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The ancient roots of calcium signalling evolutionary tree

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ABSTRACT

Molecular cascades of calcium homeostasis and signalling (Ca^{2+} pumps, channels, cation exchangers, and Ca^{2+}-binding proteins) emerged in prokaryotes and further developed at the unicellular stage of eukaryote evolution. With progressive evolution, mechanisms of signalling became diversified reflecting multiplication and specialisation of Ca^{2+}-regulated cellular activities. Recent genomic analysis of organisms from different systematic positions, combined with proteomic and functional probing invigorated expansion in our understanding of the evolution of Ca^{2+} signalling. Particularly impressive is the consistent role of Ca^{2+}-ATPases/pumps, calmodulin and calcineurin from very early stages of eukaryotic evolution, although with interspecies differences. Deviations in Ca^{2+} handling and signalling are observed between vertebrates and flowering plants as well as between protists at the basis of the two systematic categories, Unikonta (for example choanoflagellates) and Bikonta (for example ciliates). Only the B-subunit of calcineurin, for instance, is maintained to regulate highly diversified protein kinases for stress defence in flowering plants, whereas the complete dimeric protein, in vertebrates up to humans, regulates gene transcription, immune-defence and plasticity of the brain. Calmodulin is similarly maintained throughout evolution, but in plants a calmoldulin-like domain is integrated into protein kinase molecules. The eukaryotic cell has inherited and invented many mechanisms to exploit the advantages of signalling by Ca^{2+}, and there is considerable overall similarity in basic processes of Ca^{2+} regulation and signalling during evolution, although some details may vary.

1. Bacterial inheritance: Ca^{2+} regulation and primaeval Ca^{2+} signalling

Early life may have emerged in the ocean or in local parts of it under alkaline conditions that favoured relatively low (in a 100 s nM range) Ca^{2+} concentrations ([1,2] this special issue). At this early stage Ca^{2+} permeation into ancestral cells, Ca^{2+} handling and Ca^{2+} influence on energetic (and in particular the requirement of low free Ca^{2+} for ATP metabolism [3]) made Ca^{2+} ions critical for life and for signalling processes.

Bacteria maintain Ca^{2+} homeostasis [4,5] although resting levels of free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) are somewhat higher than in eukaryotes [6] and although specific mechanisms employed by different bacterial species are yet to be characterised in detail. Some bacteria express primary and secondary active transporters including P-type Ca^{2+}-transport ATPases of which several resemble the Sarcoplasmic and Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) of eukaryotes [7,8]. These pumps, together with mechanosensitive channels [9], Ca^{2+}-activated channels [10], cation exchangers, such as Ca^{2+}/H^+ and Ca^{2+}/Na^+ exchangers, an array of Ca^{2+}-binding proteins (CaBP), and a battery of Ca^{2+}-activated enzymes, which all are present in bacteria ([11] this special issue) formed the primordial Ca^{2+} homeostatic and Ca^{2+} signalling system. In bacteria which live today, changes in $[Ca^{2+}]_i$, regulate numerous functions such as, for example, chemotaxis [6,12]. Calmodulin-like proteins are found in the genome of certain Gram-positive bacteria [13–15], in addition to other CaBPs [16]. However, fast Ca^{2+} sensors with C2 domains (such as for example synaptotagmins [17]) have not been reported.

Considering the frequent occurrence of gene transfer between ancestral organisms, the early period of evolution of molecular cascades responsible for control over cellular Ca^{2+} remains rather vague and speculative. Even genuine Ca^{2+} signalling in bacteria has been debated [10]. Apart from these restrictions it appears that

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bacteria prophesied several important mechanisms that have been advanced and refined throughout the evolutionary ladder.

2. From bacteria to the eukaryote cell: requirement of Ca^{2+} for trafficking

There is considerable uncertainty about the origin of the eukaryotic cell from archaebacteria or eubacteria [18], with different scenarios being proposed [19,20]. Even the age of eukaryotes is disputed; the classical (and prevailing) view of their emergence (as witnessed by fossils) ~2 billion years ago [21–24], is not universally acknowledged with the data on the presence of eukaryotic markers in much older (~3 to ~3.5 billion years) fossils [25,26]. Based on the analysis of eukaryotic signature proteins, the emergence of a "chronocyte", the intermediate distinct from Archaea and eubacteria has been contemplated [27]. The textbook view highlights an archaebacterial ancestor whose genome has been sequestered by another cell through invagination of the cell membrane. Integration of an archaebacterium into an eubacterium is another hypothetic scenario. The Ca^{2+} regulating and Ca^{2+} regulated proteins outlined above are essentially known from eubacteria, whereas important proteins of the nucleus have orthologues in some archaebacteria [28].

By infolding of the cell membrane with ribosomes attached, the Endoplasmic Reticulum (ER) could have formed, followed by controlled blebbing and fusion of vesicular compartments, also a Ca^{2+}-dependent process, wherever it has been accessible to analysis. This must have been prerequisite to any further differentiation and trafficking. This capability, together with a cytoskeleton, has been ascribed to the chronocyte [27]. In contrast, a LAECA-(latest archaeal-eukaryote common ancestor) type organisms, endowed with high internal complexity, has been postulated to precede genuine eukaryotes [29]. Endocytosis and intracellular digestion are thought to have become important for further complexity of the LAECA-type ancestor, and the early eukaryotic cell [30,31]. The acquisition of endomembranes and intracellular compartments that could emerge in some prokaryotes was certainly associated with the transition from prokaryotes to eukaryotes and prompted new developments in Ca^{2+} signalling.

Regulation of intracellular trafficking become the special function of Ca^{2+}, which generally assumes a key role in membrane-membrane interactions, and hence complex $[Ca^{2+}]_i$ dynamics, regulated in space and time, provided a canvass for ubiquitous and versatile Ca^{2+} signalling. Ca^{2+} has outstanding properties that make it an almost ideal second messenger [32]. Since too high $[Ca^{2+}]_i$ is toxic, strict regulation and "taming" of Ca^{2+} movements is required, which have made it a molecule suitable for signalling at a low additional energy costs. The human body contains up to 1.4–2 kg of Ca^{2+} (of which 99% is present in the form of insoluble phosphates accumulated in bones). Concentration of Ca^{2+} is ~10 mM in the ocean; total Ca^{2+} concentration reaches 25 mM in plants; in mammalian cells, total Ca^{2+} concentration in the cytoplasm (free and bound) is in the millimolar range [32], whereas free concentrations of $[Ca^{2+}]_i$ are, as a rule, below ~0.1 micromolar in the resting cell [33]. The existence of a continuous concentration gradient aimed at the cytosol allows $[Ca^{2+}]_i$ to be rapidly and locally increased for signalling at defined sites, which rise is followed by reversible binding to CaBPs. These latter proteins are generally characterised by rapid binding kinetics and widely different affinity (expressed as a binding constant, K_D). High capacity/low affinity binding makes some CaBPs suitable for Ca^{2+} binding inside the organelles (universally known as dynamic Ca^{2+} stores) and for signal inactivation in the cytosol, whereas rapid activation of dynamic processes is a responsibility of low capacity/high affinity binding with a variety of membrane-bound and cytosolic CaBPs. Local regulation of Ca^{2+} controls selective and spatially restricted specific processes [33], avoids toxicity and keeps energetic costs for re-establishing homeostasis low. For the latter purpose, some high capacity/low affinity CaBPs are also present in the cytosol [34].

3. An evolutionary time scale and diversification of Ca^{2+} signalling

Ancestral eukaryotes diversified into two main branches, Unikonta (that eventually evolved into vertebrates) and Bikonta (that are at the root of angiosperms, or flowering plants [35,36]). The founders of these branches are two unicellular groups whose current main representatives date back to ~760–960 million of years (choanoflagellates [37]) and ~800 million of years (ciliates [23]). Choanoflagellates are considered as the founding group of metazoa and, therefore, deserve special interest with regard to Ca^{2+} signalling (Cai et al. [48], this special issue). Myxamoebae (*Dictyostelium*) are another well analysed unikont; their phylogenetic age is somewhat ambiguous, although they can be younger than the other groups [38]. Mammals are more than 200 million of years old being therefore older than flowering plants that are believed to emerge 130–190 million of years ago [23,39].

Molecules participating in trafficking have greatly diversified during evolution, which could be extrapolated from comparative genomic studies. There are about 20 SNAREs (soluble N-ethyl maleimide sensitive attachment protein receptors) in the Ur-eukaryote, whereas they are about twice as many in mammals [40]. The number of Rab-type GTPases increased from an estimated 20 in ancestral eukaryote [41] to 163 in human [42]. Insights from cells living today suggests that already in early times Ca^{2+} must have been "hired" for signalling purposes. Considering rapid diffusion, binding and deactivation of Ca^{2+}, increasingly elaborate intracellular trafficking required a strict localisaton of Ca^{2+} signals. This in turn requires Ca^{2+} stores, with high capacity/low affinity CaBPs in their lumen, and mechanisms for Ca^{2+} uptake and local release [43,44]. All these components, including primary and secondary active Ca^{2+} transport mechanisms and Ca^{2+} release channels (CRC) are abundant in protozoa in one or the other form, as found in *Paramecium* [45,46], and to some extent in *Dictyostelium* [47] and in choanoflagellates ([48], this special issue). The first two genera represent the major phylogenetic lines and are frequently used for studies in cell biology, whereas choanoflagellates are currently only analysed by molecular biology, although with important predictions. The increasing importance of Ca^{2+} during evolution is highlighted by an increase in the number of CaBPs, which rises from ~70 in bacteria to 3640 in mammals [16,49]. Substantial increase in numbers and diversity of CaBP in eukaryotes reflects a rising capability of fine tuning Ca^{2+} signals [50]. A rather different way of diversification of Ca^{2+} signalling, however, is observed in plants ([51–53] in this special issue).

It is now generally acknowledged that evolutionary improvement of cell energetic is associated with endocytosis and domestication of eubacteria with respiratory activity, that become mitochondria, about 1.5 billion years ago [54]. Considering the high proportion of energy investment in ionic balance in modern eukaryotes, one may assume that acquisition of mitochondrial precursors was an important step in advancement of Ca^{2+} signalling ([2], this special issue). The uptake of Ca^{2+} by mitochondria, achieved in modern eukaryotes by an uniporter [55] is swift and it stimulates ATP production by activating dehydrogenases in the mitochondrial matrix [56]. A homologue of Ca^{2+} uniporter is present already in bacteria [57], and in choanoflagellates ([48], this special issue), while a mitochondrial calcium uniporter (MCU) is conserved from protozoa to human, no MCU homologues, however, were found in various parasitic protozoa [57,58]. The essential

MCU regulator, EMRE, is absent in some protozoa analysed, including *Dictyostelium* and *Tetrahymena* [58], thus probably indicating lower capability for adjustment of Ca^{2+} transport. In summary, during evolution, mitochondria are actively engaged regulating and exploiting energetically Ca^{2+} signals, but with some variability.

Considering the great age of the most ancient forms of eukaryotes we have to concede that cells of modernity are remote descendants, with ample chances for parallel evolution, possibly also including gene transfer, thus disguising images of the distant past. However, the multitude of Ca^{2+} regulating and Ca^{2+} regulated activities suggest significant original evolutionary inheritance of a remarkable inventory of molecules relevant for Ca^{2+} regulation.

4. Ca^{2+} signalling in cells of modern era

Can modern protists (protozoa and algae) provide clues to the evolution of Ca^{2+} signalling? As already mentioned, unikonts include myxamoebae (with *Dictyostelium* being the best known example [59]), and choanoflagellates, whereas bikonts are represented by alveolates, including ciliates like *Paramecium* and *Tetrahymena*. Experimental data on Ca^{2+} signalling are available for *Dictyostelium* and *Paramecium* [46]. Placing choanoflagellates at the roots of metazoan evolution [60] is based on a variety of molecular aspects, mainly Tyr phosphorylation and occurrence of cell adhesion molecules [61]. Whether and how these molecules bind extracellular Ca^{2+} remains to be clarified. Both ciliates and choanoflagellates, possess several Ca^{2+} influx channels, including transient receptor potential channels, cyclic nucleotide gated channels and voltage-gated channels. Remarkably ciliary localisation of voltage-dependent Ca^{2+} channels is also found in ctenophores [62].

Ca^{2+} is fundamental for regulation of multiple and distinct processes operating on widely different time scale not only in "higher" eukaryotes such as mammals [63], but also in protozoa. These cellular processes regulated by Ca^{2+} include gene transcription, exocytosis, endocytosis, vesicle trafficking, amoeboid movement and chemotaxis, ciliary and flagellar beat etc. When time domain is concerned, in ciliates Ca^{2+}-regulated reactions can occur in submillisecond times in case of membrane fusion, in sub-second times in ciliary beat [64] and may also last hours in the regulation of gene transcription [65]. In subsequent sections we shall narrate a considerable congruence of different cell biological phenomena in low and high eukaryotes, respectively, with considerable deviations, however, in higher plants. A summary of certain important aspects is presented in Fig. 1.

5. Ca^{2+} signalling toolkits in protozoa

In the modern world Ca^{2+} normally occurs in sufficiently high concentration in the environment; Ca^{2+} concentration in the body fluids (that is extracellular Ca^{2+}, $[Ca^{2+}]_o$) is also at the millimolar range being thus 20,000 times in excess over resting $[Ca^{2+}]_i$ [5]. Ongoing diffusion through the plasmalemmal pores requires counter-regulation (i.e. Ca^{2+} efflux) in all cell types studied so far. Active transport by Ca^{2+}-pumps and cation exchangers, possibly inherited from bacterial ancestors, execute this task. In all eukaryotes, a variety of plasmalemmal channels make Ca^{2+} available locally, but not distantly. Therefore, eukaryotic cells are additionally endowed with intracellular Ca^{2+} release channels, CRCs, pumps and antiporters localised to the membranes of Ca^{2+}-storing organelles in animal [33,44,66] and plant cells [51,52]. Ca^{2+} is contained not only in dedicated Ca^{2+} stores, such as the Endoplasmic and Sarcoplasmic Reticulum (ER/SR), but also in organelles of the different trafficking pathways. In essence, this arrangement is maintained throughout eukaryotic cell evolution.

5.1. Ca^{2+} influx

The spectrum of plasmalemmal Ca^{2+} influx channels, as well as of Ca^{2+} activated cation (Na^+, K^+ etc.) channels operating in the cell membrane steadily increases in evolution, from bacteria onwards [10]. There are considerable differences in Ca^{2+} channels between myxamoebae (*Dictyostelium*) and ciliates (*Paramecium*) [10]. The mechanosensitive cationic channels with Ca^{2+} permeability are present from bacteria to human, as are ligand-gated and voltage-dependent Ca^{2+} channels. Specific subtypes of mechanosensitive channels are abundant in many of pathogenic protozoa, although Piezo subunits are not found in Apicomplexa [67], and yet they occur in the genome of related ciliates [68]. In many protozoa, including ciliates and parasites (Apicomplexa and Trypanosomatids), there is a diversified interaction between cyclic nucleotides and Ca^{2+} [69]. Evidence for purinergic Ca^{2+}-permeable channels is available from protozoa – in choanoflagellates and in *Dictyostelium* [3,70]. In *Dictyostelium* Ca^{2+} release by the $P2X_A$ channel mediates vesicle fusion at the level of the contractile vacuole [71]. Higher plants have mechanosensitive and nucleotide-gated channels [9] as well as ionotropic purinoceptors [72], but miss voltage-dependent Ca^{2+} channels ([73,53] this special issue). The higher up in the animal kingdom, the more differentiated become ligand-gated Ca^{2+} influx channels [74]. In summary, there is a variability of plasmalemmal Ca^{2+} channels depending on the systematic position of a species (for instance, significant differences are found between algae and flowering plants [73]), but some channels are preserved more or less throughout evolution.

5.2. Ca^{2+} pumps

The plasmamembrane Ca^{2+}-ATPase (PMCA) also known as plasmalemmal Ca^{2+} pump contributes to keep $[Ca^{2+}]_i$ low despite permanent influx from the outside medium, as does the SERCA pump; both are P-type ATPases. Faster Ca^{2+} removal can be achieved by secondary active transporters, such as antiporter systems. Both primary and secondary active mechanisms of Ca^{2+} regulation are already known from bacteria, including SERCA-related pumps. The PMCA and SERCA are also present in protozoa being thus the old regulatory mechanisms maintained during evolution (Fig. 1). In *Dictyostelium*, according to sequencing data, a putative PMCA (pat1), ~120 kDa large, is reported to lack the typical calmodulin-binding domain [75]. Later sequence data suggested the presence of such a domain in three paralogues, including patA, although for all three isoforms localisation to the ER and/or the plasmamembrane has been left open [47]. The plasmalemmal presence, however, is supported by the colocalisation with calmodulin [75]. In *Paramecium* the PMCA is ~130 kDa in size and shows a conserved 21 aminoacids-long potential calmodulin-binding domain [76], in contrast to the SERCA of the same species [77]. Thus, despite some uncertainties in myxamoebae, it appears that Ca^{2+} extrusion and sequestration mechanisms are generally preserved over evolution. The PMCA-type pumps are operational not only in the cell membrane of animal cells, but also in flowering plants. The autoinhibitory domain of the PMCA is situated in the amino-terminal part in plants and in the carboxy-terminal part in mammals [78]. De-inhibition occurs by binding calmodulin. In plants, autoinhibited forms called ACA (for auto-inhibited Ca^{2+}-ATPase), are slightly different and they occur not only in the plasmalemma but also in the vacuole membrane, also with a calmodulin-binding domain ([53,79,80] this special issue). During evolution, the inventory of pumps can be complemented by Ca^{2+}-pyrophosphatase (CaPPase) in acidocalcisomes and in the contractile vacuole complex of some protozoa. This is of particular importance for protozoan parasites (see [81,82], this special issue).

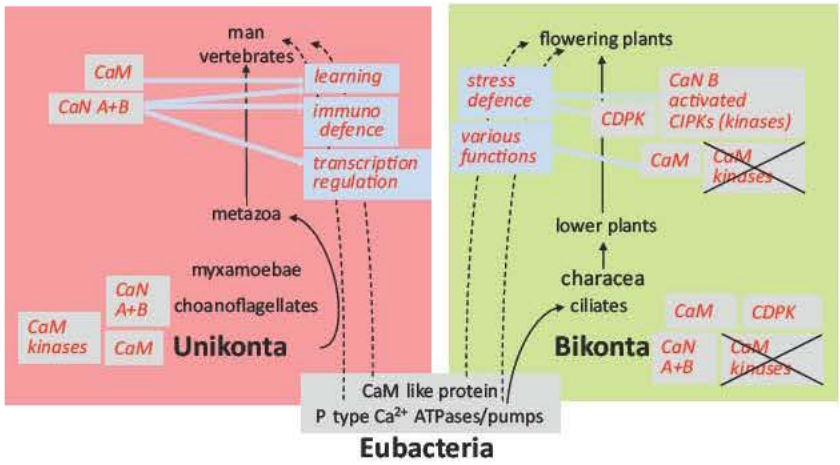

Fig. 1. This scheme highlights the transfer of some highly important molecules, or precursors thereof, through evolution, according to data cited in the text. It also reveals some essential differences between the two main lineages, Unikonta and Bikonta. P-type Ca^{2+}-ATPases/pumps and calmodulin-like proteins are found already in bacteria. They are most elaborate already in protists of the mono- and bikont lineage. They acquire high significance on the way up to man where calmodulin regulates ion channels and the activity of the multifunctional dimeric protein phosphatase calcineurin (CaN). This in turn regulates gene transcription, immune defence and long term potentiation (learning). In higher plants, only the B subunit of calcineurin is maintained which regulates calcineurin B-activated protein kinases (CIPKs = CBL-interacting protein kinases) in an extremely high number of combination of isoforms. The scheme also shows that ciliates and plants of different evolutionary level contain Ca^{2+}-dependent protein kinases (CDPK, with integrated calmodulin-like EF-hand motifs), much more than – if any – Ca^{2+}/calmodulin-activated protein kinases (CaM kinases). Beyond that, in plants calmodulin also regulates many processes, some of which are plant specific.

5.3. Exchangers

Various Ca^{2+} exchangers are present from archaebacteria onwards [83], up to higher plants [51,52] and mammals [33]. In the absence of specific inhibitors, fictional expression of these exchangers is difficult to verify in protozoa; convincing evidence exists only for the different parasites ([81,82], this special issue). Antiporter systems for Ca^{2+} are frequently supported by a V-type Ca^{2+}-ATPase/pump. Although little is known about antiporters in free-living protozoa and although in ciliates antiporters have not been identified at a molecular level, their occurrence can be derived from functional observations. When in *Paramecium* the V-type H^+-ATPase, a salient feature of the contractile vacuole/osmoregulatory complex, is blocked, $[Ca^{2+}]_i$ recovery after stimulated Ca^{2+} increase is retarded by about 10 times [84] (in agreement with the permanent Ca^{2+} extrusion by the organelle) just as after knock-out of cortical centrin [85].

5.4. Intracellular stores: Lumenal CaBPs

Little is known about lumenal high capacity/low affinity CaBPs in vesicular stores of protists. Genes encoding calreticulin and calsequestrin have not been found in the *Paramecium* database, although there are some hints for their existence [86]. Difficulties in finding gene sequences (see [87], this special issue), can be explained by the abundance of acidic aminoacid residues, rather than of specific motifs [88]. However, the ER-resident forms, calreticulin and calnexin have been identified in the database of *Dityostelium*, where knockout experiments resulted in inhibition of phagocytosis [89]. In that latter study, calreticulin sequences were also detected in *Trypanosoma* and *Leishmania*.

5.5. Intracellular stores: $InsP_3R$/RyR calcium release channels

The inositol 1,4,5-trisphosphate ($InsP_3$) Ca^{2+} release channels generally referred to as $InsP_3$ receptors ($InsP_3Rs$) have been identified in genomic studies in *Dictyostelium* [90] and in the choanoflagellate, *Monosiga* [61,91]. Although not studied in detail at the molecular level, $InsP_3R$ null-mutants of *Dictyostelium* are available and, thus, $InsP_3$ was shown to contribute to the motility responses to shear stress, together with Ca^{2+} influx activated by trimeric G-protein [92]. In *Paramecium*, genomic analysis revealed $InsP_3Rs$ [93], and a second type of CRC resembling a ryanodine receptor (RyR), defined as RyR-LPs [94], or CRCs with mixed features ([45,87,95] this special issue). When compared with their mammalian counterparts [96,97] RyR-LPs of *Paramecium* were remarkably different in size [45]. Two of *Paramecium* CRCs have been thoroughly characterised at a cellular and functional level, including $InsP_3$ binding [93] and stimulation with RyR agonists [94]. Genomic analysis of *Monosiga* supports the presence of $InsP_3Rs$, whereas sequences indicating RyRs are detected only for *Salpingoeca* ([48], this special issue). Sequences with variable similarity can be found in the genetic databases of different organisms, even where such CRCs have not been described in any more detail [94,98]. However, this aspect has to be considered with caution before any proteomic and functional analyses are made.

Although it is impossible to extrapolate to the Ur-eukaryote, identification of both these CRC types and intermediates in ciliates, led to an assumption of a common ancestor of both, $InsP_3R$ and a RyRs already at the level of protozoa [94,98]. According to genomic analyses, also in choanoflagellates, the most important CRCs have been formed very early, similarly to ciliates. This includes not only $InsP_3R$ and possibly also homologues of RyRs, but also two-pore channels, TPCs ([48], this special issue). In evolution, plants either have never acquired, or lost all these channels ([53], this special issue). A common ancestral channel for both, $InsP_3Rs$ and RyRs, has been proposed particularly based on similar size, on the pore domain with six transmembrane domains, and the aminoacid signature within its selectivity filter [94,98].

5.6. Other CRCs and additional Ca^{2+} stores

The complexity of Ca^{2+} stores and Ca^{2+} release channels may be further complemented by additional organelles capable of storing Ca^{2+} and by additional Ca^{2+} release channels, which were observed already in unicellular organisms. Additional Ca^{2+} stores, such as acidocalcisomes, have been recently discovered in various protozoa including *Dictyostelium* [99], in flagellate parasites, as well as in human ([81,100], this special issue). The big vacuole of higher plants may be similarly considered as a Ca^{2+} storage organelle.

There is indirect evidence from microinjection studies that some metabolites, such as cADPR and NAADP known to release Ca^{2+} from intracellular stores in higher eukaryotes are also effective in triggering Ca^{2+} release in *Paramecium* [84]. The cADPR is discussed as the physiological activator of RyRs [101], whereas NAADP activates two-pore-channels (TPCs) in acidic compartments [102,103], such as lysosomal and endosomal compartments in mammalian cells [104]. Although TPCs have not been hitherto identified in ciliates at a molecular level, their presence can be expected from the binding of NAADP with a K_D of 3.3 nM [84] and the reaction of cells to microinjected NAADP [105]; see also ref. [106], this special issue.

Several types of Ca^{2+} channels disappeared and some were newly formed during evolution. Ca^{2+} is involved in several steps of chemotaxis and a variety of Ca^{2+}-dependent key molecules are conserved from *Dictyostelium* to human [107]. In *Dictyostelium*, as mentioned, putative $InsP_3Rs$ may contribute to the motility in response to shear flow [92]. In the myxamoebae, certain sequences suggest the appearance of additional putative CRCs, of mucolipin type (late endosomes/lysosomes), or polycystin-2 type (these being equivalent to polycystin cation channel PKD2) or TRPP2 and a TPC in the contractile vacuole complex [47,108]. The PKD2 serves for the perception of fluid flow [108]. In *Dictyostelium*, Ca^{2+} permeable purinoceptors of P2X type, serve as CRCs localised to the contractile vacuole complex [71,109] where they modulate osmoregulation [110]. In plants, $InsP_3Rs$ and TRPs seem to have disappeared ([53], this special issue). TRPs and TPC channels have not been found in ciliate databases [111] although there are functional hints to support functional expression of TPCs, as outlined above.

Besides dedicated Ca^{2+} stores (ER, SR), many trafficking organelles (endosomes, lysosomes, phagosomes and some secretory organelles) also contain Ca^{2+} [112]. This organelles are likely to facilitate local signalling for vesicle interaction and fusion. Not only $InsP_3Rs$ and RyRs may be involved, but also some other types of CRCs. A characteristic example is Ca^{2+} release by a P2X purinoceptors contained in the contractile vacuole membrane of *Dictyostelium* at the contact site to the cell membrane where both membrane fuse by exocytosis for contents release [71]. The principle of heterogeneous distribution of CRCs between different Ca^{2+} storing organelles is realised also with other proteins relevant for trafficking [87,95]. For instance, acidification can determine targeting by a trans-membrane signal through a conformational change of the V-type H^+-ATPase complex and ensuing attachment of Rab-GTPase modulating proteins [113]; similarly a targeting sequence in human TPC2 interacts with Rab GTPases [114]. Escorters of different kinds can also achieve selective deposition of membrane proteins relevant for Ca^{2+} signalling (see [115] for details).

5.7. Release channels in other Bikonta

How is Ca^{2+} handled in green plants, from algae to angiosperms? In flagellated algae, such as *Euglena gracilis* [116], and in the complex alga, *Chara* [117], positioned at the roots of multicellular plant evolution, as well as in higher plants, $InsP_3R$ effects have been experimentally detected. In *Euglena*, cADPR applied to subcellular fractions was also effective [116], but no RyR-type channels have been detected so far. This is in line with electrophysiological recordings with reconstituted membranes isolated from the *Bryonia* plant (climbing on bushes in central Europe, [118]) and with biochemical studies with vacuolar membranes isolated from cauliflower [119]. Nevertheless, neither $InsP_3Rs$, nor voltage-gated Ca^{2+} channels nor TRP channels were identified in plants, in contrast to mechanosensitive, ATP-gated P2X-like purinoceptors, cyclic nucleotide-gated and two-pore channels ([53,72,73], this special issue).

In essence, $InsP_3Rs$ are widely distributed in lower eukaryotes (though not in all), where RyRs also appear in the form of RyR-LPs, as documented for *Paramecium*. In contrast, such channels could not be ascertained in higher-level bikonts, such as plants.

Most recently, molecular and bioinformatic analysis of representatives of unikonts and bikonts revealed the presence of Ca^{2+}/H^+ exchangers and of TPCs in both groups ([48], this special issue). This also includes a complex flagellar Ca^{2+} channel, CatSper, initially discovered as a sperm-specific cation channel responsible for motile activity of spermatozoa [120]. These molecules, therefore, seem to belong to a common original heritage. Analysis of a primitive bikont, *Aurantiochytrium limacinum* revealed, in addition, sequences of voltage-gated Ca^{2+} influx channels, $InsP_3Rs$ (though functionally not tested), Ca^{2+} exchangers, purinergic receptors, TPCs, TRPCs, MCU and regulator MICU, in addition to PMCA and SERCA ([48], this special issue). These are promising predictions, which however, require further scrutiny.

5.8. Store-operated Ca^{2+} entry

Release of Ca^{2+} from intracellular stores, with their consequent depletion, in many types of mammalian cells induces the store-operated Ca^{2+} entry, SOCE [121], initially defined as a capacitative Ca^{2+} entry [122]. The content of the ER store is monitored by the endomembrane protein, stromal interacting molecule (Stim), which, upon store depletion, oligomerises, migrates to ER-plasmalemmal junctions and opens the Ca^{2+}-release-activated Ca^{2+} (CRAC) channels assembled from Orai proteins [123,124]. None of these molecules have been detected in *Paramecium* [46]. Only in some algae sequences indicative of Orai have been found, whereas choanoflagellates possess Orai and Stim; none of them occurs in *Arabidopsis* and in *Dictyostelium* or the protozoa whose database has been evaluated ([48,111], this special issue). The SOCE can be also mediated by plasmalemmal TRP channels that can be, arguably, activated by oligomerised Stim [125,126]. The TRP channels are represented by five metazoan forms, which exist already in the genome of choanoflagellates [127] and are highly diversified in further evolution to mammals [128].

A store-operated Ca^{2+} entry, however, is operational in *Paramecium* [129], where it is linked to a Ca^{2+} release mediated by RyR-like channels [46]. Considering the manifold types of ER-cell membrane connections that become increasingly known [130–133], a functional equivalent of Orai/Stim may not be required for the coupling of cortical stores with the plasma membrane in lower eukaryotes providing other proteins take over this function. In *Dictyostelium* the situation seems to be different. Here, a CICR was reported to be coupled to contractile vacuole (an acidic compartment) activity as analysed in a mutant devoid of the putative $InsP_3R$ [134]. It would be interesting to see whether a NAADP signalling pathway could be involved here. In summary, plasmalemmal Ca^{2+} influx coupled to store activation via SOCE or perhaps also to CICR (if verified) appear evolutionary old mechanisms.

6. Evolution of Ca^{2+} binding proteins

Bacteria contain many CaBPs or their elements found also in eukaryotes (Fig. 1), including for example a calmodulin-like protein present in eubacteria. This and other bacterial CaBPs all contain EF-hand motif [16,49], thus suggesting a conserved role for EF-hand CaBPs throughout evolution all the way to flowering plants [80] and mammals [135]. At the same time the data on bacterial expression of centrin (also with EF-hand motives, [136]) or CaBPs with C2 domains are missing. Centrin is present from protozoa, including ciliates [137] to human and from *Chlamydomonas* to angiosperms [138,139a].

In animals, the number of EF-type CaBPs exceeds C2-domain CaBPs by 3 times, but only by about two times in plants [16,49].

The CaBPs with two C2 domains, as present in synaptotagmin of metazoans, are not known from protozoa, but they occur in plants [139b]. In animals, synaptotagmin is the metazoan Ca^{2+} sensor required for membrane fusion. By its fast conformational change synaptotagmin allows for rapid membrane fusion during exocytosis [140,141], as well as during endocytosis [142]. In *Paramecium*, the only comparable protein with eight C2 domains can be detected in the database (R. Kissmehl and H. Plattner unpublished observations) although the exocytosis in this species is very fast [64]. Similar proteins, called extended (E-) synaptotagmins, with up to six C2 domains have been detected also in mammalian cells where they can substitute for synaptotagmin for rapid vesicle fusion [143,144]. As in other protozoa, proteins with C2 domains [145] remain to be scrutinised also in *Dictyostelium*. In mammalian cells, exocytosis kinetics differs substantially between cell types [146,147], which can be possibly associated with different sensors [144]. In summary, scarce information about C2-type Ca^{2+} sensors in lower eukaryotes entails the salient question: How is membrane fusion mediated in these cells?

In summary, calmodulin contributes to the assembly of exocytotic membrane fusion sites from protozoa [148] to mammals [149,150]. Similarly, from protozoa onwards calmodulin activates the PMCA, although reportedly not in all species, as discussed above. Centrin expression is well established in protozoa; in *Paramecium* for instance, binding of Ca^{2+} to cortical centrin after strong stimulation of exocytosis is sufficiently fast and efficient to provide for fast $[Ca^{2+}]_i$ recovery [85].

Copines and annexins represent additional groups of CaBPs, either with C2 or with alternative Ca^{2+} binding motifs. After detection in *Paramecium* [151] copines were found ubiquitously, from *Dictyostelium* [152] and *Arabidopsis* [153] up to the mammalian brain [154]. By their C2 domains copines bind to membranes in Ca^{2+}-dependent manner and, hence, may contribute to vesicle trafficking [153]. Annexins are widely distributed Ca^{2+}/phospholipid binding proteins with typical domains, of neither of the EF- nor of the C2-type [155]. For coordinative Ca^{2+} binding only smaller stretches are available. After a long debate about the fusogenicity of annexins, e.g. during exocytosis, they are now considered as membrane-to-membrane links [155].

In summary, the substantial inventory of CaBPs in bacteria and protozoa provide a background for considerable increase in higher plants and animals. However, in protozoa, Ca^{2+} sensors mediating membrane fusion remain to be characterised.

7. An evolutionary selection for Ca^{2+} sensors

All eukaryotic cells contain enzymes regulated by Ca^{2+} binding. The *Dictyostelium* kinome contains a battery of Ca^{2+}/calmodulin-dependent kinases ("CaM-kinases") [156]. The ciliates [157], their relatives (Apicomplexa, e.g. *Plasmodium*) [158] as well as plants from green algae to land plants [159,160] all express Ca^{2+}-dependent protein kinases (CDPK), which contain an integrated calmodulin-like domain, rather than binding a free Ca^{2+}/calmodulin complex, as is the case for CaM-kinases in higher animal cells. From chlorophyceae (*Chlamydomonas*) to angiosperms the CDPK family is broadly represented [161] particularly in the course of transition to terrestrial life where it supports developmental processes and stress resistance [160]. Modern flowering plants also contain a genuine "CaM kinase" or Ca^{2+}/calmodulin activated protein kinase ([53], this special issue).

Other Ca^{2+} sensor functions include calcineurin (protein phosphatase 2B, PP2B) which, during animal evolution, has developed a signalling network based on conserved substrate motifs [162]. In mammals, calcineurin is involved in immune defence by activating transcription factor NFAT during T-cell activation as well as in long-term potentiation in Purkinje neurones [163]. Calcineurin also appears in myxamoebae [164] where it regulates development and differentiation. *Dictyostelium* and *Paramecium* contain both subunits of calcineurin, A and B [165,166], as is the case with other protozoa [166]. In flowering plants, only the regulatory Ca^{2+}-binding subunit B is found; it serves for stress defence (Fig. 1; [53,159], this special issue). Thus, calcineurin/PP2 is another system developed at early stages of Ca^{2+} signalling and maintained up to the very top of the evolutionary ladder. Being a CaBP itself, it also regulates Ca^{2+} dynamics, for example SOCE, via Orai and Stim [167]. Homer is another protein maintained from the choanoflagellate precursors of metazoans on [168]. In mammalian nervous system Homer serves as a scaffold for the formation of postsynaptic densities; remarkably, in choanoflagellates and in metazoan astrocytes it resides in the nucleus as a Homer/Flotillin (Reggie) complex [168].

8. Special aspects in apicomplexan parasites and plants

8.1. The parasites

Parasites are notorious for evolving survival strategies. It appears that apicomplexan parasites (*Plasmodium* and *Toxoplasma)*, close relatives of ciliates [36,158], have functionally transformed the equivalent of the alveolar sacs from a Ca^{2+} store in ciliates to the "inner membrane complex" which, in contrast to alveoloar sacs of *Paramecium*, have extremely low (i.e. below detection threshold) Ca^{2+} concentration [84]. These organelles are, arguably, dedicated to the mechanics of host cell penetration, rather than serving as a Ca^{2+} store [84,169]; the infectious attack is facilitated by the release of nearby docked dense core-secretory vesicles, rhoptries and micronemes (remotely resembling *Paramecium's* trichocysts). Consequently, these parasites rely on other Ca^{2+} stores (Lourido and Moreno [82] this special issue). Though $InsP_3$ effects relevant for Ca^{2+} signalling and host cell interaction have been established [170–172], the $InsP_3$Rs have not yet been identified [45,173]. In contrast, molecular and functional identification of $InsP_3$Rs has been achieved in other protozoa of the bikont branch, the parasitic flagellates, *Trypanosoma brucei* [174] and *Trypanosoma cruzi* [175]. In *T. brucei*, $InsP_3$Rs have been shown to be located in the acidocalcisome membrane ([81], this special issue).

8.2. Comparison with higher bikonts, the plants

Handling of, and signalling by Ca^{2+} in the immobile green plants differs considerably from that in animals. Ecological aspects, such as selection of non-calciferous locations, as well as gross regulatory mechanisms, for example release of an excess of Ca^{2+} ions by guttation from leaves, can help the plant cell to avoid stress. Limiting stress also requires regulation of Ca^{2+} at a cellular level [51]. Stress defence activates a system of CBL-interacting protein kinases (CIPKs) that are activated by calcineurin B [176,177], the regulatory subunit containing a Ca^{2+} binding site. In plant cell membrane and in the membrane of the big vacuole, primary active and secondary active transport processes occur through exchangers connected to the H^+-ATPase/pump. Here the most extensive diversification of calcineurin B-LPs (CBL) is observed; the CBL in conjunction with CIPKs, regulate many functions of the vacuole and of the plasma membrane [178]. No intracellular CRCs of the $InsP_3$R/RyR supergroup have been unambiguously demonstrated in plants although some reports advocated for $InsP_3$Rs-related physiological effects, as discussed above. In contrast, numerous influx channels and CaBPs have been identified in plants ([53], this special issue). Essentially plants may have lost $InsP_3$Rs as well as voltage-gated and TRP channels [73] using other channels instead.

8.3. Ca^{2+} signalling and transition to multicellularity

Transition to multicellularity called for cell adhesion proteins, including $Ca^{2+}{}_o$-dependent cadherins or their predecessors. The attachment of *Dictyostelium* to the substrate, similarly to mammalian cells, requires Ca^{2+} [92]. Cadherins are absent from *Dictyostelium* and their capability to form a multicellular stalk has another molecular basis [179]. The choanoflagellates genomic data mining suggests cadherin-based cell-to-cell connections ([60], and [48] this special issue). Proteins involved in intercellular contact formation during conjugation in ciliates are not related to metazoan cell adhesion molecules [180]. Cadherins were maintained throughout metazoan evolution, but have not been found in plant databases [181]. This indicates a clear separation between unikont and bikont organisms.

9. Conclusions

Fundamental elements of cellular Ca^{2+} homeostasis and Ca^{2+} signalling are conserved from bacteria to human. These include Ca^{2+}-pumps and exchangers, Ca^{2+} influx channels as well as some CaBPs, such as calmodulin, which all are present in prokaryotes. The Ca^{2+}/calmodulin-activated protein phosphatase PP2B (calcineurin) is engaged in manifold signalling from different protozoa up to human where it regulates complex functions, such as immune-defence and long term potentiation [182]. However, some gaps in knowledge remain; for instance there is a discrepancy between overt $InsP_3$ effects observed and the failure to identify $InsP_3R$-type genomic sequences in some organisms. The recent focus on choanoflagellates as ancestors of metazoans calls for the extension of molecular biology data to cell physiology. Interpretation of sequences in an evolutionary context can also be hampered by horizontal, and possibly also by vertical gene transfer that is common among protozoa [183]. Nevertheless, many data on Ca^{2+} signalling throughout evolution yield a quite consistent picture (Fig. 1). The present issue of Cell Calcium presents an interim balance.

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