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The IP_3 receptor and Ca^{2+} signaling in trypanosomes

Roberto Docampo[*], **Guozhong Huang**

Center for Tropical and Emerging Global Diseases, and Department of Cellular Biology, University of Georgia, Athens, GA, 30602, USA.

Abstract

Trypanosoma cruzi, and the *T. brucei* group of parasites cause neglected diseases that affect millions of people around the world. These unicellular microorganisms have complex life cycles involving an insect vector and a mammalian host. Both groups of pathogens possess an inositol 1,4,5-trisphosphate (IP_3)/diacylglycerol (DAG) signaling pathway, and an IP_3 receptor, but with lineage-specific adaptations that make them different from their mammalian counterparts. The phospholipase C (PLC), which hydrolyzes phosphatidyl inositol 4,5-bisphosphate (PIP_2) to IP_3 is N-terminally myristoylated and palmitoylated. Acidocalcisomes, which are lysosome-related organelles rich in polyphosphate, are the main intracellular Ca^{2+} stores. The inositol 1,4,5-trisphosphate receptor (IP_3R) localizes to acidocalcisomes instead of the endoplasmic reticulum. The trypanosome IP_3R is stimulated by luminal phosphate and pyrophosphate, which are hydrolysis products of polyphosphate (polyP), and inhibited by tripolyphosphate ($polyP_3$), which is the most abundant polyP in acidocalcisomes. Ca^{2+} signaling is important for host cell invasion and differentiation and to maintain cellular bioenergetics.

Keywords

Acidocalcisome; calcium; inositol phosphates; IP_3 receptor; mitochondria; polyphosphate

1. Trypanosomes and acidocalcisomes.

T. cruzi is the etiologic agent of Chagas disease or American trypanosomiasis, which is vector-borne disease endemic from the South of the United States to the South or Argentina and Chile. However, cases of this disease have been detected all over the world because of migratory activity [1]. Its life cycle involves vector stages (epimastigote, and metacyclic trypomastigote) and mammalian stages (bloodstream trypomastigote, and intracellular

[*]Corresponding author; rdocampo@uga.edu.

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amastigote). When the infected vector (triatomine) takes a blood meal it releases metacyclic trypomastigotes in its feces near the site of the bite wound. These trypomastigotes enter the host through the wound or through intact mucosal membranes, such as the conjunctiva, and invade nucleated cells where they transform into intracellular amastigotes. After replicating for 4–5 days, amastigotes are converted to cell-derived trypomastigotes, which are released into the circulation to infect other cells or to be ingested by another vector. In the vector, cell-derived trypomastigotes differentiate into epimastigotes, duplicate by binary fission in the midgut, and transform into metacyclic trypomastigotes in the hind gut to start a new cycle. The *T. brucei* group of parasites includes subspecies that cause human African trypanosomiasis (sleeping sickness), and nagana in cattle, and their occurrence is limited to the African continent. Two main stages of these parasites are usually studied: procyclic trypomastigotes (vector stage) and bloodstream trypomastigotes (mammalian stage). When the infected vector (tsetse fly) takes a blood meal it releases metacyclic trypomastigotes into the skin. The parasites enter the lymphatic system and pass into the bloodstream where they transform into bloodstream trypomastigotes, which reach different tissues and replicate while in circulation. The tsetse fly gets infected with bloodstream trypomastigotes when taking a blood meal on the mammalian host. The parasites transform into procyclic trypomastigotes in the midgut of the vector, multiply by binary fission, leave the midgut, and transform into epimastigotes. The epimastigotes reach the salivary glands and continue multiplication by binary fission to finally differentiate into metacyclic trypomastigotes to close the cycle.

Trypanosomes are characterized by possessing acidocalcisomes as their main Ca^{2+} storage organelle. Acidocalcisomes are acidic calcium stores first described in trypanosomes [2, 3]. Acidocalcisomes of trypanosomes possess a Ca^{2+}-ATPase for Ca^{2+} uptake [4, 5] and an IP_3 receptor for Ca^{2+} release [6, 7], and several other membrane pumps, channels, and exchangers (Fig. 1) [7, 8]. Acidification of the organelles is through two electrogenic pumps: a vacuolar H^+-ATPase [2, 3, 8, 9] and a vacuolar H^+-pyrophosphatase [10, 11]. Other transporters include orthologs to the yeast vacuolar iron transporter (VIT), a polyamine transporter [8], and a zinc transporter [8, 12], as well as channels: an aquaporin in *T. cruzi* [13, 14] and a K^+ channel in *T. brucei* [15]. There is also physiological evidence of the presence of Na^+/H^+ and Ca^{2+}/H^+ exchangers [16]. Acidocalcisomes are rich in P_i, PP_i, and polyphosphate (polyP) [17–19] and they possess an enzymatic complex named vacuolar transporter chaperone complex (VTC) for the synthesis and translocation of polyP, formed by at least two subunits, VTC1 and VTC4 [20–22]. There is also evidence for the presence of enzymes possibly linked to polyP hydrolysis within acidocalcisomes: an exopolyphosphatase [18], a vacuolar soluble pyrophosphatase (VSP) [23–26], and an acid phosphatase [8]. VSP has pyrophosphatase activity in the presence of Mg^{2+} and exopolyphosphatase activity in the presence of Zn^{2+} [24]. There is also a Na^+/phosphate symporter orthologue to PHO91 of yeast, involved in Na^+ and P_i release [8, 27–29]. PolyP, a polymer of three to hundreds of orthophosphate units is abundant within acidocalcisomes, could reach molar concentrations, and is complexed with cations, such as calcium, magnesium, potassium, sodium, zinc, and iron [27]. This chemical composition is probably the reason for the high electron-density of acidocalcisomes when examined by electron microscopy. They are spherical, have an average diameter of 0.2–0.3 μm and can also be

identified by staining with DAPI, that labels polyP, or dyes that accumulate in acidic compartment, such as acridine orange or LysoTracker [30]. Functions of the acidocalcisomes include cation and Pi storage [27], osmoregulation [14, 31, 32], parasite persistence [24], Ca^{2+} signaling [33], and regulation of cell bioenergetics [34].

2. The IP_3/diacylglycerol signaling pathway

This pathway was first described in *T. cruzi* [35] and later found in *T. brucei* [36]. Using [^{32}P]P_i and [^{3}H]inositol as precursors it was possible to detect phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2), and their derivatives inositol phosphate (IP), inositol 1,4-bisphosphate (IP_2) and IP_3, respectively, in epimastigotes of *T. cruzi*. Calcium stimulated the formation of IP_2, IP_3, and diacylglycerol in permeabilized cells [35]. The use of [^{3}H]inositol labeling also identified the presence of IP, IP_2 and IP_3 in *T. cruzi* amastigotes [37] and trypomastigotes [38], and in procyclic and bloodstream forms of *T. brucei* [36, 39].

A novel phosphoinositide-specific phospholipase C (PI-PLC), which is responsible for the hydrolysis of PIP_2 to IP_3 was also identified in *T. cruzi* [31, 34, 40–43] and *T. brucei* [44, 45]. The *T. cruzi* enzyme is different from mammalian PI-PLCs: a) it is stimulated by [Ca^{2+}] at above 10 nM; b) lacks a pleckstrin homology (PH) domain; c) has a highly charged linker region between the catalytic X and Y domains; d) has an N-myristoylation consensus sequence at its amino-terminal end, and is dually acylated in vivo by myristate, and palmitate or stearate; and e) is targeted to the plasma membrane [40] (Fig. 2A). Myristoylation is in a glycine residue in the 2nd position while palmitoylation or stearylation is in the cysteine in the 4th position of the protein [31]. The amino-terminal 20 amino acids of TcPI-PLC are necessary and sufficient to target a fused GFP to the surface of epimastigotes and amastigotes [31]. Interestingly, TcPI-PLC localizes to the outer surface of amastigotes, and dual acylation is required for this localization [31].

TcPI-PLC shows two peaks of surface expression in amastigotes, the first immediately after differentiation of trypomastigotes into amastigotes and the second before differentiation of amastigotes to trypomastigotes [41].

Two potential roles of the surface expression of TcPI-PLC are: 1) a role in shedding GPI-anchor proteins; and 2) a role in cell signaling in the host. Concerning the first role, a number of GPI-anchored proteins from *T. cruzi* (*trans*-sialidases, mucins, Ssp4) are shed to the medium by an endogenous PLC [46–48], and it has been shown that the presence of ceramide in the lipid portion of the GPI anchor of these proteins is related to the shedding by endogenous PLC [48]. In this regard, TcPI-PLC is equally capable of cleaving phosphatidylinositol and inositol phosphoceramide, which is the lipid portion of these GPI-anchored proteins [49]. Concerning the second role, surface expression and secretion of TcPI-PLC coincide with PIP_2 depletion in the host cell membrane and increase in IP_3, suggesting a role of TcPI-PLC in cell signaling in the host [41].

TcPI-PLC expression and production of IP_3 are induced during in vitro differentiation of trypomastigotes to amastigotes at low pH [40, 42]. This differentiation is inhibited by

treatment of the cells with antisense oligonucleotides against *TcPI-PLC*, and stimulated by *TcPI-PLC* overexpression, suggesting that this pathway is involved in this differentiation step [42].

T. brucei PI-PLC (*TbPI-PLC*) is structurally similar to the *T. cruzi* enzyme. It lacks a PH domain, has a negatively charged region between the catalytic regions X and Y, and an amino-terminal N-myristoylation consensus sequence that is important for targeting it to intracellular vesicles close to the plasma membrane in procyclic forms (Fig. 2A). The enzyme is active at Ca^{2+} concentrations below the cytosolic levels, suggesting that it is constitutively active. Its downregulation by RNAi did not result in growth inhibition in procyclic forms and its overexpression increased the activity of lysates, demonstrating its function. Overexpression of *TbPI-PLC* in bloodstream forms, which is localized to the inner face of the plasma membrane, has no effect on growth but derepresses silent telomeric expression sites (ES) leading to the expression of multiple variant surface glycoproteins (VSG) and this effect is ablated by mutation of its catalytic site [45]. Endogenously tagged TbPI-PLC in procyclic forms [44] and overexpressed TcPI-PLC in bloodstream forms [45] co-localize with their substrate PIP_2.

In conclusion, the IP_3/diacyglycerol signaling pathway is present in both *T. cruzi* and *T. brucei*. The PI-PLCs of both trypanosomatids have similar structural characteristics that distinguishes them from the mammalian enzymes. While TcPI-PLC appears to have roles in differentiation of trypomastigotes to amastigotes, and potential roles in shedding of GPI-anchor proteins and host cell signaling, TbPI-PLC is somehow involved in repression of telomeric ES.

3. The IP_3 receptor

The IP_3 receptor was first characterized in *T. brucei* [6]. Sequence analysis using the InterPro and TMHMM servers predicted 5 transmembrane domains (TMDs) in the C-terminal of the IP_3R of *T. brucei* (6) and *T. cruzi* (34), as also described in TriTrypDB (http://tritrypdb.org/tritrypdb/). Structural studies will be needed to reveal whether this change affects the topology of the receptor as compared with the mammalian orthologs that possess 6 TMDs. The receptor has a series of conserved domains including a suppressor domain-like (SD), ryanodine receptor IP_3R homology (RIH), and RIH-associated (RIAD) domains [50] (Fig. 2B). A Ca^{2+}-selectivity filter (GGGVGD), similar to those present in the mammalian IP_3Rs, is in the intraluminal loop between TMDs at the C-terminal region. Only 5 of the 10 residues that form the basic pocket that binds IP_3 [51, 52] are present in $TbIP_3R$ [6]. IP_3 was able to stimulate Ca^{2+} release from DT40–3KO cells transfected with $TbIP_3R$ in a dose-dependent manner [6]. DT40–3KO cells are chicken lymphocytes in which the three endogenous vertebrate IP_3Rs have been knocked out. IP_3 was also able to stimulate Ca^{2+} release from digitonin-permeabilized procyclic forms or from isolated acidocalcisomes, demonstrating its activity in vitro [6]. UV light photolysis of caged IP_3 increased intracellular Ca^{2+} in live procyclic trypanosomes. Knock down of $TbIP_3R$ in bloodstream forms produced growth defects in vitro and reduced mouse infectivity in vivo [6].

Experiments with DT40–3KO cells expressing $TbIP_3R$ revealed that IP_3-mediated Ca^{2+} release depends on Ca^{2+} but not on ATP concentration, is inhibited by heparin, caffeine, and 2-aminomethoxydiphenyl borate (2-APB), and stimulated by adenophostin A [53]. In contrast to the results with DT40–3KO cells expressing rat type I IP_3R, DT40–3KO, excised patch clamp recordings from nuclear membranes of DT40–3KO cells expressing $TbIP_3R$ showed that luminal orthophosphate (P_i) or pyrophosphate (PP_i) and neutral or alkaline pH stimulated IP_3-generated currents. However, long chain polyphosphate or acidic pH did not generate currents, while tripolyphosphate ($polyP_3$), the most abundant polyP of acidocalcisomes, inhibited these currents [53]. When extrapolated to what could occur in acidocalcisomes, these results would explain that under the normal acidic conditions of the organelle, the IP_3R channel would be closed but when intra-organelle pH increases, for example by increased amino acid catabolism and production of NH_3 and its sequestration in acidic compartments as ammonium ($NH4^+$), the channel could open and favor Ca^{2+} release. Alkalization would also activate polyP hydrolysis by the vacuolar soluble pyrophosphatase, which hydrolyze polyP to P_i and PP_i (Fig. 1), favoring the opening of the channel. In agreement with this suggestion it was found that alkalization of acidocalcisomes in permeabilized cells by exposure to ionophores or NH_4Cl led to Ca^{2+} release [53].

T. cruzi IP_3R ($TcIP_3R$) is structurally very similar to $TbIP_3R$ sharing 45% of amino acid identity. It was also functionally expressed in the ER of DT40–3KO cells to demonstrate IP_3-mediated Ca^{2+} release [34, 54]. Knockdown of $TcIP_3R$ led to defects in growth, metacyclogenesis and infectivity in vitro and reduced parasitemia in mice [54]. Recent work using the CRISPR/Cas9 technique to knockout the expression of *$TcIP_3R$* showed that the channel is required for Ca^{2+} uptake by mitochondria, regulation of pyruvate dehydrogenase dephosphorylation, and mitochondrial O_2 consumption [34]. Knockout mutants increased ammonia production and the AMP/ATP ratio, and increased autophagy, revealing a modulatory activity of $TcIP_3R$-mediated acidocalcisome Ca^{2+} release on cell bioenergetics [34] (Fig. 3A), which is favored by the presence of acidocalcisome-mitochondrion membrane contact sites [55] (Fig. 3B).

4. Ca^{2+} signaling

The best evidence for the involvement of Ca^{2+} signaling in *T. cruzi* has been the detection of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase in Fura 2-labeled trypomastigotes upon contact with host cells [56], and the prevention of host cell invasion by preincubation of the parasites with intracellular Ca^{2+} chelators [56, 57]. In agreement with these results, increasing intracellular Ca^{2+} by treatment with ionomycin led to a higher host cell invasion [57]. The trypomastigotes receptor that triggers this Ca^{2+} increase has not been identified although there is some evidence that Ca^{2+} release from acidocalcisomes could be involved in this process [34]. Ablation of the gene that encodes the $TcIP_3R$ led to decreased host cell invasion [34]. Similarly, treatment of parasites with a combination of ionomycin plus NH_4Cl or nigericin, which are known to deplete acidocalcisome Ca^{2+} [2], reduces the infectivity of metacyclic forms [33].

Attachment and invasion of host cells is a process that requires energy [58] and it has been shown that IP_3-mediated Ca^{2+} release from acidocalcisomes is important for mitochondrial

Ca^{2+} uptake and generation of ATP [34]. Knockout of the genes involved in mitochondrial Ca^{2+} uptake [59, 60] or in mitochondrial activation of bioenergetic metabolism [61], inhibit host cell invasion.

A role for Ca^{2+} signaling in metacyclogenesis was also inferred after detection of a rise in $[Ca^{2+}]_i$ in epimastigotes by addition of *Triatoma infestants* (one of the vectors) homogenates. Both Ca^{2+} rise and metacyclogenesis were blocked by treatment with an intracellular Ca^{2+} chelator (BAPTA-AM) but not by extracellular EGTA [62], suggesting an intracellular origin of Ca^{2+}.

Evidence for Ca^{2+} signaling during differentiation of *T. brucei* is more indirect and based on changes in $[Ca^{2+}]_i$ during the differentiation from long slender to short stumpy bloodstream forms [63].

Finally, IP_3-dependent Ca^{2+} release from acidocalcisomes is important to maintain cellular bioenergetics through mitochondrial Ca^{2+} uptake and stimulation of energy generation in both T*. cruzi* [34] and *T. brucei* [6, 64].

Outlook

Trypanosomes have distinct acidic Ca^{2+} stores named acidocalcisomes that have a Ca^{2+}-ATPase for Ca^{2+} uptake and an IP_3 receptor for Ca^{2+} release. IP_3 is formed by hydrolysis of PIP_2 catalyzed by a peculiar phospholipase C that lacks a PH domain for plasma membrane localization, and instead is N-terminal myristoylated and palmitoylated. Surface expression of PI-PLC could have potential roles in shedding of GPI-anchored proteins from the parasite and cell signaling in the host. The IP_3 receptor is also peculiar with only 5 TMD, and only 5 of the 10 amino acids described in the mammalian enzymes needed for binding IP_3. The inositol phosphate/diacylglycerol pathway is important for Ca^{2+} signaling, which is required for *T. cruzi* host cell invasion and metacyclogenesis, potentially for *T. brucei* differentiation, and for maintaining cellular bioenergetics in both parasites. In conclusion, differences between trypanosomatid and mammalian IP_3 signaling represent good targets for the development of new strategies to combat these organisms.

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Highlights

- Acidocalcisomes are acidic Ca^{2+} stores rich in polyphosphate
- The IP_3 receptor of trypanosomes is in the acidocalcisomes
- The IP_3 receptor is luminally stimulated by polyphosphate hydrolysis products
- The trypanosome PI-PLC is lipid modified and surface-localized
- Ca^{2+} in trypanosomes is important for differentiation, autophagy, and infectivity

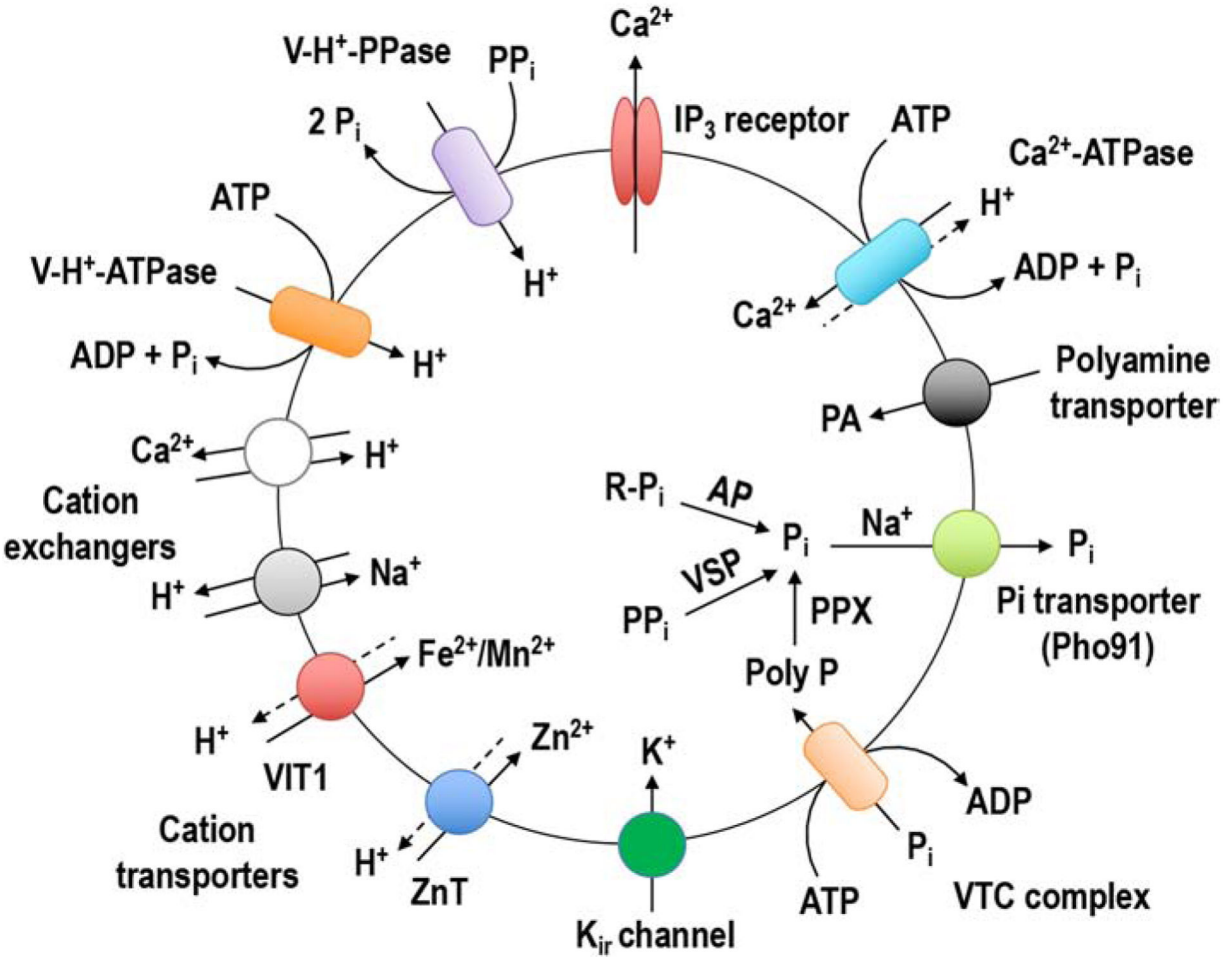

Figure 1.

Schematic representation of the acidocalcisome of *T. brucei*. In the *T. brucei* acidocalcisomes, Ca^{2+} is taken up by a H^+-counter-transporting Ca^{2+}-ATPase and released by the inositol 1,4,5,trisphosphate (IP_3) receptor. H^+ is pumped in electrogenically by either the V-H^+-PPase or the multisubunit V-H^+-ATPase. Ca^{2+}/H^+ and Na^+/H^+ exchangers could be used for Ca^{2+} release in exchange for Na^+ uptake. A vacuolar iron transporter (VIT1) can be used for either Mn^{2+} or Fe^{2+} uptake and a Zn^{2+} transporter (ZnT) for Zn^{2+} uptake. There is also a polyamine (PA) transporter. A VTC with at least two subunits (Vtc1 and Vtc4) synthesizes polyP using ATP and translocates it into the organelle. A Na^+/P_i symporter (Pho91) releases Na^+ and Pi from acidocalcisomes. Within acidocalcisomes there is a vacuolar soluble pyrophosphatase (VSP), an exopolyphosphatase (PPX) and an acid phosphatase (AP). An inward rectifier potassium channel (K_{ir}) also localizes to the acidocalcisome.

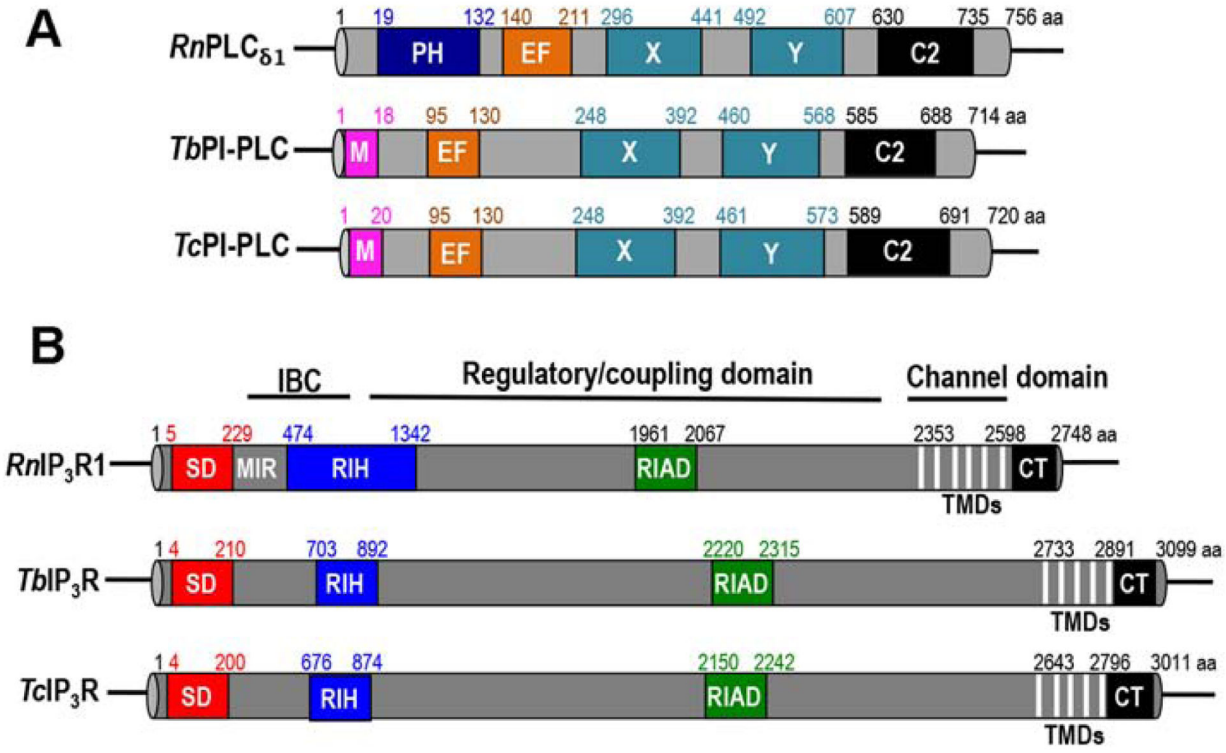

Figure 2.

(A) Schematic domain organization of PLC proteins, from *Trypanosoma brucei* (*Tb*PI-PLC, Tb927.11.5970), *Trypanosoma cruzi* (*Tc*PI-PLC, TcCLB.504149.160), and *Rattus norvegicus* (*Rn*PLC$_{\delta 1}$, NP_058731.1). M, myristoylation domain; PH, Pleckstrin homology domain; EF, EF-hand domain; X and Y, catalytic domains; C2, C2 domain. (B) Schematic domain organization of IP$_3$R proteins, from *T. brucei* (*Tb*IP$_3$R, Tb927.8.2770), *T. cruzi* (*Tc*IP$_3$R, TcCLB.509461.90), and *R. norvegicus* (RnIP$_3$R1, NM_001007235.2). SD, suppressor domain; MIR, (mannosyltransferase, IP$_3$R and RyR) domain; RIH, (RyR and IP$_3$R homology) domain; RIAD, RIH-associated domain; TMDs, transmembrane domains (also called as channel or pore-forming domain); CT, cytosolic-terminus domain (also called as gate keeper domain); IBC, IP$_3$ binding core domain. The conserved functional domains of the PLC or IP$_3$R proteins were identified with InterPro (ebi.ac.uk/interpro).

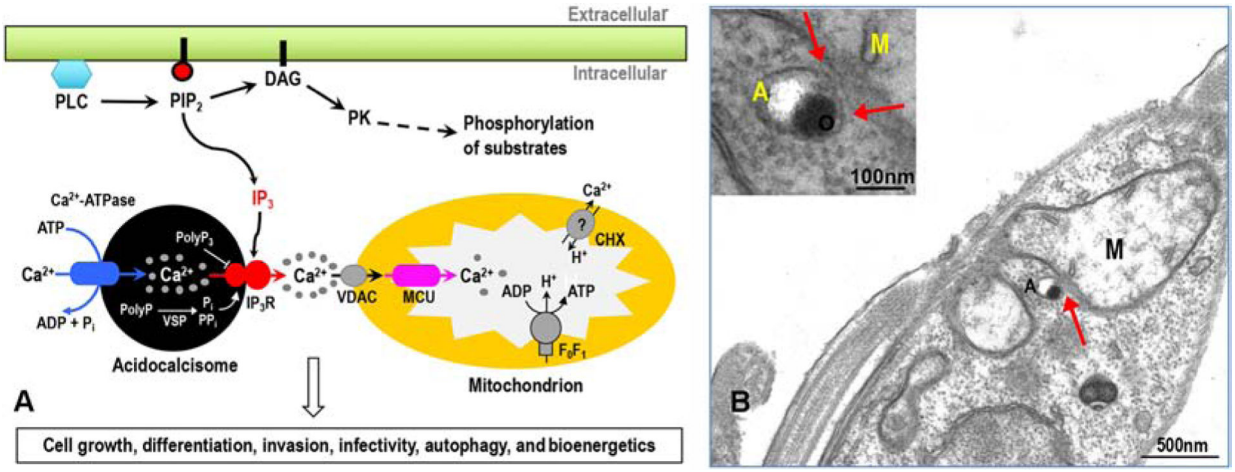

Figure 3.

(A). Schematic representation of IP_3-mediated Ca^{2+} signaling pathway in trypanosomes. Inositol 1,4,5-trisphosphate (IP_3) is generated by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), catalyzed by phosphoinositide-specific PLC (PI-PLCs), which also generates diacylglycerol (DAG), possibly activating protein kinase (PK) that catalyzes phosphorylation of a variety of intracellular proteins. IP_3 binds to IP_3 receptor (IP_3R) and stimulates Ca^{2+} release from acidocalcisomes through the IP_3R into the cytosol, thereby activating various Ca^{2+} regulated signal molecules, which regulate the parasite's cell growth, differentiation, invasion, and infectivity. The IP_3-mediated Ca^{2+} release from acidocalcisome can also be modulated by the activation of the luminal orthophosphate (P_i) or pyrophosphate (PP_i) and by the inhibition of tripolyphosphate ($polyP_3$). Importantly, when Ca^{2+} release from acidocalcisomes is stimulated, Ca^{2+} permeates a mitochondrial voltage-dependent anion-selective channel (VDAC) through microdomains of high Ca^{2+} concentration (*gray balls*) present in their vicinity, and subsequently Ca^{2+} can be efficiently taken up by the mitochondrial calcium uniporter (MCU), thereby regulating mitochondrial metabolism, autophagy, and cell bioenergetics. CHX, Ca^{2+}/H^+ exchanger. (B) Transmission electron microscopy of procyclic forms (PCF) of *T. brucei* shows membrane contacts between acidocalcisome (A) and mitochondrion (M) of this parasite, which may function for efficient Ca^{2+} transfer as described in (A). An acidocalcisome appears as a rounded organelle containing electron-dense material that adheres to one side of the membrane, and is seen adjacent to the mitochondrion double membrane. Reproduced with permission from reference 55.