

Review Article

Phospholipases A in Trypanosomatids

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Phospholipases are a complex and important group of enzymes widespread in nature, that play crucial roles in diverse biochemical processes and are classified as A₁, A₂, C, and D. Phospholipases A₁ and A₂ activities have been linked to pathogenesis in various microorganisms, and particularly in pathogenic protozoa they have been implicated in cell invasion. Kinetoplastids are a group of flagellated protozoa, including extra- and intracellular parasites that cause severe disease in humans and animals. In the present paper, we will mainly focus on the three most important kinetoplastid human pathogens, *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania spp.*, giving a perspective of the research done up to now regarding biochemical, biological, and molecular characteristics of Phospholipases A₁ and A₂ and their contribution to pathogenesis.

1. Introduction

Kinetoplastids are a group of flagellated protozoans distinguished by the presence, in their single large mitochondrion, of a DNA-containing region known as kinetoplast. These unicellular organisms have a similar genomic organization and cellular structures and undergo morphological changes during their life cycles, being transmitted by different insect vectors. The members of this group include extra- and intracellular parasites that cause severe diseases in humans and animals, as well as various free-living forms. Herein, we will mainly focus on the three most important human pathogens *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania spp.*

T. brucei and its subspecies are extracellular parasites transmitted by tsetse flies and responsible of human African trypanosomiasis (HAT), also known as African sleeping sickness, and Nagana in cattle. The life cycle of African trypanosomes is complex and represented by extracellular stages found in blood, lymph, and spinal fluid in the mammal [1]. The disease threatens over 70 million people and uncounted numbers of cattle in 36 countries of sub-Saharan Africa, having a devastating impact in human health and economy in affected areas [2]. HAT symptoms occur in two stages; in the first, haemolympathic phase, parasite invasion

of the circulatory and lymphatic systems is associated with severe swelling of lymph nodes. If untreated, the disease overcomes the host's defences and can cause more extensive damage. The second stage, neurological phase, begins when the parasite invades the central nervous system by passing through the blood-brain barrier; without treatment, it is fatal and the damage caused can be irreversible [1].

T. cruzi in contrast, is an intracellular parasite that invades all types of nucleated cells in the mammalian host. This protozoa, transmitted through the faeces of bloodsucking insects of the Triatominae family, enters the mammalian host via damage to the skin and causes Chagas disease in humans, a chronic inflammatory condition characterized by cardiomyopathy, megacolon, and mega-esophagus [3]. The disease is prevalent throughout America and according to WHO estimations, 25 million people are at risk and 10 million are infected worldwide [4]. Chemotherapy against Chagas disease is limited and unsatisfactory. The two available drugs, nifurtimox and beznidazole, are capable of curing at least 50% of recent infections and both produced side effects [5].

The genus *Leishmania* comprises 20 species of intracellular protozoan that are transmitted by phlebotomine sandflies and infect specifically cells of the mononuclear phagocyte system in mammals. These parasites cause various diseases

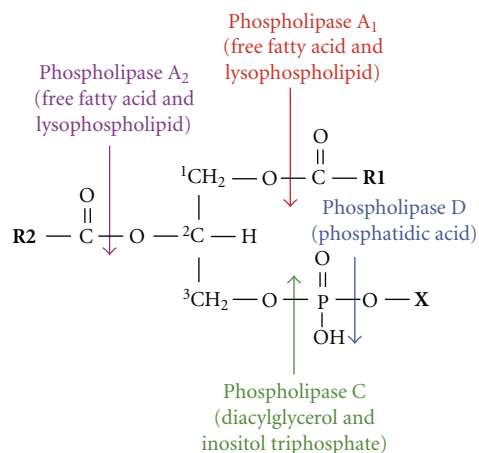


FIGURE 1: Hydrolysis of phospholipids by phospholipases. Arrows indicate the sites of attack for hydrolytic cleavage of phospholipases type A₁, A₂, C, and D. The main products generated by their action are also shown. R₁/R₂: free fatty acids in *sn*-1 or *sn*-2 positions; X: choline, ethanolamine, serine, inositol, and so forth.

ranging from self-healing cutaneous leishmaniasis, mucocutaneous leishmaniasis, with partial or total destruction of the mucous membranes, to severe and lethal (if untreated) visceral leishmaniasis, also known as kala-azar. Leishmaniasis is wide spread in Southern Europe, Africa, Asia, and America, threatening 350 million people in 88 countries around the world and represents an important global health problem that results in a significant economic burden [6].

One of the major components of biomembranes present in all living organism are phospholipids (PL), which form the lipid bilayer and serve as hydrophobic anchors of membrane proteins. These compounds can be enzymatically modified by the action of phospholipases (PLAs), with generation of bioactive lipid molecules that can act as second messengers and also modulate the immune response [7–9]. Moreover, PLAs have been considered virulence factors for many pathogenic bacteria like *Escherichia coli*, *Helicobacter pylori*, *Neisseria spp.*, *Yersinia spp.*, and so forth [10–14].

PLAs are a complex and important group of enzymes, widespread in nature, that play crucial roles in diverse biochemical processes and are classified as A₁, A₂, C, and D, depending on the site of hydrolysis [15]. These enzymes cleave cell membrane and intracellular PL releasing a variety of products such as lysophospholipids (LPL), free fatty acids (FFA), diacylglycerols (DG), choline phosphate, phosphoinositides and phosphatidic acid, among others (Figure 1).

Phospholipase A₁ (PLA₁) (EC 3.1.1.32) specifically hydrolyses acyl groups from PL at the *sn*-1 position, producing FFA and LPL (Figure 1) [15]. PLA₁ activities have been detected in various cell types and tissues from a wide range of organisms by measuring hydrolysis of phosphatidylcholine (PC) to lysophosphatidylcholine (LPC) [15]. But despite their apparent ubiquity and diversity, up to now a limited number of PLA₁s have been cloned and characterized [16–18]. Increasing evidence indicates that some PLA₁s are capable of generating bioactive lipids, a role traditionally

assigned to PLAs A₂, C, and D, though the biological relevance of this particular PLA deserves to be deeply studied [16, 19, 20].

At present time, nine PLA₁ molecules are known in mammals, being six extracellular and three intracellular enzymes, sharing no sequence homology between them and probably having distinct functions [7]. The extracellular PLA₁s belong to the pancreatic lipase gene family, that is conserved in an extensive range of organisms from insects to mammals and have been biochemically characterized and classified, according to their substrate specificities, structures, expression patterns, and possible functions [7]. Some PLA₁s have a broad substrate specificity and might also have triacylglycerol lipase activity (EC 3.1.1.3) [21]. Others, such as phosphatidylserine-specific PLA₁s from rat platelets and membrane-associated phosphatidic acid PLA₁α and PLA₁β, show strict substrate specificity [20]. The latter PLA₁s have specific roles in producing bioactive LPL, lysophosphatidylserine, and lysophosphatidic acid [7].

Phospholipases A₂ (EC 3.1.1.4), in contrast to PLA₁, are the most widely studied. Great advances in understanding the structure and function of the superfamily of Phospholipase A₂ (PLA₂) has occurred in the last decades [9, 22–25]. This superfamily includes fifteen groups, comprising four main types including secreted PLA₂ (sPLA₂), cytosolic PLA₂ (cPLA₂), Ca²⁺-independent PLA₂ (iPLA₂), and platelet activating factor acetyl hydrolase/oxidized lipid lipoprotein associated PLA₂ (LpPLA₂) [9]. The classification is based upon the following characteristics: source, secreted, or cytosolic, availability of structural information, molecular weight, cofactors, and inhibitor specificity [9]. sPLA₂s have a low molecular weight (~14 kDa) and contain a large number of disulphide bridges, consistent with their extracellular environment, and require millimolar concentrations of Ca²⁺ for optimum catalytic activity [9]. cPLA₂s have a high molecular weight (~85 kDa) and preferentially hydrolyze PL containing arachidonic acid, therefore playing a key role in the biosynthesis of eicosanoids, precursors of prostaglandins and thromboxane [26]. Full activation of these enzymes requires Ca²⁺ binding to an N-terminal C2 domain and phosphorylation on serine residues [23]. iPLA₂s have a high molecular weight (85–88 kDa), contain seven to eight ankyrin repeats, one of the most common sequence motif, and the consensus lipase motif GX SXG, being detected mainly in human tissues [27].

Considering the important and various roles that PLAs possess, in the present review, we will summarize the research done to date in Trypanosomes regarding biochemical, biological, and molecular characteristics of PLAs and their contribution to pathogenesis.

2. Biochemical and Biological Characteristics of the Trypanosomatids Phospholipases A

2.1. Phospholipase A₁. In 1978, Tizard et al. described the presence of a haemolytic activity in *T. congolense*, due to the FFA generated by the action of PLA on endogenous PC, meanwhile in the nonpathogenic *T. lewisi*, no FFA generation



FIGURE 2: Multiple sequence alignment of trypanosome PLA₁. Alignment of the protein sequences of cloned *T. brucei*, *T. cruzi*, and *L. braziliensis* PLA₁ and *T. congolense* and *T. vivax* putatives PLA₁ was performed with NTI 10 Software (Invitrogen) using the Clustal W algorithm. The lipase consensus pattern is underlined. Letters indicate: identical (red), conserved (blue), similar (green), weakly similar (grey), and nonsimilar (black) amino acids. *Leishmania braziliensis* PLA₁ (TriTrypDB LbrM31_V2.2750), *Trypanosoma brucei* PLA₁ (TriTrypDB Tb.927.1.4830), *Trypanosoma congolense* putative PLA₁ (TriTrypDB TcIL3000.1.2010), *Trypanosoma vivax putative* PLA₁ (TriTrypDB TvY486_0102170), and *Trypanosoma cruzi* PLA₁ (TriTrypDB Tc00.1047053510679.100).

was observed and therefore, nonhaemolytic activity was detected [28]. Further, it was determined in four different species of African trypanosomes that Ca²⁺-independent PLA₁ was the predominant PC-degrading activity. The levels of PLA₁ varied widely, with very high activity in the pathogenic *T. brucei* and relatively low activity in the nonpathogenic *T. lewisi* species [29]. Other authors found that *T. brucei* bloodstream forms possess high levels of PLA₁ activity, whereas in the procyclic culture forms PLA₁ specific activity was only 15% of that of bloodstream forms, suggesting an important physiological role for the enzyme in the mammalian stage [30]. Bloodstream trypomastigotes are covered with a dense layer of Variant Surface Glycoprotein (VSG), which protects the parasite from lysis by host complement via the alternative pathway [31]. It has been

suggested that the high activity of PLA₁ in these forms, may play a role in the acquisition of fatty acids for synthesis of the VSG and also provide a source of myristate that can be employed for remodelling the lipid anchor of the VSG [32]. PLA₁ was purified from *T. brucei* bloodstream forms, where the major portion was found as a soluble activity in the cytosol and the minor as a particle-bound activity associated with lysosomal markers. Both enzymes had optimal activity at acid pH and were activated by Triton X-100 [30]. Although cultured procyclic trypomastigotes also possess PLA₁ activity, the levels were significantly reduced compared to bloodstream forms, due to a decrease in soluble PLA, similar levels of lysosomal activity were present in both stages [30]. Other authors reported that PLA₁ activity eluted together with a lysophospholipase activity (LPLA)

(EC:3.1.1.5), suggesting that a single enzyme displays both activities [33].

T. brucei PLA₁ (TbPLA₁) has been recently cloned and expressed as recombinant protein [34]. This intracellular enzyme is localized in the cytosol and has optimal activity at neutral pH and a predicted size of 34.8 kDa. TbPLA₁ deacylates choline-containing PLs, with greater efficiency than those containing ethanolamine, inositol, serine, or just phosphorous at the *sn*-3 position [34]. The enzyme also displayed LPLA activity towards LPC, as previously reported [33].

As regards *T. cruzi*, we previously determined that when epimastigotes were grown at 28°C and then transferred to 37°C, a fatty acid exchange occurs between PL and neutral lipids [35]. This mechanism of membrane lipid adaptation suggested for the first time the action of PLA activity as part of a deacylation-reacylation cycle in *T. cruzi* [35]. Other authors reported membrane PLA₁ and PLA₂ activities in epimastigotes, but acting only on anionic PL such as inositolphospholipids and inositolphosphoceramides [36]. We further determined the presence of a PC-PLA₁-degrading activity in all parasite stages, being up to 20-fold higher in the infective amastigotes and trypomastigotes than in the noninfective epimastigotes, as occurs in *T. brucei*, where the mammalian stages possess the highest levels of PLA₁ activity [30, 37]. Interestingly, in both infective stages membrane-bound PLA₁ activity was remarkably higher than those detected in organelle bound or soluble fractions [38]. This localization does not appear to have a similar counterpart in *T. brucei*, where the major proportion of activity (more than 90% of the total) corresponds to a soluble cytosolic fraction [30]. In *T. cruzi* epimastigotes, in contrast, the enzyme was only detected in lysosomes [37]. It is remarkable that only infective stages secreted PLA₁ to the extracellular media [38], similarly to other enzymes that participate in *T. cruzi* endocytic pathway, such as cruzipain and transsialidase [39–42]. We purified *T. cruzi* PLA₁ (TcPLA₁) from epimastigote and amastigotes, obtaining in both cases a unique band of ~38 kDa. These enzymes proved to be independent of the bivalent cations Ca²⁺, Mn²⁺, and Mg²⁺, had an optimum acidic pH, and were activated by Triton X-100. The biochemical characteristics of TcPLA₁ activities were similar to those reported for TbPLA₁ and other PLA₁s from mammals [43, 44]. As previously demonstrated in *T. brucei* we also determined the presence of LPLA activity in autolysing parasites [17, 33, 45].

As concerns secreted TcPLA₁, we determined during metacyclogenesis, process in which epimastigotes differentiate into the infective metacyclic trypomastigotes, an increase in secreted enzyme activity simultaneously with the appearance of metacyclic forms, as expected [38]. Accordingly, it has been reported that membrane PLA₁, A₂, and C, may act in remodelling reactions needed for plasma membrane transformation during *T. cruzi* differentiation; these enzyme activities may be acting in remodelling reactions leading to the anchor of the mature glycoproteins of *T. cruzi* [46].

In the case of *Leishmania spp.*, preliminary results of our laboratory have also demonstrated in *L. braziliensis* promastigotes a PLA₁ activity hydrolyzing PC. Moreover, we

detected by Immunoblot two bands of ~37 and 41 kDa, using polyclonal antibodies against both TcPLA₁ and TbPLA₁ [47]. These antibodies were obtained in our laboratory, since no commercial antibodies against any PLA₁ were available until this year [48].

2.2. Phospholipase A₂. The eukaryotic PLA₂ were the first of the PLAs to be recognized. The pancreatic PLA₂ has been known to degrade PC since 1878, and at the turn of the century cobra venom was shown by Keyes in 1902 to have haemolytic activity directed towards the membranes of erythrocytes [49]. Secreted and membrane-bound PLA₂ activity has been described in Bacteria, Fungi and Protozoa [50–54], but in the case of Trypanosomes just a few reports are available.

PLA₂ was isolated from *T. congolense*; the enzyme appeared to exist in a dimeric form with subunit molecular weights of 16.5 and 18 kDa, had optimum pH of 6.8, and showed specificity for 1,2-dimyristoyl-*sn*-PC and 1,2-dioleoyl-*sn*-PC [55]. Inhibition studies implicated a thiol group at the catalytic site of the enzyme, which was stable to heat treatment and possessed haemolytic and anticoagulating properties [55].

In *T. brucei* bloodstream forms, other authors reported a PLA₂ activity that could be stimulated by Ca²⁺ or by the amphiphilic peptide melittin, and that was responsible of the release of arachidonic acid, a prostaglandins precursor, being a pivotal enzyme in the control of Ca²⁺ influx [56, 57]. In addition, it was demonstrated in *T. brucei* procyclic forms, that the arachidonic acid generated endogenously could induce both Ca²⁺ entry and Ca²⁺ release from the intracellular compartments acidocalcisomes, suggesting that PLA₂ activity participates in *T. brucei* signalling events [58]. On the other hand, in *L. donovani* promastigotes and *T. cruzi* amastigotes, these authors found that arachidonic acid only induced Ca²⁺ entry, possibly due to low generation of arachidonic acid or to the low amount of releasable Ca²⁺ in the acidocalcisomes of these cells [58].

Previous reports in *T. cruzi* suggested that PLA₂ could mediate the association between the parasite and macrophages, but the authors did not clearly establish the source of the enzyme [59]. In epimastigotes, it has been described a haemolytic activity that destabilize *in vitro* red blood cells membranes and that could be attributed to PLA₂ activity [60]. However under our experimental conditions using zwitterionic PL such as PC or PE as substrates, no secreted PLA₂ was detected in the supernatants of living epimastigotes [38]. In this concern, other authors have described in this protozoa a membrane-bound PLA₂ activity acting only on anionic PL such as inositolphospholipids and inositolphosphoceramides [36].

PLA₂ degrading activities have been also reported in *L. major*, and they could be involved in the biosynthesis of lipophosphoglycan, the main macromolecule on the surface of the procyclic promastigote [61]. Other authors showed that parasite pretreatment with a low dose of pachymatismin, a glycoprotein extracted from a marine sponge, increased PLA₂ activity, however macrophage invasion was partially inhibited [62].

TABLE 1: Phospholipase A₂ putative genes found in TriTrypDB.

Gene	Organism	Product	Syntenic	Comments
LbrM34_V2.2930	<i>L. braziliensis</i>	phospholipase A ₂ -like protein, putative	yes	no
LinJ35_V3.3070	<i>L. infantum</i>	phospholipase A ₂ -like protein, putative	yes	no
LmjF35.3020	<i>L. major</i>	phospholipase A ₂ -like protein, putative	yes	no
LmxM34.3020	<i>L. mexicana mexicana</i>	phospholipase A ₂ -like protein, putative	yes	no
Tbg972.9.7760	<i>T. brucei gambiense</i>	phospholipase A ₂ -like protein, putative	yes	no
TcLL3000.0.00740	<i>T. congolense</i>	product unspecified	no	no
Tc00.1047053510743.60	<i>T. cruzi CL Brener Esmeraldo-like</i>	phospholipase A ₂ -like protein, putative	yes	no
Tc00.1047053510659.250	<i>T. cruzi CL Brener Non-Esmeraldo-like</i>	phospholipase A ₂ -like protein, putative	yes	no
TvY486_0906130	<i>T. vivax</i>	phospholipase A ₂ -like protein, putative	yes	no

On the other hand, in *L. amazonensis* the modification of PL composition of infected macrophages has been described, with increasing levels of LPC, an effect that may reflect indirectly, the action of an endogenous/parasite PLA₂ on the macrophage [63]. Furthermore, other studies showed PLA₂ activity in supernatants and lysates of *L. (L.) amazonensis* promastigotes and suggested that this enzyme may be a progression factor for cutaneous leishmaniasis [64].

In summary, there are increasing evidences of the presence and possible roles of PLA₂ in the pathogenic Trypanosomes, so far however, these enzymes have not been purified or characterized in deep.

3. Phospholipases A of Trypanosomatids and Pathogenesis

PLA activity has been linked to pathogenesis in various microorganisms such as *Escherichia coli*, *Yersinia spp*, *Helicobacter pylori*, *Neisseria spp*, *Legionella spp.*, and *Campylobacter spp.*, which cause different disease syndromes; however, the exact mechanism of the PLA action has not been definitively determined [10–14, 65, 66]. PLA toxicity has been associated to cytolytic activity resulting from the accumulation of membrane-destabilizing products or by the extensive destruction of membrane phospholipids [10]. In pathogenic protozoa PLAs have been implicated in cell invasion [54, 67, 68]; in *Toxoplasma gondii* it has been described that PLA₂ inhibition protected human monocytic cells from parasite invasion [53]; in *Entamoeba histolytica*, PLA₂ is one of the several factors related to virulence [54] and in *Cryptosporidium parvum* the use of PLA₂ inhibitors as well as specific anti PLA₂ antibodies significantly reduced invasion of human enterocytes [68].

The role of PLA₁ in the pathogenesis of African trypanosomiasis has been intensively studied [29, 45, 69, 70]. Hambrey et al. described in the tissue fluids of *T. brucei* infected rabbits large amounts of PLA₁ activity that increased

with parasite burden, whereas in blood plasma this activity was also detected, but at a considerably lower level [70]. The enzyme seemed to be of trypanosomal origin, being either secreted by living parasites or released from dying organisms [70]. In intravascular locations PLA₁ could contribute to the pathology of trypanosomiasis by causing cell membrane damage and could account for some or all of the connective tissue cell destruction, which is a prominent feature of infections with *T. brucei* [71].

The high level of PLA₁ found in *T. congolense* and *T. brucei*, in comparison to other pathogens like *Escherichia coli* (1000 times fold higher) [33], and its relatively low level in the nonpathogenic rat trypanosome *T. lewisi*, suggested the importance of the enzyme in the pathology of African trypanosomiasis [28, 29, 45, 69, 72]. Given that *T. lewisi* and *T. congolense* are restricted almost entirely to the blood stream of the host, whereas *T. brucei* develops mainly in the connective tissues [71], it was suggested that PLA₁ could help the latter to penetrate blood vessels endothelium and other barriers hindering and contributing to tissue damage [29].

In the pathogenic *T. brucei* and *T. congolense* it has been determined that PLA₁ activity increased greatly during the autolytic process and large quantities of FFA were accumulated, whereas the non pathogenic *T. lewisi* failed to increase the enzyme activity even on prolonged autolysis [69]. PLA₁ yields FFA and LPC, which is then further degraded by the LPLA to yield more FFA and glycerophosphorylcholine. FFA are cytotoxic and haemolytic as a result of their detergent-like properties [73], and they could account for the immunosuppression and the structural disturbances in lymphoid organs observed in African trypanosomiasis [69]. These observations deserve to be updated and deeply studied.

The first evidences related to phospholipid degrading enzymes in *T. cruzi*, was associated to the inflammatory responses that appear surrounding degenerating amastigote nests in various tissues of Chagas' disease patients [74]. This finding strongly suggested that autolytic processes generate factors, possibly PL-breakdown products, which

cause inflammation [11]. In this regard, it was demonstrated that FFA and LPL released from killed trypomastigotes have toxic effects on culture cells [12]. These facts are in agreement with the pathogenic mechanism proposed for African trypanosomiasis [29, 69]. Accordingly, we determined in all *T. cruzi* stages the rapid and extensive breakdown of endogenous PL in autolysing parasites [37]. A major increase in FFA was observed, significantly higher than the generation of LPC, indicating not only the presence of PLA₁ activity but also LPLA activity [37]. We also found that living *T. cruzi* infective stages were able to hydrolyze LPC, confirming the presence of a LPLA activity (Belaunzarán et al. unpublished observations). It is well known that LPC is potentially toxic for the cells [75, 76] though this activity would thereby contribute significantly to the parasite self-protection against lysocompounds. Similarly, in living *T. brucei* it has been demonstrated that PLA₁ is active against LPL [77]. Other authors reported that bloodstream forms can acquire substantial amounts of exogenous LPL through a pathway consisting of three enzymes associated with the plasma membrane: PLA₁, acyl-CoA ligase, and LPC acyl-CoA-acyl transferase [32]. These cytotoxic compounds can change the ionic permeability of the plasma membrane, though they are rapidly metabolized to ensure tolerable levels in the cell. Thus a membrane-bound PLA₁ would protect *T. brucei* against the high levels of plasma LPC [32].

In *T. cruzi*, we already showed the involvement of PLA₁ in the early events of parasite-host cell interaction preceding parasite invasion. We demonstrated that either intact infective parasites or purified PLA₁ significantly modified the host cell lipid profile with generation of second lipid messengers (DG, FFA, and LPC) and concomitant protein kinase C activation [38], an enzyme that has been implicated in the upregulation of *T. cruzi* invasion [78].

With respect to *Leishmania spp.*, it has been observed that LPC, which is scarce in the macrophage, increased significantly after infection with *L. amazonensis* [63]. As LPC and arachidonic acid are the products of PC cleavage by PLA₂, the increase in the levels of LPC may suggest the action of the enzyme on the macrophage PC, producing prostaglandin E₂ [63]. In this regard, it has been shown that this lipid mediator is increased after 1-2 hours of infection with *L. donovani* and can exacerbate the infection [79]. Nevertheless, whether the LPC generation was due to parasite PLA₂ or to the activation of macrophage PLA₂ remains unclear [63]. It is possible that the LPC could also be generated by a PLA₁ activity, similarly to that we detected in *L. braziliensis* [47].

4. Bioinformatic Analysis of the Trypanosomatid Genomes for Phospholipases A

The publication of the genomes of the kinetoplastid parasites *T. brucei* [80], *T. cruzi* [81], *Leishmania spp.* [82], and other related organisms, allowed the scientific community to perform comparative analyses giving insight into the evolutionary similarities/differences among trypanosomatids.

T. brucei PLA₁ (Tb.927.1.4830) has been cloned and characterized and the analyses of its protein sequence indicated that this enzyme is not homologous to neutral lipases [34]. The only similarity to them was in the amino acid sequence that contains a lipase consensus pattern harbouring a conserved GX SXG motif, a marker of the serine hydrolase superfamily [34]. In these enzymes, the catalytic triad is typically constituted by a base residue (Histidine), an acid (Aspartic), and a nucleophile (Serine), belonging to the latter to the GX SXG motif. No eukaryotic homologues of TbPLA₁ were found in *T. cruzi* and *Leishmania spp.*, but orthologues of this enzyme were identified in *T. congolense* (TcIL3000.1.2010) and *T. vivax* (TvY486_0102170) [34]. Interestingly, TbPLA₁ resembled a putative PLA₁ homologue from *Sodalis glossinidius*, a proteobacterium endosymbiont of tsetse flies. These findings suggested that a *T. brucei* ancestor acquired the PLA₁ gene through horizontal gene transfer after/during its adaptation to a parasitic lifestyle in the insect vector [34].

Regarding *T. cruzi*, we previously reported the presence in the *T. cruzi* data base (<http://www.tcruidb.org/>) of at least sixteen different genes encoding putative lipases and the identified sequences presented a high degree of similarity among them (70–80%), may be haplotype variants [38]. When we further performed a search in the Kinetoplastid Genomic Resource TriTrypDB (<http://tritrypdb.org/tritrypdb/>) using only the lipase consensus pattern of TbPLA₁ and considering the biochemical characteristics of *T. cruzi* PLA₁, the number of putative genes was reduced to eight [38]. One of them (Tc00.1047053510679.100) was cloned and expressed in *E. coli*, being the recombinant enzyme recognized by both anti-TcPLA₁ and anti-TbPLA₁ antibodies [48]. The eight sequences are currently under study in our laboratory, to elucidate the identity of each of these genes that codify for *T. cruzi* PLA₁ and to obtain the active recombinant enzyme.

We extended these analyses to *Leishmania spp.*, searching in the TriTrypDB database for homologues of *T. cruzi* PLA₁ putative genes and have identified in *L. braziliensis*, *L. infantum*, and *L. major*, three, nine and eight putative genes with the conserved lipase motif, respectively. One of the putative genes from *L. braziliensis*, LbrM31.V2.2750, was cloned and expressed in *E. coli*, being the recombinant protein recognized by both anti-TcPLA₁ and anti-TbPLA₁ antibodies [47]. At present, we are running assays with the aim of obtaining and characterizing the active recombinant enzyme.

The alignment of the protein sequences corresponding to the cloned *T. brucei*, *T. cruzi*, and *L. braziliensis* PLA₁ (Tb.927.1.4830, Tc00.1047053510679.100, and LbrM31.V2.2750, resp.), with the putative PLA₁s proteins of *T. congolense* and *T. vivax*, (TcIL3000.1.2010 and TvY486_0102170), shows that the sequences of *T. brucei*, *T. congolense*, and *T. vivax* are closely related, whereas *T. cruzi* and *L. braziliensis* only share with all of them the lipase motif (Figure 2). The fact that *T. cruzi* and *L. braziliensis* PLA₁ protein sequences do not share significant homologies with African trypanosomes, particularly with TbPLA₁, is in

agreement with that previously observed by Richmond and Smith [34].

Although PLA₂ activity was detected in *T. brucei*, *T. congolense*, *T. cruzi*, *L. major*, and *L. amazonensis* years ago, still little is known about the identity of the genes that codify for them [36, 55, 56, 61, 63, 83]. We have performed a search in the TriTrypDB database and identified at least 9 putative PLA₂-like proteins in the different Trypanosomes species (Table 1), but at the moment no PLA₂ of trypanosomal origin has been identified in the genomes or cloned.

5. Inhibitors of Trypanosomatid Phospholipases A

As described above, parasite PLAs participate in diverse and relevant cellular processes such as membrane remodelling, modification of membrane permeability, generation of lipid second messengers and parasite invasion. All these facts emphasize the interest of these enzymes as potential chemotherapeutic targets that could contribute to the control of parasite proliferation and survival.

A number of compounds with potential inhibitory activity on parasite PLA₁ have been investigated. TbPLA₁ activity was inhibited by several heavy metals through an undefined mechanism, being the most potent at lower concentrations cadmium and copper. Iron produced partial to total inhibition depending on the concentrations employed, whereas moderate inhibition was detected in the presence of relatively high concentrations of nickel and zinc [34]. As the active-site residue for TbPLA₁ is Serine 131, the active-site serine modifiers iPr2P-F (di-isopropyl fluorophosphate), PMSF (phenylmethylsulfonyl fluoride), and E-600 (diethyl-*p*-nitrophenyl phosphate) were also assayed. Relatively little inhibition of the enzyme activity was observed but at very high concentration of inhibitors, suggesting that the catalytic triad active site of TbPLA₁ is buried inside the enzyme and sheltered by a lid domain, a property shared with other lipases [34].

Other compounds with potential inhibitory activity on TcPLA₁ were investigated in our laboratory, including the antimalarial drugs quinine and chloroquine, the antiarrhythmic drugs amiodarone and chlorpromazine, and the local anaesthetics dibucaine, procaine, and xylocaine. Among all of them, only chlorpromazine had an inhibitory effect, but at concentrations that induce cell toxicity [37].

As previously mentioned, in *T. brucei* Ca²⁺ influx can be regulated by PLA₂ [56, 58]. Various inhibitors of this enzyme such as thioetheramide-PC, manoalide, arachidonyl trifluoromethyl ketone, and aristolochic acid were tested in this protozoa, being the most effective in blocking Ca²⁺ influx 3-(4-octadecyl)-benzoylacrylic acid (OBAA), a potent inhibitor of secreted PLA₂ [56]. On the other hand, *T. brucei gambiense* and *T. brucei brucei* PLA₂s were inhibited in a noncompetitive fashion when using organotin compounds like fatty acid derivatives of dibutyltin dichloride [83]. In the case of *T. cruzi*, it has been suggested that quinacrine, which inhibited erythrocyte lyses, blocked PLA₂ activity [60].

Concerning *Leishmania spp.*, up to now, there are no reports about the use of specific PLA inhibitors. However,

it has been reported that the lysophospholipid analog (LPA) miltefosine, affects lipid metabolism in *Leishmania donovani* promastigotes, with reduction in PC and enhancement in PE and LPC, a process in which PLAs could participate among other enzymes [84]. The usefulness of lipid biosynthesis inhibitors has gained great interest in the last years to fight parasitic Trypanosomes [5, 85–88]. These compounds, initially developed to be antitumor agents, have proved to be highly effective in the treatment of visceral leishmaniasis [87, 89, 90]. Although their effectiveness is known, the mode of action against this parasite is not completely understood [91]. In *T. cruzi*, the synergy of the LPAs edelfosine, ilmofosine, and miltefosine with the ergosterol biosynthesis inhibitor, ketoconazole, induced alterations in the plasma membrane, reservosomes, and mitochondrion, indicating that these organelles are potential targets of these drugs, probably through interference with lipid metabolism [92].

Considering that PLAs are present in both, trypanosomes and mammalian host, it will be of relevance to achieve the knowledge of their three-dimensional structures to determine the differences/similarities among them. This would allow the rational design of specific inhibitors that could be employed as potential chemotherapeutic agents in the diseases caused by kinetoplastid pathogens.

6. Concluding Remarks

In summary, as presented in this paper PLAs of pathogen trypanosomes mediate a variety of processes in both protozoan and host cell lipid metabolism, being also considered virulence factors. However, the knowledge of these enzymes is far from complete, though in the future, continued biochemical, biological, and structural research are needed to obtain a full understanding of the molecular mechanism in which these enzymes participate. Unravelling the differences between parasite and host PLAs may contribute, besides, to the design of specific enzyme inhibitors that could be used in the treatment of the neglected diseases that trypanosomes cause.

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