Ca^{2+} signalling early in evolution – all but primitive

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Summary

Early in evolution, Ca^{2+} emerged as the most important second messenger for regulating widely different cellular functions. In eukaryotic cells Ca^{2+} signals originate from several sources, i.e. influx from the outside medium, release from internal stores or from both. In mammalian cells, Ca^{2+}-release channels represented by inositol 1,4,5-trisphosphate receptors and ryanodine receptors (InsP_3R and RyR, respectively) are the most important. In unicellular organisms and plants, these channels are characterised with much less precision. In the ciliated protozoan, *Paramecium tetraurelia*, 34 molecularly distinct Ca^{2+}-release channels that can be grouped in six subfamilies, based on criteria such as domain structure, pore, selectivity filter and activation mechanism have been identified. Some of these channels are genuine InsP_3Rs and some are related to RyRs. Others show some – but not all – features that are characteristic for one or the other type of release channel. Localisation and gene silencing experiments revealed widely different – yet distinct – localisation, activation and functional engagement of the different Ca^{2+}-release channels. Here, we shall discuss early evolutionary routes of Ca^{2+}-release machinery in protozoa and demonstrate that detailed domain analyses and scrutinised functional analyses are instrumental for in-depth evolutionary mapping of Ca^{2+}-release channels in unicellular organisms.

Key words: Ca^{2+}, Calcium, Ca^{2+}-release channels, Protist, Protozoa

Introduction

In metazoan cells, intracellular Ca^{2+}-release channels (CRCs) are instrumental for local signalling on which specific membrane-to-membrane interactions are known to depend (Berridge et al., 2000; Berridge et al., 2003; Burgoyne and Clague, 2003; Petersen et al., 2005; Verkhratsky, 2005; Clapham, 2007; Hay, 2007; Dodd et al., 2010). This is particularly true for inositol 1,4,5-trisphosphate receptors and ryanodine-receptor type (InsP_3R and RyR, respectively) CRCs. Evolutionary roots of these intracellular Ca^{2+} channels are of particular interest because their development most probably defined the polarisation and spatial specificity of Ca^{2+} signalling. Molecules (i.e. channels) that are responsible for Ca^{2+} entry and extrusion through the cell membrane have been identified in prokaryotes, and are extensively developed in most primitive metazoans, such as for example choanoflagellates (Cai, 2008). The origins of CRCs, however, are much less characterised. These intracellular channels have not been identified unambiguously in protozoa or in plants, despite several reports describing the physiological effects of, for instance, InsP_3 in parasitic and non-parasitic protists (Table 1). The InsP_3R-like protein (encoded by the *iplA* gene) was, for example, identified in *Dictyostelium discoideum*, and has been linked to autophagy, chemotaxis and, possibly, the regulation of Ca^{2+} signalling (Schaloske et al., 2005; Lam et al., 2008, Lusche et al., 2012). There is, however, no conclusive evidence that this protein acts as an intracellular Ca^{2+}-release channel. Hitherto, CRCs have been extensively characterised only in the ciliate *Paramecium tetraurelia* (Box 1) – and this was through detailed domain analysis rather than analysis of overall similarities, gene silencing and monitoring physiological effects, including Ca^{2+} dynamics (Ladenburger et al., 2006; Ladenburger et al., 2009; Ladenburger and Plattner, 2011). The CRCs family in *P. tetraurelia* is represented by 34 members (in contrast to only six members in vertebrates), which are surprisingly diverse in their molecular structure, subcellular distribution and physiology. Such a great diversification of CRC could have been instrumental for evolutionary fine-tuning of Ca^{2+}-signalling pathways. Considering the occurrence of fragments of related sequences with variable similarity in many protozoa, the expression of functional CRCs among unicellular organisms might be much more widespread than previously thought. In this Commentary, we shall systematically discuss the structure and function of CRCs in the context of the evolution of the Ca^{2+}-signalling machinery and outline strategies that may help to identify Ca^{2+}-release channels in other protozoa, including parasitic species.

Prokaryotic foundation of Ca^{2+} signalling

The tight regulation of intracellular Ca^{2+} concentration is a common feature of all life forms investigated thus far. This is most likely to reflect the importance of this ion for the form of life that emerged on earth some 3.5 billion years ago, and it has been speculated that Ca^{2+} is linked to ATP-centered energetic and the stability of DNA or RNA (Jaiswal, 2001; Case et al., 2007). At high concentrations (i.e. >1–10 μM), Ca^{2+} triggers the precipitation of phosphates, alters the integrity of lipid membranes and causes aggregation of proteins and nucleic acids (Jaiswal, 2001; Case et al., 2007; Williams, 2007); therefore, intracellular Ca^{2+} levels are kept at a relatively low concentration ($[Ca^{2+}]_i$ ~100 nM) compared with the extracellular fluid. The evolutionary basis for this might lie in the chemical

Table 1. Signalling trough CRCs in organisms of different evolutionary levels

Organism		Effect of InsP_3	Sequences similarities with InsP_3Rs	Sequences similarities with RyRs
Multicellular metazoa				
Mammals	*Mus musculus*	+	+	+
Echinoderms	*Asterina pectinifera*	+	+	+
Insects	*Drosophila melanogaster*	+	+	+
Nematodes	*Caenorhabditis elegans*	+	+	+
Flowering plants				
Thale cress	*Arabidopsis thaliana*	+	–	–
Fungi				
Ascomycetes	*Saccharomyces cerevisiae*	+	–	–
	Neurospora crassa	+	–	–
	Candida albicans	+	–	–
Oomycetes	*Phythophthora infestans*	+	+[a]	–
Unicellular organisms				
Algae				
Green algae	*Chlamydomonas reinhardtii*	+	+[a]	–
Chrysophytes	*Aureococcus anophagefferrens*	n.d.	+[a]	–
Red algae	*Cyanidioschyzon merolae*	n.d.	–	–
Euglenozoa	*Euglena gracilis*	+	–	–
Free-living protozoa				
Choanoflagellates	*Monosiga brevicollis*	n.d.	+[a]	-
Slime moulds	*Dictyostelium discoideum*	+	+[b]	-
Ciliates	*Paramecium tetraurelia*	+	+	+[c]
	Tetrahymena thermophila	+	+[a]	–[d]
Parasitic forms				
Apicomplexa	*Plasmodium falciparum*	+[e]	–	–
	Toxoplasma gondii	+[e]	–	–
Flagellates	*Leishmania major*	n.d.	+[a]	–
	Leishmania infantum	n.d.	+[a]	–
	Trypanosoma brucei	+[f]	+[f]	–
	Trypanosoma cruzi	+[g]	+[g]	–

For further details see Ladenburger et al., 2009 and references therein. n.d., not done.
[a]Only partial characterization available.
[b]Characterized by overall similarity without domain analysis (Traynor et al., 2000); pore domain not conserved.
[c]Characterized by domain and functional analysis (Ladenburger et al., 2009; Ladenburger and Plattner, 2011); see text for details.
[d]No detailed analysis available.
[e]For references, see review by Plattner et al., 2012.
[f]For recent analysis see Huang et al., 2013.
[g]For recent analysis see Hashimoto et al., 2013.

composition of the highly alkaline prebiotic ocean, which contained Ca^{2+} only at very low concentrations that are comparable with its characteristic cytosolic levels (Kazmierczak et al., 2013). Thus the primordial biochemistry evolved under conditions of low Ca^{2+}. Over the first billion years of evolution, prokaryotic life forms experienced a slow increase in the Ca^{2+} content of the ocean, therefore, initiating the development of molecular signalling cascades aimed at preserving low cytosolic Ca^{2+}. This, in turn, gave rise to the Ca^{2+} homeostatic machinery and its evolution. These environmental changes also lead to the appearance of Ca^{2+} gradients between membranes within a cell that are associated with dynamic intracellular Ca^{2+} stores, which comprise Ca^{2+} pumps and intracellular Ca^{2+}-release channels.

Ca^{2+} homeostasis and signalling systems are composed of cooperating transporters and aqueous channels that are well developed even in the most primitive life forms. In prokaryotes, the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ is maintained below 100 nM (Gangola and Rosen, 1987), which is achieved by intracellular buffering and the activity of several types of Ca^{2+} pump (Gambel et al., 1992; Kanamaru et al., 1993; Berkelman et al., 1994; Shemarova and Nesterov, 2005) and, possibly, ion exchangers (Ivey et al., 1993; Shemarova and Nesterov, 2005). In addition, prokaryotes also possess Ca^{2+}-permeable channels that allow for Ca^{2+} entry – a main source for their Ca^{2+} signalling, it seems (Matsushita et al., 1989; Durell and Guy, 2001; Shemarova and Nesterov, 2005). Ca^{2+} channels in prokaryotes are functionally similar to their eukaryotic analogues; for example, bacterial Ca^{2+} channels have comparable voltage-dependence and pharmacology. Similarly, prokaryotes already possess diverse Ca^{2+} channels, and low-voltage-activated channels that resemble T-channels in eukaryotes have been indentified in *Escherichia coli* (Tisa et al., 2000). Incidentally, the bacterial Na^+ channel NaChBac (cloned from *Bacillus halodurans*) is structurally similar to eukaryotic L-type Ca^{2+}-channels (Ren et al., 2001), which may indicate an early evolutionary appearance of Ca^{2+} channels. At the same time, bacteria contain Ca^{2+}-dependent K^+ channels, which most probably act as membrane sensors of Ca^{2+} signals (Franciolini and Petris, 1989). Against this background, CRCs may have evolved early in eukaryotic evolution.

Eukaryotic revolution: appearance of intracellular Ca^{2+} stores

During the evolution of eukaryotes, Ca^{2+} has become the most universal second messenger. Ca^{2+} signals are mounted in response to widely different stimuli and are represented by

Box 1. Structures and molecules that contribute to Ca^{2+} signalling in lower eukaryotes

Alveolata belong to a group that encompasses ciliates, Apicomplexa (parasites) and dinoflagellates.

Alveolar sacs are cortical Ca^{2+} stores that are endowed with SERCA-type Ca^{2+}-pumps and with CRCs of the type *P. tetraurelia* CRC-IV (which are distributed over the entire peripheral part of alveolar sacs membranes) and, to a lesser extent, with *Pt*CRC-V (which are restricted to contact sites between adjacent alveolar sacs).

Ca^{2+}-ATPases/pumps in *P. tetraurelia* were cloned and, by using immuno-labelling, found localized in the ER and to alveolar sacs.

Ciliates belong to a phylum of protozoa with numerous cilia and alveolar sacs. Ciliates, such as the model organisms *P. tetraurelia* and *Tetrahymena*, are closely related to the pathogenic phylum of Apicomplexa (e.g. *Plasmodium*, *Toxoplasma*).

Contractile vacuole complex (CVC) is a highly dynamic organelle with a complicated structure that is present in many protozoa. It comprises a contractile vacuole that swells periodically before it expels its fluid by exocytosis at the pore. The fluid is collected by radial arms (connecting canals). An H^+-gradient formed by the V-type H^+-ATPase serves for the chemiosmotic extrusion of water and ions such as Ca^{2+}. To fine-tune $[Ca^{2+}]$ the CVC in *P. tetraurelia* contains the InsP_3R CRC-II.

Ca^{2+}-release channels (CRCs) provide for the release of Ca^{2+} from stores and are located in various organelles. Important types of CRC in *P. tetraurelia* are the CRC-II, which acts as an InsP_3R, and the CRC-IV, which – in ciliates – can be activated by caffeine or by 4-chloro-*m*-cresol, and is similar to RyRs in other organisms, including mammals.

Food vacuoles are phagosomes that digest food particles (e.g. bacteria). Indigestible material is released at the cytoproct. Recycling vesicles provide for the formation of a new food vacuole. These require a local Ca^{2+} signal for interaction and fusion with the food vacuole.

Oral cavity is the site of food vacuole (phagosome) formation.

RyR–InsP_3R homology (RIH) domains are sequences that are conserved in both types of Ca^{2+}-release channel.

Selectivity filter is a pore region between the last two C-terminal transmembrane domains within the CRCs discussed in this Commentary. A short sequence of amino acids is the basis for their selectivity for bivalent cations. In higher eukaryotes, the amino acid sequences GVGD and GIGD have been established for InsP_3Rs and RyRs, respectively. In lower eukaryotes the sequence GIGD also occurs in InsP_3Rs.

Store-operated Ca^{2+}-influx (SOC) is activated by the stimulation of trichocyst exocytosis that causes the release of Ca^{2+} from alveolar sacs. These two components (Ca^{2+} release and Ca^{2+} influx) have been separated in quench-flow experiments combined with energy-dispersive X-ray microanalysis (EDX) employing analytical electron microscopy.

Trichocysts are dense-core secretory vesicles in *Paramecium*.

local increases in $[Ca^{2+}]_i$ by approximately two orders of magnitude. These localised changes in $[Ca^{2+}]_i$ regulate numerous different functions, from secretion (exocytosis), locomotion (including ciliary beat regulation) to cell division (Berridge et al., 2000; Clapham, 2007). Particularly intriguing is the spatial segregation of Ca^{2+} effects in association with local $[Ca^{2+}]$ (Neher, 1998). Such a mode of Ca^{2+} signalling is well established for mammals (Berridge et al., 2000; Berridge et al., 2003; Clapham, 2007), plants (Dodd et al., 2010) and protozoa, such as the social amoeba *D. discoideum* (Allan and Fisher, 2009) and the ciliate *P. tetraurelia* (Plattner and Klauke, 2001).

In contrast to prokaryotes, the Ca^{2+} signal in eukaryotes originates from Ca^{2+} entry through the cell membrane and from the release of intracellular Ca^{2+}. This reflects the most important evolutionary change, the appearance of organelle-associated Ca^{2+} stores in eukaryotes, which became the sources of intracellular Ca^{2+} for signalling. However, the exact origin of most of the organelles remains mostly speculative (Hedges, 2002). Invagination of the cell membrane either allowed for uptake and preservation of a bacterial genome, or sequestration of an endogenous genome, thus forming a nuclear compartment with increasing genomic information. This, in turn, allowed for the differentiation of structural elements and regulation processes and – with the nuclear envelope being connected with the endoplasmic reticulum (ER) – the ER evolving to be the predominant intracellular Ca^{2+} store throughout the subsequent evolution. Additional budding processes led to the appearance of diverse vesicles, some with considerable Ca^{2+} storage capacity (Burgoyne and Clague, 2003; Hay, 2007; Zampese and Pizzo, 2012). This allowed for increasingly differentiated interactions between vesicles and membranes, and necessitated regulators such as soluble N-ethylmaleimide-sensitive attachment protein receptor (SNARE) proteins and CRCs, thus matching the requirement of local signalling. However, whereas the evolution of SNAREs can be traced back to the common predecessor or 'Ur-eukaryote' (Kloepper et al., 2008), no such evolvement is known for Ca^{2+}-release channels.

The generation of extra- and intracellular Ca^{2+} signals is already present in protozoa; examples are *D. discoideum* (Allan and Fisher, 2009) and the ciliate *P. tetraurelia* (Machemer, 1988; Plattner and Klauke, 2001; Saimi and Kung, 2002). Some mechanisms of Ca^{2+} regulation, such as the inhibition of voltage-dependent Ca^{2+}-influx channels by Ca^{2+}/calmodulin (Brehm and Eckert, 1978; Saimi and Kung, 2002) have been maintained throughout evolution up to neuronal systems (Levitan, 1999). However, in contrast to the high number of data that stem from research of Ca^{2+}-influx channels at the plasmalemma, intracellular Ca^{2+}-release channels have remained elusive in the entire group of protozoa.

In mammalian cells, CRCs are represented by several subtypes; the most important CRCs are the InsP_3R and the RyRs (reviewed by Berridge et al., 2000; Berridge et al., 2003; Verkhratsky, 2005; Clapham, 2007). Both types of CRC are closely related and share some characteristic features (Taylor et al., 2004; Taylor et al., 2009; Boehning, 2010; Seo et al., 2012). However, CRCs have not been identified in many unicellular organisms or in plants (Gillaspy, 2011). Considering that choanoflagellates are at the roots of metazoans, it is of interest that database mining suggests for *Monosiga brevicollis* the absence of RyRs but the presence of InsP_3Rs (Cai, 2008). Similar suggestions emerged from database searches of a variety of protozoans. However, a more detailed analysis of protein domains, such as a near-N-terminal InsP_3-binding domain, a RyR-InsP_3R homology (RIH) domain and a C-terminal channel domain may deliver more stringent criteria in the future and help to identify CRCs in protozoans.

By using the *P. tetraurelia* genomic database (Aury et al., 2006; Arnaiz et al., 2007), genomic sequences that are indicative of CRCs with characteristic domain structures have been identified (Ladenburger et al., 2006; Ladenburger et al., 2009; Ladenburger and Plattner, 2011) (Fig. 1). Some of these included an InsP_3-binding domain, a RyR–InsP_3R homology (RIH) domain, and a channel domain with the pore and its selectivity

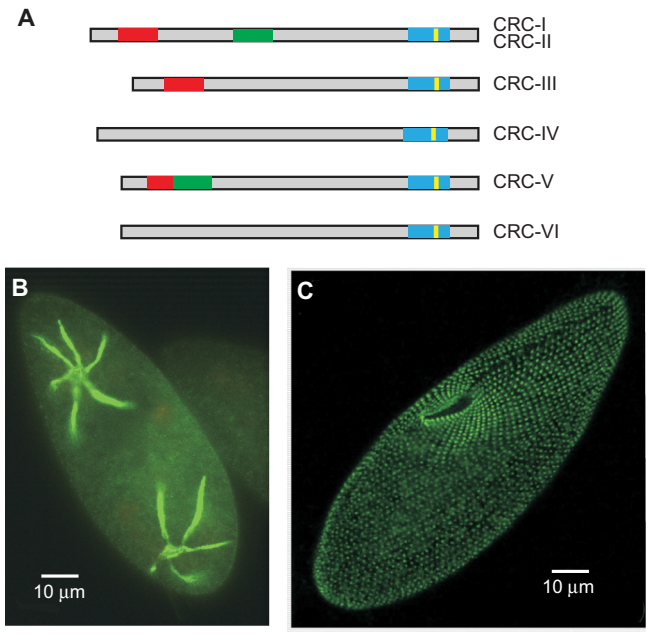

Fig. 1. Different Ca^{2+}-release channels in *P. tetraurelia*. (**A**) Schematic of the different CRCs in *P. tetraurelia*, created by using data from Ladenburger and Plattner (Ladenburger and Plattner, 2011). Receptor domains were identified by sequence similarity. InsP_3-binding domain (red), RyR–InsP_3R homology (RIH) domain (green). These domains can be absent from some of the paralogues of the respective group or subfamily, i.e. in members of the CRC-III and CRC-V groups (not specified here in detail) (for more information, see Ladenburger and Plattner, 2011). Blue, C-terminal part with transmembrane domains (TMDs) as predicted by using the TOPCONS algorithm (Bernsel et al., 2009); yellow, pore with selectivity filter within the TMD. (**B**,**C**) Micrographs of *P. tetraurelia* immunostained for (B) CRC-II (an InsP_3R) in the contractile vacuole complex and (C) CRC-IV (a RyR or RyR-like protein) in the alveolar sacs. In panel C, the repetitive arrangement of the cortical Ca^{2+} stores can be clearly identified. B was previously published in *Journal of Cell Science* (Ladenburger et al., 2006); C was reproduced from Ladenburger et al., 2009 with permission from American Society for Microbiology (Ladenburger et al., 2009).

filter (Fig. 2). All in all, six groups of putative CRCs have been cloned from *P. tetraurelia* (Fig. 1A), some of which show characteristics of InsP_3Rs (Ladenburger et al., 2006), others of RyRs (Ladenburger et al., 2009). Both groups of CRCs were, thus, unambiguously identified for the first time in protozoa, although an InsP_3R had been previously assigned to *D. discoideum* on the basis of overall similarity (Traynor et al., 2000). The CRCs in *P. tetraurelia* show some, but not all of the characteristic features of either receptor and/or release-channel type (Ladenburger and Plattner, 2011). Considering the ancient evolutionary origin of ciliates ~850 million years ago (Douzery et al., 2004) and of alveolate precursors between ~1650 and ~2000 million years ago (Hedges, 2002, Hedges et al., 2004, respectively), as well as the age of eukaryotes of ~2200 million years ago (Feng et al., 1997; Hedges, 2002; Hedges et al., 2004), one can assume that some of these channels are ancestral or close to primeval forms.

Ca^{2+}-release channels in *P. tetraurelia*

The diversification of *P. tetraurelia* CRCs into six subfamilies is without precedent. In total, its 34 InsP_3R-like and RyR-like CRCs (Fig. 1A) can be divided in six groups CRC-I to CRC-VI (Ladenburger and Plattner, 2011). The emergence of the number of paralogs in the six groups can be explained by several whole-genome duplications (Aury et al., 2006). Transcripts of characterised *P. tetraurelia* CRCs correspond to full-length proteins with two exceptions: CRC-VI-3 might, evolutionary, become a pseudogene, and CRC-VI-1b is a putative pseudogene (Ladenburger and Plattner, 2011). The remaining 32 CRCs are functional. As with other gene families in *P. tetraurelia*, this unexpected variation might predominantly serve gene amplification.

Remarkably, all *P. tetraurelia* CRC subfamilies have six transmembrane domains (TMDs), even those that are devoid of an InsP_3-binding domain and have functional characteristics of a RyR-like protein (Ladenburger and Plattner, 2011). The presence of six TMDs is considered typical of InsP_3Rs (Boehning, 2010; Taylor et al., 2004), whereas for RyRs only four TMDs are typically assumed (Williams et al., 2001), although alternative views also exist (Hamilton, 2005; Zalk et al., 2007). Our analysis of CRCs in *P. tetraurelia* was (1) carried out on the basis of on hydrophobicity plots (Kyte and Doolittle, 1982) and (2) derived from analysis using the TOPCONS algorithm (Ladenburger and Plattner, 2011). This algorithm is based on large-scale evaluation of proteins with established TMDs (Bernsel et al., 2009). These domains are also preferred for evolutionary aspects, because they diverge much more slowly than regions localised outside of membranes (Oberai et al., 2009).

The selectivity filter that defines the Ca^{2+} permeability of CRCs in mammalian cells has specific features in InsP_3Rs and RyRs (Boehning, 2010) and can be compared with the sequences of CRCs found in *P. tetraurelia* (Fig. 2). In a detailed analysis of the selectivity filter of mouse, fly and worm (on the basis of comparative sequence analysis and site-directed mutagenesis studies performed by different laboratories), the sequence GVGD was considered typical of InsP_3Rs, whereas the sequence GIGD was assigned to RyRs (Boehning, 2010). The sequence GVGD occurs in the mouse InsP_3R type 1 (Fig. 2, *Mm*IP3R1) and the *P. tetraurelia* CRC isoforms CRC-I-1a, CRC-I-1b and CRC-I-1c (with a predicted, although not experimentally verified, InsP_3-binding domain), whereas the GIGD motif occurs not only in metazoan RyRs, but also in worm and fly InsP_3Rs (Fig. 2, *Ce*TR1 and *Dm*InsP_3R, respectively), and the *P. tetraurelia* CRC isoforms CRC-II-1a, CRC-II-1b, CRC-II-2, CRC-II-3 and CRC-II-5, as well as CRC-III-2 (see Fig. 2). Thus, it appears that the GIGD motif is widely distributed in InsP_3Rs of lower eukaryotes and, therefore, may not be specific for RyRs at that stage of evolution. Isoforms CRC-IV-1a, CRC-IV-1b, CRC-IV-2 and CRC-IV-3b in *P. tetraurelia* also contain the GIGD sequence in the absence of an InsP_3-binding domain. The activation mechanism of CRC-IV-type channels – as described below – is similar to that of mammalian RyRs. Mutation analysis of the selectivity filter has revealed that, although its conductivity is maintained, its properties are altered (Boehning, 2010), but further experimental analysis is required. Nevertheless, the occurrence of *P. tetraurelia* CRCs with mixed characteristics (i.e. sharing properties of InsP_3Rs and RyRs), already suggests a common ancestor of the two types of CRC.

Intracellular distribution and local effects of CRCs in *P. tetraurelia*

The intracellular distribution of CRCs in *P. tetraurelia* is highly heterogeneous (Fig. 3) (Plattner and Klauke, 2001; Plattner et al., 2012). As expected, the ER contains CRCs, some with traits of InsP_3Rs (CRC-I members) and some resembling RyRs (CRC-IV-1)

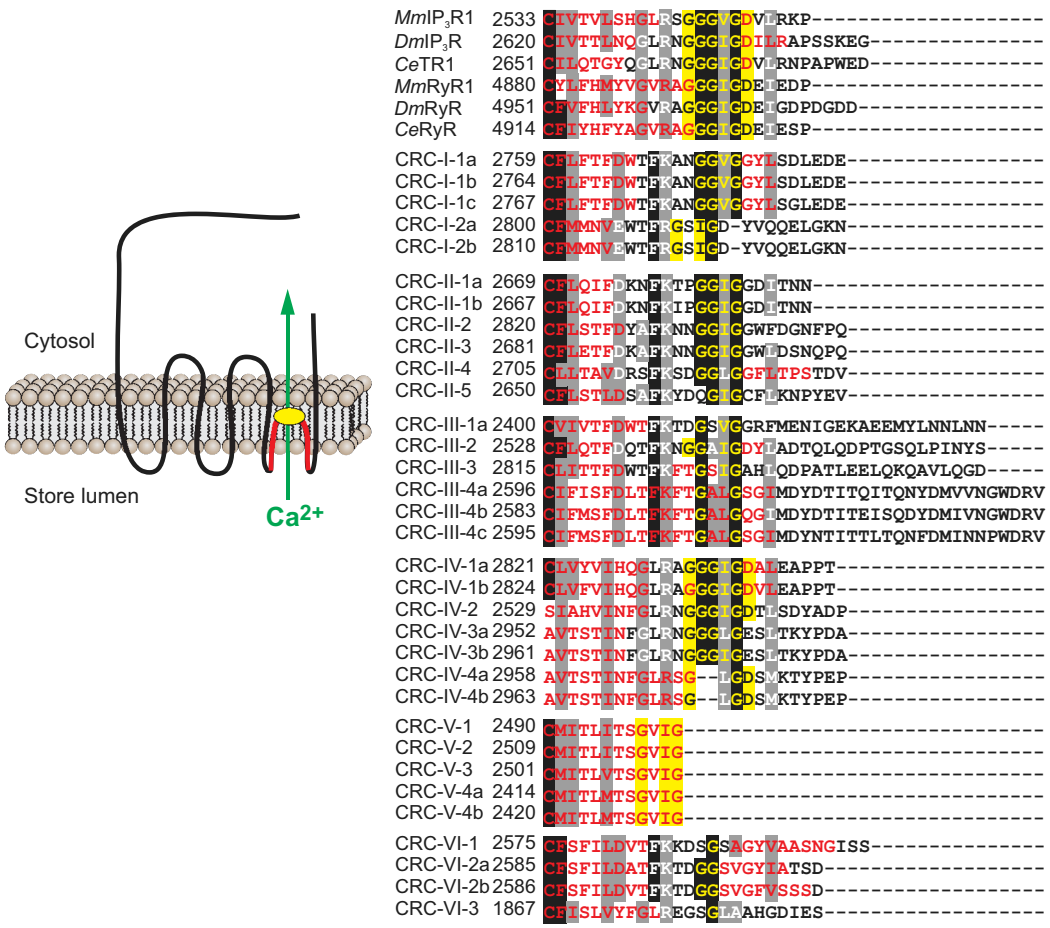

Fig. 2. Predicted pore regions with adjacent transmembrane segments. The selectivity filter that defines the Ca^{2+} permeability of CRCs in mammalian cells has specific features in RyRs and InsP_3Rs (Boehning, 2010), and can be compared with the sequences found in CRCs of *P. tetraurelia*. Sequences are aligned according to InsP_3Rs and RyRs in *Caenorhabditis elegans* (*Ce*), *Drosophila melanogaster* (*Dm*) and *Mus* mouse musculus (*Mm*) (Clustal algorithm). Black background indicates identity, grey background indicates similarity. Localised between TMD5 and TMD6 (predicted for all proteins shown as indicated in the text), the pore region is shown in red, with the putative selectivity filter highlighted in yellow. Also, note the insertion of several amino acids between the filter region and TMD6 in CRC-III molecules. Sequences are reproduced from Ladenburger and Plattner with permission from PLoS ONE (Ladenburger and Plattner, 2011).

(Ladenburger and Plattner, 2011). The CRC types II and IV are, respectively, localised to the contractile vacuole complex and to alveolar sacs (Figs. 1B,C and Fig. 3), which are the osmoregulatory system and the subplasmalemmal Ca^{2+} stores, respectively.

CRC-II members – who are restricted to the contractile vacuole complex (Ladenburger et al., 2006) (Fig. 1B) – possess an InsP_3-binding domain that contains amino-acid residues that are considered most relevant for InsP_3-binding (Yoshikawa et al., 1996). Furthermore, molecular modelling of the InsP_3-binding domain revealed striking similarity to its mammalian counterpart (Ladenburger et al., 2006), with the exception of some additional loops. The CRC-II channels are fully functional – as demonstrated by uncaging of InsP_3, which modifies spontaneous Ca^{2+} signals, and by binding of [^{3}H]InsP_3, which has been documented by using the homologously expressed InsP_3-binding domain (Ladenburger et al., 2006). In fresh-water organisms, the contractile vacuole complex serves for the expulsion of water (Allen and Naitoh, 2002) and some ions, including excessive Ca^{2+} (Stock et al., 2002). Type II CRCs may, therefore, serve to fine-tune Ca^{2+} homeostasis by partially refluxing of Ca^{2+}, thus resembling a systemic regulatory principle that is operative in kidneys. Spontaneous Ca^{2+} puffs are seen along the tubular extensions of the contractile vacuole, indicating constitutively active InsP_3Rs (Ladenburger et al., 2006). Incidentally, a similarly constitutive activity of InsP_3Rs has also been reported for leukocytes (Cárdenas et al., 2010).

CRC-III molecules are associated with recycling vesicles that are engaged in phagosome formation (Ladenburger and Plattner, 2011). CRC-III-4 members are associated mainly with some stages of phagosome and with different types of recycling vesicle that are linked to phagosomes and their formation. Considering that the formation of phagosomes requires Ca^{2+} and that the vesicles involved contain Ca^{2+} – as known from mammalian cells (Burgoyne and Clague, 2003; Hay, 2007; Zampese and Pizzo, 2012), these compartments could serve as dynamic Ca^{2+} stores for local membrane fusion events.

There is no sequence homology to an InsP_3-binding domain in CRCs of the groups IV, V and VI, with the exception of some CRC-V members (Ladenburger and Plattner, 2011) (see Fig. 1A). CRC-IV-1 channels display several functional characteristics of a RyR (Ladenburger et al., 2009) and are localised to alveolar sacs, the established subplasmalemmal Ca^{2+}-stores of *P. tetraurelia*

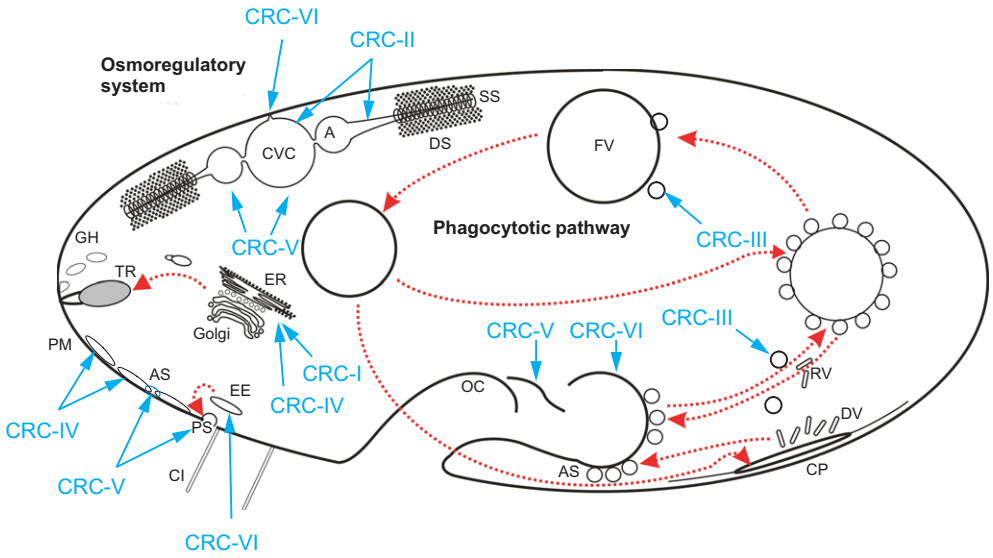

Fig. 3. Schematic distribution of the different CRCs in *P. tetraurelia*. The ER contains CRC types I and IV. Alveolar sacs (AS), which function as subplasmalemmal Ca^{2+} stores contain CRC types IV and V (type IV over the entire peripheral part, type V only in adjacent membrane regions, where stores touch each other). Parasomal sacs (PS) are clathrin-coated pits in the cell membrane and contain CRC type V; early endosomes (EE; also known as terminal cisternae) contain CRC-VI. In the phagocytotic cycle (dotted red arrows), CRC-IIIs are localised to food vacuoles (FV; also known as phagocytic vacuoles) of different stages and to vesicles associated with them. Vesicles identified as recycling vesicles (RV) clearly also contain CRC-III. This includes round RVs derived from early-stage food vacuoles (right) or from the cytoproct (CP) – the site of release of spent vacuoles – but not another type of recycling vesicle, such as discoidal vesicles (DV). RVs and DVs travel along postoral fiber bundles (not shown) to the oral cavity (OC) for membrane delivery to nascent food vacuoles. The CRC-V and CRC-VI also occur along the OC, and the OC as well as the outer surface of the cell are known to contain similar structures (coated pits and flat sacs that are reminiscent of alveolar sacs) that occur along the cell surface outside the oral cavity. The contractile vacuole complex (CVC) contains CRC-V in its central part, as well as CRC-II in more peripheral parts designated as the smooth spongiome (SS), but not in the decorated spongiome (DS) – the outermost part of the spongiome enriched with H^+-ATPase. In the pore region, the site of periodic fluid expulsion, CRC-VI is found. For simplicity the CRC-V on the membrane of micronuclei and along the cleavage furrow is not shown, neither is CRC-VI on some ill-defined cortical vesicles. See Ladenburger and Plattner, 2011 for these details, as well as for the specific paralogues that were analysed. Modified from from Ladenburger and Plattner with permission by PLoS ONE (Ladenburger and Plattner, 2011). A, ampullae; CI, cilia; PM, plasma membrane; GH, membrane 'ghosts' from released trichocysts.

(Stelly et al., 1991). By using different approaches, including electron microscopy (EM) (Knoll et al., 1991) combined with electron spectroscopic imaging (ESI) (Knoll et al., 1993) and energy-dispersive X-ray microanalysis (EDX) (Hardt and Plattner, 2000), the total Ca^{2+} content in the cortical stores was recorded within a sub-second time range of stimulation, and the decrease of Ca^{2+} during stimulation under control conditions and following replacement of Ca^{2+} with Sr^{2+} during stimulation was monitored. Using this experimental set-up, it was unambiguously revealed that a direct link exists between the depletion of cortical stores (mediated by CRC-IV channels in the alveolar sacs) and the activation of plasmalemmal Ca^{2+} influx (indicative of functional store-operated Ca^{2+} entry). Silencing of CRC-IV-1a and/or CRC-IV-1b greatly reduces stimulated exocytosis (Ladenburger et al., 2009) instigated by the RyR activators caffeine (Klauke and Plattner, 1998) or 4-chloro-*m*-cresol (Klauke et al., 2000), and obliterates Ca^{2+} signals that normally accompany exocytosis (Klauke and Plattner, 1997). A motif that is important for binding of 4-chloro-*m*-cresol in mammalian RyRs (Fessenden et al., 2006) is present in CRC-IV-1 (Ladenburger et al., 2009), although the binding of ryanodine to the isolated organelles in protozoa often differs from that in mammalian cells (Plattner et al., 2009). Nevertheless, the CRC-IV members are functionally similar to mammalian RyRs.

CRC subfamily types V and VI in *P. tetraurelia* have a more complex subcellular distribution (Fig. 3). Essentially, they localise to the contractile vacuole complex, – in addition to CRC-II – and to vesicles that are engaged in endocytosis, phagocytosis and recycling (Ladenburger and Plattner, 2011). CRC-V-4 channels are the most heterogeneously distributed in the cell and are found in the lateral parts of alveolar sacs (in addition to CRC-IV), in nuclear membrane, oral cavity, contractile vacuole and cleavage furrow. It has not been tested yet, however, whether the predicted InsP_3-binding domain of CRC-V-4 is functional. Remarkably, fusion of endo-, lyso- and phagosomes also involves Ca^{2+}-dependent vesicle delivery, as known from mammalian cells (Lindmark et al., 2002; Vinet et al., 2008). Members of the CRC-V-4 family are found in abundance at certain sections of the cell membrane, e.g. at clathrin-coated pits (parasomal sacs), which flank each ciliary basis (Ladenburger and Plattner, 2011). Incidentally, the overall number of CRCs in the outer cell membrane of *P. tetraurelia* outnumbers that of InsP_3Rs (Dellis et al., 2006) in the plasmalemma of some mammalian cells by several orders of magnitude. Functionally, the CRC-V-4 channels in *P. tetraurelia* might account for plasmalemmal leakage conductance that has been described in *P. tetraurelia* (Machemer, 1988). It might also indicate that, early in evolution, channels that now serve as intracellular CRCs have allowed Ca^{2+} influx across the cell membrane, in addition to other channels found on the cell surface of *P. tetraurelia*.

The contractile vacuole complex is the designated osmoregulatory system and is involved in several modes of

membrane trafficking, as evidenced by the presence of numerous specific SNAREs in *P. tetraurelia* (Plattner, 2010) and specific GTPase proteins in *Tetrahymena thermophila* (Bright et al., 2010). Therefore, only some of the CRCs present in this organelle may serve to fine tune $[Ca^{2+}]_i$, whereas other may function in the local delivery of Ca^{2+} to the permanent interactions between vesicles and the membrane. CRC-VI-2 and CRC-VI-3 are localised at sites of periodic exocytotic release of contractile vacuole fluid, the so-called pore (Ladenburger and Plattner, 2011). CRC-VI-2 is also associated with some ill-defined cortical vesicles.

In summary, the analysis of the distribution of specific CRC families in *P. tetraurelia* reveals a heterogeneous and distinct localisation, which points to the respective functions of different types of Ca^{2+}-release channel (Fig. 3). The variety of CRC molecules in *P. tetraurelia* indicates that protozoa have already evolved several specific intracellular Ca^{2+}-signalling pathways. The distinct intracellular distribution of CRCs in *P. tetraurelia* may serve to fulfil the local requirements for Ca^{2+}-mediated membrane–membrane interaction and membrane fusion or fission. In higher eukaryotes, most of these processes are known to require local cytosolic Ca^{2+} release from internal stores (Burgoyne and Clague, 2003; Hay, 2007). Similarly, trafficking organelles also contain Ca^{2+}, although in variable concentrations (Zampese and Pizzo, 2012). By contrast, local SNARE proteins can regulate the activity of Ca^{2+} channels, for example in the endolysosomal system (Shen et al., 2011). A tight regulation is required because of the rapid dissipation and reduction of $[Ca^{2+}]$ in cells (Neher, 1998). To appreciate the number of functional diversification of CRCs in *P. tetraurelia*, one has to keep in mind that paralogues resulting from the most recent genome duplications, although almost all expressed (Ladenburger and Plattner, 2011), might predominantly serve for gene amplification and only in part for further diversification. This leaves us at least with those six, clearly distinguishable, groups of CRC, which are the basis for the formation of specific local signals used in the different types of vesicle trafficking in *P. tetraurelia*. The distribution of CRCs in *P. tetraurelia* is even more complex than in higher eukaryotes, in which InsP_3Rs and RyRs can also coexist in the same cell or even in the same organelle (Solovyova and Verkhratsky, 2003; McCarron and Olson, 2008).

The enigma of InsP_3Rs and RyRs in protozoa

InsP_3 signalling has been recorded in many species from protozoa to humans, but the identification of InsP_3Rs in unicellular organisms is still to come (Table 1). Only in *D. discoideum* has a molecule with overall similarity, but without confirmed CRC role, been reported (Traynor et al., 2000). Even more uncertain in unicellular organisms is the existence of RyRs, because they are generally assumed to be absent from protozoa (Prole and Taylor, 2011) and algae (Verret et al., 2010). For a number of unicellular organisms, database searches reveal partial sequences with some – although variable – similarity to the InsP_3R (Fig. 4). This, however, does not necessarily exclude the presence of RyRs or RyR-like proteins, and more scrutiny including detailed domain analysis is necessary.

At the one end of the spectrum is the exuberant vesicle trafficking of *P. tetraurelia* with its clearly defined routes (Allen and Fok, 2000) that might have required a particularly complex differentiation of CRC isoforms (Fig. 3). At the other end of the spectrum, there are organisms that probably do not have any InsP_3Rs and RyRs, such as fungi, types of Ascomycota (yeast etc.), or parasites of the group Apicomplexa, such as *Plasmodium* and *Toxoplasma* (Table 1). *Toxoplasma* possess subplasmalemmal sacs, called the inner membrane complex that resembles alveolar sacs of ciliates – one of many reasons to combine both groups in the supergroup Alveolata. Apicomplexa require Ca^{2+} signalling for sequential exocytosis of special dense-core secretory vesicles (comparable with trichocysts in *P. tetraurelia*), which is a prerequisite for host cell penetration (Lovett and Sibley, 2003; Nagamune et al., 2008). Nevertheless, despite extensive searches, there is no indication for the existence of comparable CRCs in Apicomplexa (Nagamune and Sibley, 2006; Nagamune et al., 2008; Prole and Taylor, 2011; Plattner et al., 2012). Both, ascomycetes and apicomplexans are known for the secondary reduction of their genomes (Aravind et al., 2003; Roos, 2005), and they might have developed other sources of Ca^{2+} and other types of CRC. As an increasing number of other free-living unicellular organisms are being added to databases (Fig. 4), now might be a good time to intensify and renew the search for CRCs complemented by domain structure analysis.

Conclusions and perspectives

The data discussed here have been obtained for *P. tetraurelia*, and suggest that InsP_3R- and RyR-type Ca^{2+} signalling has appeared early in evolution and already in unicellular organisms, which raises the following interesting evolutionary perspectives. These CRCs might be of common evolutionary origin; the same number of TMDs (i.e. six), and a similar selectivity filter in both types of CRC, both point to a common origin of both channel types. Considering the size of RyRs in animals and comparing it with the homologous molecules in *P. tetraurelia*, RyRs or RyR-like proteins may have expanded in size during evolution. This implies that ancestral channels – although already diverging in functional properties – retained a similar size. The pore domain has been largely maintained, with a similar selectivity filter within both channel types being present in lower eukaryotes. In mammalian InsP_3Rs and RyRs, structural details within the C-terminus and conserved domain interactions – as recently determined by X-ray structure analysis (Seo et al., 2012), are also compatible with a common origin of both types of CRC. In some unicellular organisms, RyRs might have been partially lost – in contrast to *Pt*RyRs or *Pt*RyR-like proteins – from a common ancestor. All this indicates considerable changes and, possibly, even loss of some of these channels in different branches of protozoa during evolution.

Only very recently has an InsP_3R been cloned in full and analysed in functional tests within the pathogenic flagellates *Trypanosoma brucei* (Huang et al., 2013) and *Trypanosoma cruzi* (Hashimoto et al., 2013); however, the precise domain structure remains to be shown. For other protozoa, no data are available that are comparable with those of the *P. tetraurelia* CRC types CRC-II and CRC-IV (those that have been analysed most thoroughly) – although CRCs related to CRC-II, which serves for the release of Ca^{2+}, must be ubiquitously present in all related organisms according to physiological data (Table 1). The results discussed here may encourage further work with lower eukaryotes on a broader scale.

If the CRCs we described for *P. tetraurelia* would not have a common evolutionary origin, what could be the alternative? Recently, vertical gene transfer was proposed

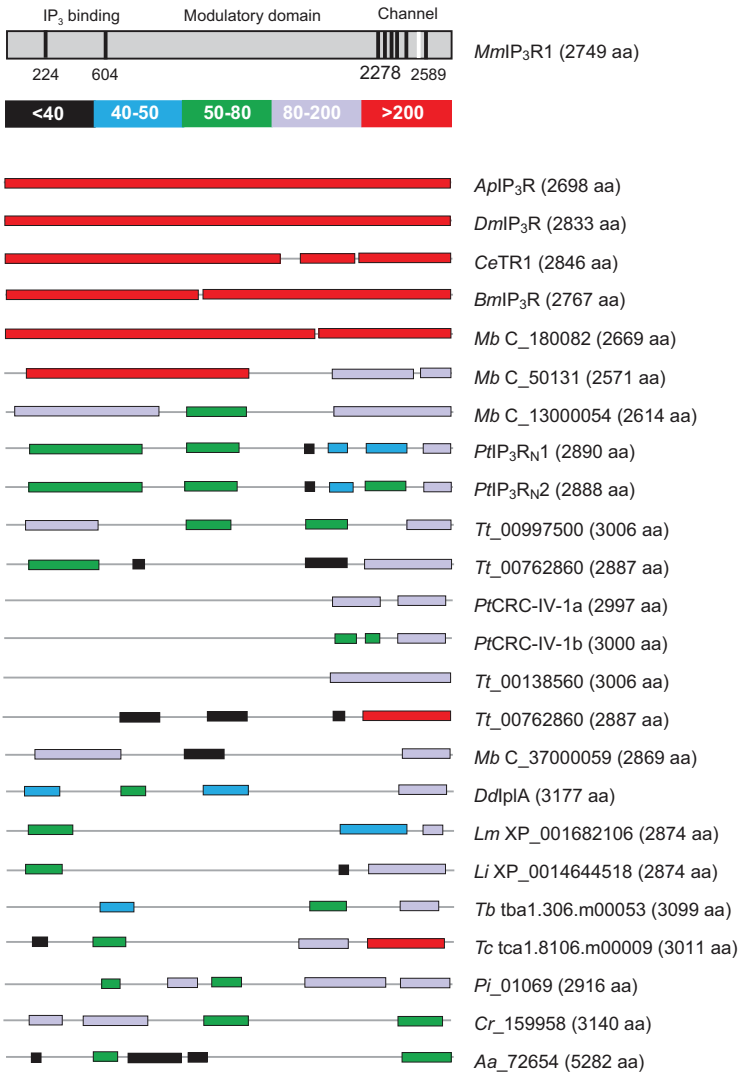

Fig. 4. CRC sequences that have been found in different organisms. Ca^{2+} channels analysed are potentially related to established RyRs and InsP_3Rs in higher organisms (genomic sequences were translated into amino acid sequences, *Pt*InsP_3R_N1 and *Pt*InsP_3R_N2 refer to the paralogs CRC-II-1a and CRC-II-1b, respectively). Colours indicate similarities of channels in different species. *Aa*, *Aureococcus anophagefferens* (chrysophysean alga); *Ap*, *Asterina pectinifera* (echinoderm); *Bm*, *Brugia malayi* (pathogenic nematode); *Ce*, *Caenorhabditis elegans* (nonpathogenic nematode); *Cr*, *Chlamydomonas reinhardtii* (chlorphycean algae); *Dd*, *Dictyostelium discoideum* (social amoeba); *Dm*, *Drosophila melanogaster* (insect); *Li*, *Leishmania infantum* (pathogenic flagellate); *Lm*, *Leishmania major* (pathogenic flagellate); *Mb*, *Monosiga brevicollis* (choanoflagellate); *Pi*, *Phytophthora infestans* (pathogenic fungus, oomycete); *Pt*, *Paramecium tetraurelia*; *Tb*, *Trypanosoma brucei* (pathogenic flagellate); *Tc*, *Trypanosoma cruzi* (pathogenic flagellate); *Tt*, *Tetrahymena thermophila* (ciliate). Reproduced from Ladenburger et al. with permission by American Society for Microbiology (Ladenburger et al., 2009). For further details, see also Table 1.

(Mackrill, 2012). Considering the multitude of Ca^{2+}-targets, Ca^{2+}-dependent and Ca^{2+}-regulating molecules in ciliates (Plattner and Klauke, 2001; Plattner et al., 2012), one would have to assume gene transfer at an unlikely large scale. In the absence of a physical interaction of these cells with other cells as potential gene donors, horizontal gene transfer is even less likely. Similarly questionable would be a transfer through bacteria serving as food. Another possibility is the parallel evolution of CRCs in ciliates and in cells that evolve into metazoans. This would take into account that precursors of ciliates may have emerged slowly after the evolution of a *UR*-eukaryote (Hedges, 2002; Hedges et al., 2004), as outlined above. The assumption of a parallel evolution that starts from a common precursor molecule is not without precedent. For instance, in *P. tetraurelia*, the number of substantially diversified SNAREs is about twice of that suggested for the *UR*-eukaryote and equals that of human SNAREs (i.e. about 40) (Plattner, 2010). The amazing diversification of the vesicle trafficking system in *P. tetraurelia* also appears to result from parallel evolution, accompanied by an equal diversification of Ca^{2+}-channels. In fact, these cells contain not only the CRCs discussed here but also voltage-dependent Ca^{2+}-influx channels, which are very similar to those in mammalian brain (Levitan, 1999).

All in all, CRCs of the InsP_3R and RyR type with clearly defined domains have already been identified in protozoa. The diversification of these CRCs matches the considerable diversification of intracellular vesicle trafficking in these cells. The observation of specific CRC types localised at specific sites in the cell may indicate their specific requirements for Ca^{2+} signalling. The occurrence of CRCs with mixed characteristics (of the InsP_3R and RyR type), together with an identical amino acid composition of the selectivity filter, can be considered as a primeval evolutionary trait.

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Note added in proof

Most recent computational analysis of the mammalian RyR also came to the conclusion of six transmembrane domains (Ramachandran et al., 2013), just as we have shown for the homologous molecule CRC-IV in *P. tetraurelia* Ladenburger et al., 2009).

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