TMDs. A more recent computational evaluation of the mammalian RyR came to the same conclusion (Ramachandran et al., 2013). Domains for tetramerization are available also in the *Pt*CRC molecules (Ladenburger and Plattner, 2011).

From their occurrence in protozoa, together with the occurrence of mixed type molecules with overlapping characteristics of IP_3Rs and RyRs, we assume that some of the *Pt*CRCs may represent ancestral CRC types, close to such primeval forms. Also the amino acids composing the selectivity filter, i.e. Gly Ile Gly Asp, are identical in both types of *Pt*CRCs (Ladenburger and Plattner, 2011). This also occurs in other lower eukaryotes (Plattner and Verkhratsky, 2013) and, thus, is in contrast to the sequence Gly Val Gly Asp in the IP_3R of metazoan cells (Boehning, 2010). In *Paramecium*, the latter sequence occurs in *Pt*CRC I 1a, 1b, and 1c, all with and IP_3 BD (Ladenburger and Plattner, 2011), though its IP_3 binding capacity has not been probed experimentally as yet. Otherwise the diversification of these channels in *Paramecium* during evolution is quite similar to the diversification of other molecules pertinent to vesicle trafficking, such as SNAREs (Plattner, 2010). This also includes more or less diversification as well as partial elimination by pseudogenization.

3.3. *Pharmacology of IP_3R and RyR type CRCs and effect of injected cADPR and NAADP on cell function in Paramecium*

Although the mechanism of AED as a secretagogue (Plattner and Hentschel, 2006) is not known in detail, some exogenous polyamines are known to induce a SOCE mechanism and exocytosis in various metazoan cells (Williams, 1997; Gamberucci et al., 1998; Plattner and Klauke, 2001; Plattner and Hentschel, 2006). By contrast, caffeine and 4CmC are established activators of RyRs up to mammalian cells (Cheek and Barry, 1993; Westerblad et al., 1998). The amino acids necessary for 4CmC binding (Fessenden et al., 2006) are found in the *Pt*CRC IV molecule (Ladenburger et al., 2009). Assays for Ca^{2+} dependent ryanodine binding to isolated alveolar sacs gave no results. This is not surprising considering the largely aberrant pharmacology of ciliates (Plattner et al., 2009) and the absence of any evolutionary pressure for this plant toxin. Also the inhibitory effect of Li^+ on phenomena related to IP_3 in *Paramecium* (Ladenburger et al., 2006) are no stringent argument considering its pleiotropic effects, whereas the usefulness of compound U73122 as an inhibitor of phosphoinositide specific phospholipase C in ciliates (Leondaritis et al., 2011) has not yet been known at the time of those analyses.

It is well known that Ca^{2+} effects are strictly locally confined and rapidly counteracted by different mechanisms (see Section 1). This explains two observations: microinjection of an excess of Ca^{2+} into a *Paramecium* cell does not cause any exocytosis (Klauke and Plattner, 1997). Similarly a diffuse Ca^{2+} influx, as achieved by sudden increase of $[Ca^{2+}]_e$, does not result in exocytosis (Erxleben et al., 1997). Also injection of a likely activator of RyRs in mammalian cells (Zalk et al., 2007), cADPR, causes no trichocyst exocytosis, as is the case with injected NAADP, the putative activator of TPCs (Galione et al., 2009; Galione et al., 2010). The localization of such channels is not known for *Paramecium*. Since either compound changes contractile vacuole pulsations (Plattner et al., 2012), organelles regulating Ca^{2+} homeostasis, one may assume the occurrence of the respective target molecules. However, the proper target may not be reached by the injected

compounds, as described above for Ca^{2+}, and the effects achieved with the contractile vacuole may be due to Ca^{2+} activation from remote organelles. With *Paramecium* homogenates a $K_d \sim 3.5$ nM for NAADP binding was determined (Plattner et al., 2012). Although a variety of acidic organelles, which could harbor TPCs, are known from *Paramecium* (Wassmer et al., 2009) acidocalci somes have not yet been identified. Also unknown is whether vacuoles containing Ca/Mg phosphate crystals (Grover et al., 1997) would be dynamic Ca^{2+} stores or just waste disposal containers.

4. Intracellular calcium channels in parasitic protozoa

4.1. IP_3R in trypanosomatids

Ca^{2+} homeostasis in trypanosomes differs significantly from that in mammalian cells. There are no orthologs for receptor operated or store operated Ca^{2+} channels although the parasites possess orthologs for a putative voltage sensitive Ca^{2+} channel that, in the case of *T. brucei*, localizes to the flagellum (Oberholzer et al., 2011). No orthologs for Na^+/Ca^{2+} exchangers are present in trypanosomes. In contrast to the plasma membrane Ca^{2+} ATPase (PMCA) of higher eukaryotes, trypanosome PMCA apparently lacks a calmodulin BD and is also localized intracellularly (Lu et al., 1998; Luo et al., 2004). The sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase (SERCA) of trypanosomatids is insensitive to inhibi tors of mammalian SERCA ATPases, such as thapsigargin (Docampo et al., 1993; Vercesi et al., 1993; Furuya et al., 2001). In addition, trypanosomes possess an important acidic calcium store, the acidocalcisomes, which is rich in polyphosphate (Docampo et al., 2005).

Recent results (Huang et al., 2013) have indicated that the IP_3R of *T. brucei* is localized to acidocalcisomes rather than to the ER (Fig. 4A). The demonstration of the localization of the IP_3R in acidocalcisomes was obtained by tagging the C terminus of *Tb*IP_3R of procyclic trypomastigotes with an hemagglutinin tag using homologous recombination with the endogenous gene locus (Huang et al., 2013). The *Tb*IP_3R partially co localized with anti bodies against *T. brucei* acidocalcisome marker vacuolar proton pyrophosphatase (V H^+ PPase, or *Tb*VP1) (Fig. 4A). An additional punctate staining of *Tb*VP1 that did not co localize with *Tb*IP_3R was detected and could correspond to trafficking vesicles. It was described before that adaptor protein 3 (AP 3) complex is involved in sorting proteins, like *Tb*VP1, to acidocalcisomes from the Golgi or from endosomes in both *L. major* (Besteiro et al., 2008) and *T. brucei* (Huang et al., 2013). No co localization with *Tb*BiP, an ER marker (Bangs et al., 1993) with a clear reticular labeling (Fig. 4B), was detected, thus ruling out ER localization of the *Tb*IP_3R. The acidocalcisome localization was confirmed using specific antibodies against *Tb*IP_3R (unpublished results).

Proteomic analysis of contractile vacuole complex (Ulrich et al., 2011) and acidocalcisome fractions (unpublished) of *T. cruzi* provided evidence of the presence of the *Tc*IP_3R ortholog in these organelles. These results coincided with the punctate and vacuolar localization reported for *Tc*IP_3R by other authors (Hashimoto et al., 2013). These authors suggested an ER localization of *Tc*IP_3R although no clear co localization with *Tb*BiP antibodies was observed.

The acidocalcisome localization of *Tb*IP_3R led to test for Ca^{2+} release by IP_3 in permeabilized cells under conditions of optimal acidocalcisome function, i.e., in the presence of pyrophosphate (PPi). Addition of PP_i is necessary to acidify acidocalcisomes by the action of *Tb*VP1. This acidification allows Ca^{2+} uptake by the acidocalcisome Ca^{2+} ATPase, which is Ca^{2+}/H^+ countertransporting (it transports Ca^{2+} in exchange for H^+). Under such conditions, IP_3 addition results in significant Ca^{2+} release (Huang et al., 2013). Similar experiments were done with isolated acidocalcisomes resulting in significant Ca^{2+} release by IP_3 (Huang et al., 2013). Previous attempts to show Ca^{2+} release by IP_3 had been unsuccessful (Moreno et al., 1992a; Moreno et al., 1992b), which was difficult to explain considering that the parasites do have a phosphoinositide phospholipase C (PI PLC) (Furuya et al., 2000; Okura et al., 2005; Martins et al., 2010), the enzyme that cleaves phosphatidylinositol 4,5 bisphosphate to gen erate diacylglycerol and IP_3, and that IP_3 was detected in both *T. cruzi* (Moreno et al., 1992b) and *T. brucei* (Moreno et al., 1992a). When those experiments were done (1991 2) the presence of acidocalci somes (Vercesi et al., 1994; Docampo et al., 1995) and an acidocalci some vacuolar H^+ PPase in trypanosomes were not known, as they were discovered much latter (Scott et al., 1998; Rodrigues et al., 1999). The reason for the lack of Ca^{2+} release in those experiments was that permeabilization results in dilution of substrates (ATP, PP_i) and alkalinization of acidocalcisomes, as a result of lack of function of the proton pumps in the absence of ATP and PP_i.

The gene coding for *Tb*IP_3R (Tb927.8.2770) shares 41% amino acid identity with *T. cruzi* IP_3R (TcCLB.509461.90), and orthologs are also present in several *Leishmania* spp. (Prole and Taylor, 2011). Structural analysis (ELM and TMHMM servers) predicted five transmembrane domains in the C terminal region of these receptors. The ORFs of *T. brucei* and *T. cruzi* IP_3Rs predict 3099, and 3011 amino acid proteins, with apparent molecular weights of 343, and 337 kDa, respectively. Trypanosome IP_3Rs possess a series of conserved domains including putative suppressor domain like (SD), ryanodine receptor IP_3R homo logy (RIH), and RIH associated (RIAD) domains (Prole and Taylor, 2011). A motif for a Ca^{2+} specific selectivity filter (GVGD) (Boehning et al., 2001; Boehning, 2010) is present in the putative intraluminal loop between transmembrane domains at the C terminal region (Huang et al., 2013). This sequence resembles that of IP_3Rs in higher

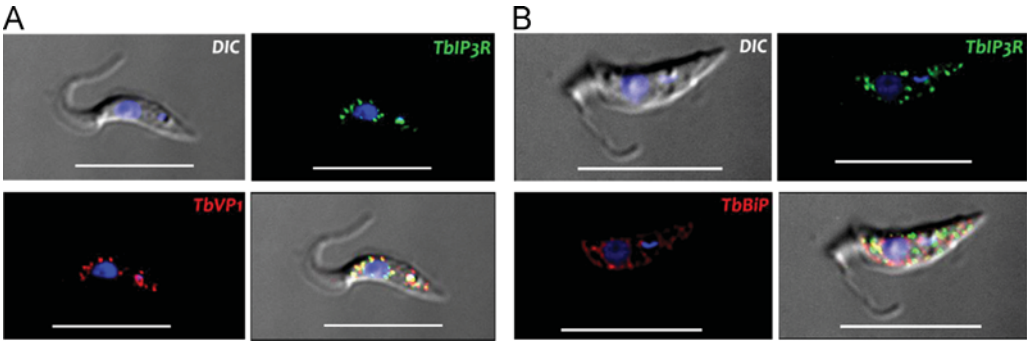

Fig. 4. Localization of *Tb*IP_3R in procyclic trypomastigotes. (A) *Tb*IP_3R partially co-localizes with *Tb*VP1 in acidocalcisomes (Pearson's correlation coefficient of 0.874), as shown by immunofluorescence microscopy analysis. The merge images show the co-localization in yellow. (B) Lack of co-localization of *Tb*IP_3R with *Tb*BiP in the endoplasmic reticulum (Pearson's correlation coefficient of 0.156). Scale bars, 10 μm. DIC, differential interference contrast. From (Huang et al., 2013).

eukaryotes (Boehning, 2010) and also occurs in *Pt*CRC I type channels (with a putative IP_3 BD), opposite to *Pt*CRC II channels (with an established IP_3 BD) (Ladenburger and Plattner, 2011; Plattner and Verkhratsky, 2013). Of the 10 residues that have been proposed to form a basic pocket that binds IP_3 (Yoshikawa et al., 1996; Bosanac et al., 2002), four are conserved in *Tb*IP_3R. Other features of trypanosomatid IP_3Rs have been described before (Prole and Taylor, 2011).

In addition to the studies on permeabilized trypanosomes (Huang et al., 2013) functional analyses of *Tb*IP_3R (Huang et al., 2013) and *Tc*IP_3R (Hashimoto et al., 2013) were also done by stable transfection of the respective genes in a chicken B lymphocyte cell line (DT40, R23 11) in which the genes for all three vertebrate IP_3Rs have been stably ablated (DT40 3KO) (Miyakawa et al., 1999). Both *Tb*IP_3R and *Tc*IP_3R1 localized to the ER of DT40 KO cells, and Ca^{2+} release by IP_3 was investigated using permeabilized cells (Huang et al., 2013), microsomal vesicles, or intact cells stimulated by anti B cell receptor monoclonal antibodies (Hashimoto et al., 2013). Microsomal vesicles from DT40 KO cells expressing *Tc*IP_3R also exhibited IP_3 binding activity (Hashimoto et al., 2013). *Tc*IP_3R was also expressed in HeLa cells, where it localized to the ER, and these permeabilized cells also exhibited Ca^{2+} release in the presence of IP_3 (Hashimoto et al., 2013). *Tb*IP_3R was found to be considerably less sensitive to IP_3 than the rat IP_3R1 (*Rn*IP_3R1) transfected in DT40 3KO cells (Huang et al., 2013). Ca^{2+} release by IP_3 was also investigated in live *T. brucei* procyclic trypomastigotes loaded with Fluo 4 AM with caged IP_3 (Huang et al., 2013). In cells loaded with caged IP_3 there were rapid Ca^{2+} increases after UV flashes to release free IP_3 (Fig. 5), and these increases were considerably reduced when using trypanosomes in which the expression of *Tb*IP_3R was downregulated by RNAi (Huang et al., 2013).

To study the importance of *TbIP$_3$R* and *TcIP$_3$R* in the biology of trypanosomes, several strategies were used. Knockdown of *TbIP$_3$R* expression was done by induction of RNAi and resulted in growth defects in both bloodstream and procyclic trypomastigotes (Huang et al., 2013). Knockdown of the expression of *TbIP$_3$R* in procyclic forms by RNAi reduced the ability of IP_3 to release Ca^{2+} from permeabilized cells and reduced the virulence of bloodstream forms in vivo (Huang et al., 2013). Knockdown of *TcIP$_3$R* was done by single knockout in epimastigotes of the Tulahuen strain. Attempts to obtain *null* mutants in this or in the Esmeraldo strain were unsuccessful, suggesting the essentiality of this gene (Hashimoto et al., 2013). *TcIP$_3$R* knockdown resulted in deficient growth of epimastigotes, deficient metacyclogenesis (transformation of epimastigotes into metacyclic trypomastigotes), deficient host cell invasion by trypomastigotes associated with reduced Ca^{2+} release upon their attachment to the host cells, deficient replication of amastigotes, increased transformation of amastigotes into trypomastigotes, and defects in virulence in vivo (Hashimoto et al., 2013). Overexpression of *TcIP$_3$R* also resulted in deficient growth of epimastigotes and amastigotes, and deficient metacyclogenesis, suggesting that an appropriate level of this receptor is necessary for these processes (Hashimoto et al., 2013). In contrast, overexpression of *TcIP$_3$R* resulted in increased host cell invasion by trypomastigotes associated with increased Ca^{2+} release upon their attachment to host cells, and decreased transformation of amastigotes into trypomastigotes, with no changes in virulence in vivo except for an early appearance of parasitemia (Hashimoto et al., 2013).

In conclusion, these works (Hashimoto et al., 2013; Huang et al., 2013) clearly established the presence of a functional IP_3 receptor in *T. brucei* and *T. cruzi*, and together with previous reports (Docampo and Pignataro, 1991; Moreno et al., 1992a; Moreno et al., 1992b; Furuya et al., 2000; Okura et al., 2005; de Paulo Martins et al., 2010; Martins et al., 2010), the function of a complete IP_3/diacylglycerol pathway in trypanosomes.

Although acidocalcisomes were initially described almost 20 years ago in *T. brucei* (Vercesi et al., 1994), the mechanism for Ca^{2+} release from these organelles was unknown until now. The localization of the IP_3 receptor in acidocalcisomes (Huang et al., 2013) provides the long sought mechanism for Ca^{2+} release from these organelles.

4.2. *Intracellular Ca^{2+} channels in Toxoplasma gondii*

Measurements of intracellular Ca^{2+} levels have been done in *T. gondii* extracellular tachyzoites using the Ca^{2+} dye Fura 2 AM (Fura 2/acetomethoxy) and values of 60 100 nM were obtained (Moreno and Zhong, 1996). The ER and acidocalcisomes were identified as the largest Ca^{2+} stores. A SERCA type Ca^{2+} ATPase, which is present in the ER (Nagamune et al., 2007) and inhibited by thapsigargin (Moreno and Zhong, 1996), is the main Ca^{2+} uptake mechanism in this compartment. In *T. gondii*, the presence of intracellular Ca^{2+} stores responsive to IP_3, ryanodine (Lovett et al., 2002) and cADPR (Chini et al., 2005; Nagamune et al., 2008), have been described but there is no genetic evidence for the presence of IP_3 or ryanodine gated channels (Nagamune and Sibley, 2006; Plattner and Verkhratsky, 2013). This is despite the evidence for the presence of enzymes involved in the generation of some of these second messengers such as a phosphoinositide phospholipase C (Fang et al., 2006) and cADPR cyclase and hydrolase activities (Chini et al., 2005). Acidocalcisomes of *T. gondii* possess a PMCA type Ca^{2+} ATPase (*Tg*A1) for Ca^{2+} uptake (Luo et al., 2001; Rohloff et al., 2011), but their mechanism of Ca^{2+}

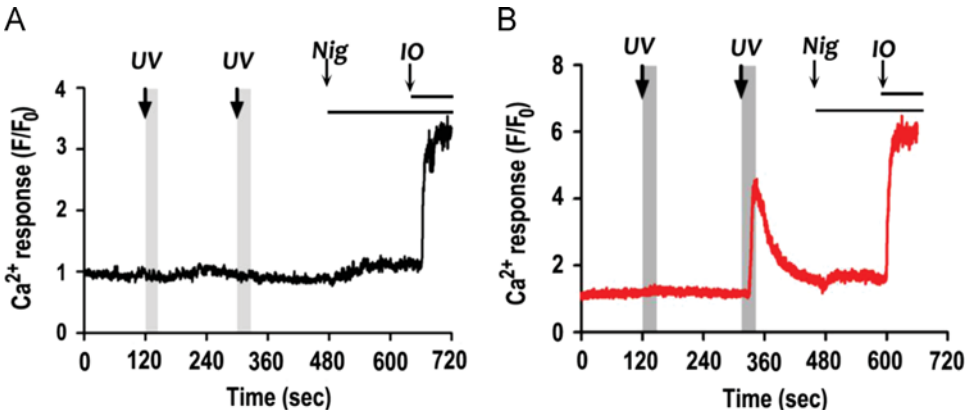

Fig. 5. Caged IP_3-dependent Ca^{2+} release in *T. brucei*. (A and B) Representative traces of Ca^{2+} responses to UV flash in control cells in the absence (A) and presence (B) of caged IP_3, respectively (first flash 3 pulses, second flash 6 pulses). Nigericin (Nig; 5 μM) and Ionomycin (IO; 5 μM) were added where indicated. From (Huang et al., 2013).

release is unknown. Evidence for a Ca^{2+}/H^{+} exchanger in these acidocalcisomes has also been reported (Rohloff et al. 2011). The recently described plant like vacuole (PLV) (Miranda et al., 2010) was also found to be rich in Ca^{2+} and also possesses the PMCA type Ca^{2+} ATPase (TgA1) for Ca^{2+} uptake. A Ca^{2+}/H^{+} exchanger is also present in the PLV (Miranda et al., 2010), but the mechanism of Ca^{2+} release is also unknown. There is no genetic evidence for the presence of a mitochondrial calcium uniporter (MCU) in any Apicomplexan parasite (Bick et al., 2012) and the role of the mitochondria in Ca^{2+} regulation is not clear. A Ca^{2+}/H^{+} antiporter is apparently present in the mitochondria of *T. gondii* (Guttery et al., 2013). Ca^{2+} entry into tachyzoites is regulated (Pace et al., submitted for publication). Other unexplored sources of Ca^{2+} in *T. gondii* are Golgi complex, apicoplast, inner membrane complex (IMC), and secretory organelles.

T. gondii is unique among parasitic protozoa in possessing an ortholog to two pore channels (TPCs), for which there are no orthologs in other Apicomplexans (Prole and Taylor, 2011). This TPC has substantial similarity to mammalian TPCs in the pore region responsible for ion conduction, suggesting that it may act as Ca^{2+} permeable channel (Prole and Taylor, 2011). However, the localization and function of this channel have not been reported. Mammalian TPCs can be Ca^{2+} channels gated by NAADP, and localized in lysosome like compartments (Brailoiu et al., 2009; Calcraft et al., 2009; Zong et al., 2009). Their function as Ca^{2+} channels and their stimulation by NAADP have been disputed (Wang et al., 2012; Cang et al., 2013), although the reason for this discrepancy is apparently that tagging the N terminal region of the channel abolishes its sensitivity to NAADP (Churamani et al., 2013). Fig. 6 shows a schematic representation of Ca^{2+} distribu tion in tachyzoites.

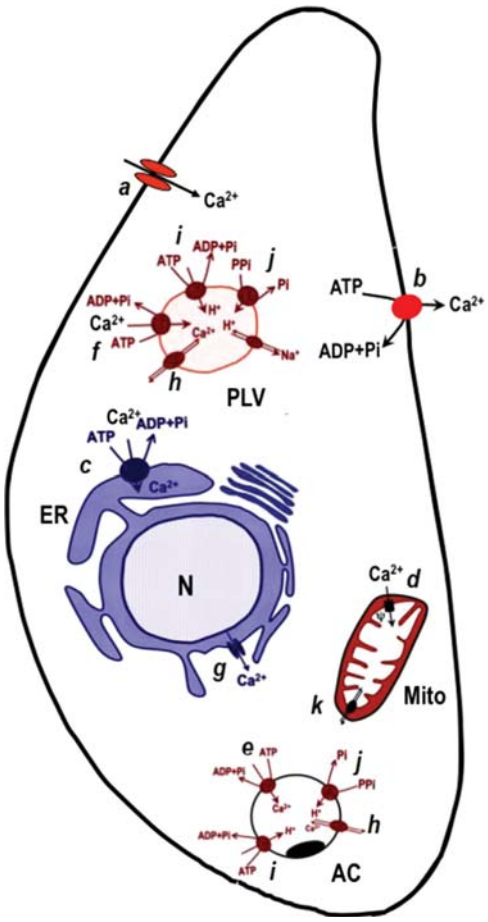

Fig. 6. Schematic representation of the distribution of Ca^{2+} in a *T. gondii* tachyzoite. Ca^{2+} entry is probably through Ca^{2+} channels (a). Once inside the cells, Ca^{2+} can be translocated back to the extracellular environment, primarily by the action of the PMCA (b). In addition, Ca^{2+} will interact with binding proteins or become sequestered by the ER by the action of the SERCA-Ca^{2+}-ATPase (c), passively sequestered by the mitochondrion (d), or sequestered by the acidocalcisome (e) or the PLV (f) by the action of a Ca^{2+}-ATPase (*Tg*A1). Ca^{2+} appears to diffuse freely into the nucleus. Calcium could also be released into the cytoplasm from the internal stores, such as the ER, through an uncharacterized channel, which appears to respond to IP_3, ryanodine and caffeine (g). It may also be released from the PLV and the acidocalcisome (AC) through a Ca^{2+}/H^{+} exchanger (h). Acidic compartments such as the acidocalcisome and the PLV contain enzymes involved in their acidification e.g. the H^{+}- ATPase (i) and the vacuolar-H^{+}-pyrophosphatase (j). Mitochondrial Ca^{2+} release is through a Ca^{2+}/H^{+} exchanger (k).

4.3. Mitochondrial calcium uniporter

Mammalian mitochondria have been shown to have a central role in intracellular Ca^{2+} homeostasis, and it is well established that intramitochondrial Ca^{2+} concentration can rapidly reach tens or hundreds micromolar values upon cytosolic Ca^{2+} rises of a few micromolar (Rizzuto et al., 1993; Montero et al., 2000). This is because mitochondria are exposed to microdomains of high Ca^{2+} concentration in proximity to sites of Ca^{2+} release at the ER, or to Ca^{2+} channels at the plasma membrane (Rizzuto et al., 1993; Rizzuto et al., 1998; Csordas et al., 1999; Montero et al., 2000; Csordas et al., 2010; Giacomello et al., 2010). This Ca^{2+} uptake is important for shaping the amplitude and spatio temporal patterns of cytosolic Ca^{2+} increases (Boitier et al., 1999; Hajnoczky et al., 1999; Tinel et al., 1999) and for regulating the activity of three intramitochondrial dehydrogenases that result in increased ATP generation (Denton and McCormack, 1990; McCormack et al., 1990; Hajnoczky et al., 1995; Jouaville et al., 1999; Voronina et al., 2010), as well as in stimulating the activity of the mitochon drial ATP synthase (Balaban, 2009). Ca^{2+} also regulates mitochon drial transporters in the inner membrane (Lasorsa et al., 2003; Satrustegui et al., 2007). Excessive Ca^{2+} uptake, however, favors the formation of the "permeability transition pore" leading to the release of pro apoptotic factors in the cytosol and cell death (Kroemer et al., 2007).

The ability of mitochondria to take up Ca^{2+} was discovered more than 50 years ago (De Luca and Engstrom, 1961; Vasington and Murphy, 1962) and the channel biophysical properties were well characterized in a patch clamp study of mitoplasts (mitochondria devoid of the outer mitochondrial membrane) (Kirichok et al., 2004). However, the molecular nature of the uniporter remained unknown for decades.

For many years after the discovery of the MCU in mammalian mitochondria, it was thought that less complex life forms such as plants, insects and other invertebrates, or unicellular organisms such as yeast, lacked a specific mitochondrial calcium uptake pathway (Carafoli and Lehninger, 1971). However, in 1989 it was reported (Docampo and Vercesi, 1989a,b) that epimastigotes of *T. cruzi* possesses a MCU with characteristics similar to those described in mammalian mitochondria: electrogenic transport, sensitivity to ruthenium red, and low affinity for the cation. The evidence of the presence of a MCU in trypanosomes but its absence in yeast was the key to the discovery of the molecular identity of MCU. An elegant study by Perocchi and coworkers (Perocchi et al., 2010) first identified a gene called *mitochondrial calcium uptake 1* or *MICU1* as encoding a potential regulator of the uniporter. The study was based on the observation that the Ca^{2+} uniporter was detected in *T. cruzi* (Docampo and Vercesi, 1989a,b) and *Leishmania donovani* (Vercesi and Docampo, 1992) yet not measurable in the yeast *Saccharomyces cerevisiae* (Balcavage et al., 1973). From a library of 1000 mouse mitochondrial proteins, 18 candidate genes were identified that have homologs in vertebrates and trypanosomes but not in yeast (Perocchi et al., 2010). Using

short hairpin (sh)RNA silencing of 13 selected genes in a commercially available HeLa cell line that stably expresses a mitochondria targeted aequorin (mt AEQ) as a reporter of Ca^{2+} uptake, they identified MICU1 as an important component in Ca^{2+} uptake. Based on the finding of Perocchi et al. (Perocchi et al., 2010), De Stefani et al. (De Stefani et al., 2011) and Baughman et al. (Baughman et al., 2011) used a similar strategy of comparing between mitochondrial genomes of trypanosomes and yeast and performing RNAi experiments of the identified genes and found a gene encoding a protein with all the characteristics of the mitochondrial calcium uniporter (MCU).

The MCU was also found in other trypanosomatids including *T. brucei* (Docampo and Lukes, 2012). The finding of a MCU uniporter in the bloodstream stage of *T. brucei* (Vercesi et al., 1992) was surprising because these stages lack a respiratory chain. However, mitochondrial Ca^{2+} uptake can also be energized by ATP in the absence of respiration, in which case it is inhibited by oligomycin, and not by inhibitors of the respiratory chain (Lehninger et al., 1963). This phenomenon also occurs in blood stream trypomastigotes: the mitochondrial membrane potential is dependent on hydrolysis of ATP by the ATP synthase which acts as an ATPase (Nolan and Voorheis, 1992; Vercesi et al., 1992; Schnaufer et al., 2005; Brown et al., 2006) allowing for Ca^{2+} to still be electrophoretically transported by the MCU (Vercesi et al., 1992). Ca^{2+} uptake by bloodstream forms of *T. brucei* has three characteristics: (1) it occurs until the ambient free Ca^{2+} concentration is lowered to 0.6 0.7 μM; (2) it is inhibited by oligomycin; and (3) it is associated with the depolarization of the inner membrane energized by ATP. These results indicate that Ca^{2+} uptake is mediated by the ATPase dependent energization of the inner mitochondrial membrane (Vercesi et al., 1992).

Although present in trypanosomatids and other protozoa, such as *Tetrahymena thermophila,* and *Naegleria gruberi,* the MCU is absent in Apicomplexan parasites such as *T. gondii* or malaria parasites (Bick et al., 2012).

The roles of mitochondrial Ca^{2+} in trypanosomes are apparently more limited than in mammalian cells. None of the dehydrogenases stimulated by Ca^{2+} in vertebrates have been studied in detail in trypanosomatids and there is no evidence of their stimulation by Ca^{2+} (Docampo and Lukes, 2012). Experiments using aequorin targeted to the mitochondria of *T. brucei* procyclic trypomastigotes revealed that intramitochondrial Ca^{2+} concentrations can reach values much higher than cytosolic Ca^{2+} rises when Ca^{2+} influx through the plasma membrane or Ca^{2+} release from acidocalcisomes are stimulated (Xiong et al., 1997). These results suggest a very close proximity of these organelles and the presence of microdomains of high Ca^{2+} concentration in the vicinity of the plasma membrane and acidocalcisomes (Xiong et al., 1997) and are in agreement with the presence of an IP_3R in acidocalcisomes (Huang et al., 2013). Because the ER type Ca^{2+} ATPase (SERCA) of *T. brucei* is insensitive to thapsigargin, a microdomain of high Ca^{2+} concentration between the ER and the mitochondria could not be established in those studies (Xiong et al., 1997). However, these results suggest that one of the main functions of the MCU in procyclic trypomastigotes would be to shape the amplitude and spatio temporal patterns of cytosolic Ca^{2+} increases. No similar studies have been done with bloodstream trypomastigotes. Fig. 7 shows a scheme of the close contact that would exist between acidocalcisomes and the unique mitochondrion of trypanosomes.

Mitochondrial Ca^{2+} could also be a contributor to programmed cell death, or apoptosis like death, in trypanosomatids. Trypanosomatids lack some of the key regulatory or effector molecules involved in apoptosis in mammalian cells, such as the tumor necrosis factor (TNF) related family of receptors, Bcl 2 family members, and caspases (Ridgley et al., 1999; Smirlis et al., 2010; Kaczanowski et al., 2011). Mitochondrial Ca^{2+} overload with changes in mitochondrial membrane potential, reactive oxygen species (ROS) generation and release of cytochrome *c* have been observed upon different triggers of cell death in some trypanosomatids (Smirlis and Soteriadou, 2011). In *T. brucei* procyclic trypomastigotes, the production of ROS impairs

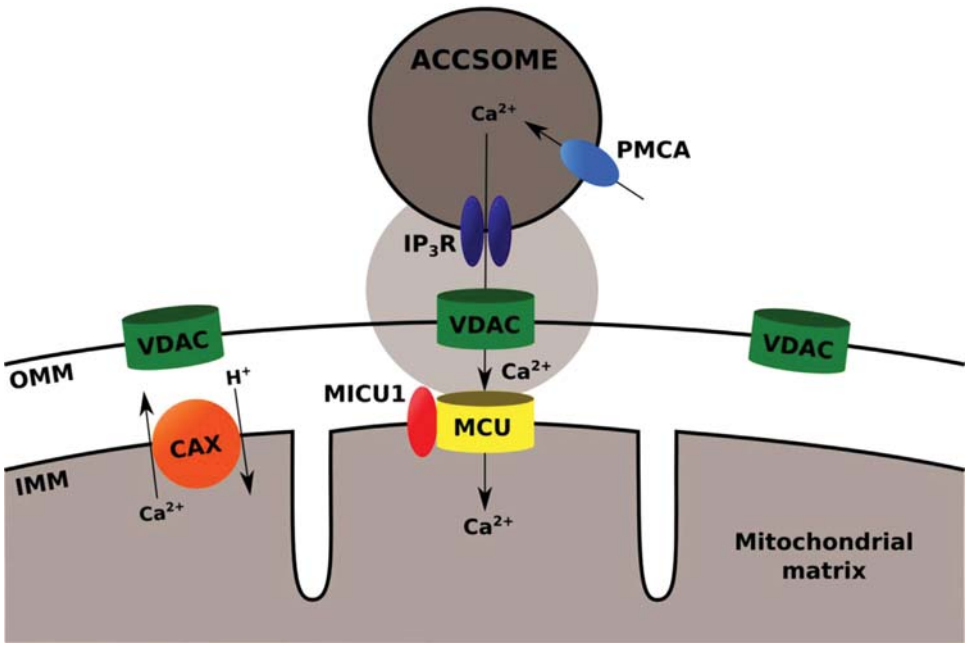

Fig. 7. Scheme of the potential contact between acidocalcisomes and mitochondrion in trypanosomes. The scheme depicts the molecules mediating Ca^{2+} influx (MCU, MICU1) and efflux (Ca^{2+}/H^+ exchanger, CAX) across the inner mitochondrial membrane (IMM) at an area of acidocalcisome (ACCSOME)-mitochondrial association. The shades of gray represent the [Ca^{2+}]: dark gray: > 500 μM; white, 100 nM; PMCA, plasma membrane-type Ca^{2+}-ATPase; OMM, outer mitochondrial membrane; VDAC, voltage-dependent anion-selective channel; IP_3R, inositol 1,4,5-trisphosphate receptor.

mitochondrial Ca^{2+} transport, leading to its accumulation in the nucleus, and causing cell death (Ridgley et al., 1999).

In summary, mitochondrial Ca^{2+} uptake in trypanosomes appears to have a role in shaping the amplitude of cytosolic Ca^{2+} increases after influx through the plasma membrane or release from acidocalcisomes, and in apoptosis like death, but it is not known whether it has a role in the regulation of ATP production.

4.4. Pharmacology of CRCs in parasitic protozoa

TbIP_3R does not respond to 10 μM NAADP or 1 μM cADPR when expressed in DT40 3KO cells (Huang et al., 2013). cADPR was also ineffective in producing Ca^{2+} release in permeabilized HeLa cells transfected with *TcIP$_3$R* (Hashimoto et al., 2013). In contrast, the IP_3 agonist adenophostin A (0.5 μM) showed a Ca^{2+} release that was comparable to that of 10 μM IP_3 in the *TbIP$_3$R* expressing DT40 3KO cells or in permeabilized *T. brucei procyclic* stages (Huang et al., 2013).

There is pharmacological evidence for the presence of channels responsive to IP_3 in *T. gondii* and malaria parasites (Passos and Garcia, 1998; Lovett and Sibley, 2003; Alves et al., 2011) although there are no gene orthologs to the mammalian IP_3R or RyR in any of the Apicomplexan genomes (Nagamune and Sibley, 2006; Plattner et al., 2012). There is an ER Ca^{2+} pool, sensitive to thapsigargin, and the Ca^{2+} ATPase involved in pumping Ca^{2+} from the cytosol into the ER has been characterized (Nagamune et al., 2007).

4.5. Calcium signaling and function in parasitic protozoa

Although Ca^{2+} signaling appears to be important for several functions in *T. cruzi* such as host cell invasion (Moreno et al., 1994), multiplication and differentiation (Lammel et al., 1996), osmo regulation (Rohloff et al., 2003), and programmed cell death (Irigoin et al., 2009), there is much less information on the role of Ca^{2+} in *T. brucei*. Based on the use of Ca^{2+} ionophores, roles for Ca^{2+} in the release of the bloodstream stage surface coat (Bowles and Voorheis, 1982), and in the maintenance of the cytoskeleton (Selzer et al., 1991) have been proposed. Changes in cytosolic Ca^{2+} levels have also been reported during differentiation from blood stream to procyclic stages of *T. brucei* (Stojdl and Clarke, 1996). Results obtained with *TbIP$_3$R* (Huang et al., 2013) and *TcIP$_3$R* (Hashimoto et al., 2013) knockdowns indicate that Ca^{2+} signaling through the trypanosome IP_3Rs has roles in growth in vitro and in vivo, as well as in cell differentiation. The localization of the *Tb*IP_3R in acidocalcisomes (Huang et al., 2013) also supports a role for acidocalcisomes in Ca^{2+} signaling.

Several studies have looked into the role of Ca^{2+} signaling during the lytic cycle of *T. gondii*. Using Ca^{2+} ionophores, Ca^{2+} chelating agents and ethanol, a link between Ca^{2+} and conoid extrusion was demonstrated (Mondragon et al., 1994; Mondragon and Frixione, 1996; Del Carmen et al., 2009), although no direct parallel Ca^{2+} measurements were reported. The effect of Ca^{2+} on gliding motility was studied on trypsin permeabilized tachyzoites (Mondragon and Frixione, 1996) or by measuring Ca^{2+} oscillations in extracellular tachyzoites loaded with the Ca^{2+} dye Fluo 4 AM and studying their correlation with gliding motility (Lovett and Sibley, 2003; Wetzel et al., 2004). The role of Ca^{2+} in microneme secretion was studied in extracellular tachyzoites following the effects of Ca^{2+} ionophores or chelators on protein secretion as evaluated by western blot analyses (Carruthers et al., 1999). A role for Ca^{2+} signaling in invasion was postulated on the basis of the analysis of changes in Ca^{2+} occurring in parasites loaded with Fura 2 AM upon their attachment to host cells (Vieira and Moreno, 2000) or by detecting the cessation of Ca^{2+} oscillations in extracellular tachyzoites loaded with Fluo 4 AM (Lovett and Sibley, 2003). These and more recent studies have provided indirect evidence that Ca^{2+} signaling is part of the pathways that result in the stimulation of conoid extrusion, gliding motility, microneme secretion, and invasion. These traits, which are impor tant steps of the lytic cycle of the parasite are enhanced by extracellular Ca^{2+} (Pace et al., submitted for publication). Ca^{2+} signaling was also proposed to be part of the pathway leading to parasite egress from the host cells based on the effect of Ca^{2+} ionophores, which stimulate egress, although it was never demon strated that $[Ca^{2+}]_i$ increases in tachyzoites before egress (Endo et al., 1982; Garrison et al., 2012). In summary, most of the studies on Ca^{2+} signaling were done with extracellular tachyzoites (con oid extrusion, gliding motility, microneme secretion, invasion) measuring indirectly the involvement of Ca^{2+} (using Ca^{2+} chela tors or ionophores) or detecting Ca^{2+} changes with fluorescent dyes independently of the phenomena examined.

5. Conclusions and perspectives

On the one hand there are many similarities in Ca^{2+} signaling in the protozoa discussed here, but on the other hand there also occur considerable differences. Differences concern the drug sensitivity of the SERCA, the absence or the occurrence of a calmodulin BD in the PMCA (typical for higher eukaryotes) and the occasional occurrence of this pump in Ca^{2+} storage organelles. Interestingly, a SOCE mechanism has been verified with some protozoa analyzed so far, based in part on the activation of RyR LPs (Ladenburger et al., 2009). There is, however, no evidence of the presence of Orai or Stim orthologs in either *Paramecium* (Ladenburger and Plattner, 2011) or parasitic protozoa (EuPathDB.com). Acidocalcisomes are present is some (trypanosomatids, Apicomplexan) but not in other parasitic protozoa (like *Giardia*, *Trichomonas*, *Entamoeba*) (Docampo et al., 2005) while it is not yet known if they are present in *Paramecium* (Plattner et al., 2012). IP_3R orthologs are present in trypanosomatids (Huang et al., 2013; Hashimoto et al., 2013) and *Paramecium* (Ladenburger et al., 2006; Plattner and Verkhratsky, 2013) but unknown in Apicomplexa.

The range of CRC molecules, type IP_3Rs/RyR in *Paramecium* and of IP_3Rs in *Monosiga brevicollis,* and trypanosomatids indicates that protozoa already have evolved this Ca^{2+} signaling pathway. Except in ciliates, only IP_3Rs have been unambiguously identified; none have been identified in Apicomplexa in striking contrast to their response to IP_3 (which, thus, still awaits elucidation). In ciliates some IP_3Rs and RyR LPs display mixed features of the two. Concomitantly, in *Paramecium* some of the CRCs can be clearly attributed to either IP_3Rs and to RyR LPs, all CRCs are differentially localized and as far as analyzed can be differentially activated. In *Paramecium* and other protozoa the selectivity filter in both types of CRCs, IP_3R and RyR like channels (or their putative homologs) is identical for most paralogues (Ladenburger and Plattner, 2011; Plattner and Verkhratsky, 2013). By contrast the parasitic flagellates have the same amino acid sequence in the selectivity filter of their IP_3R (Huang et al., 2013) as occurring in metazoa (Boehning, 2010). Evidently selective enrichment of Ca^{2+} in the stores can suffice to guarantee selective release of Ca^{2+} as a second messenger. A RyR type CRC seems to be absent from Apicomplexa; none has been identified in choanoflagellates and in the parasitic flagellates discussed here, whereas their occur rence in other protists has to be left open for the time being (Ladenburger and Plattner, 2011; Plattner and Verkhratsky, 2013). Also the number of transmembrane domains in RyR LPs, five or six being proposed depending on the database analysis method applied, requires experimental verification.

The presence of the mitochondrial calcium uniporter in a number of protozoa indicates that mitochondrial regulation of Ca^{2+} signaling is also an early appearance in evolution. There is only circumstantial or sequence evidence for the occurrence of TPCs, TRPCs and of intracellular mechano sensitive Ca^{2+} channels in *Paramecium* and in parasitic protozoa. This remains a vast field for further explorations.

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