Molecular & Biochemical Parasitology 203 (2015) 14–24

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Phosphatidylinositol kinase activities in *Trypanosoma cruzi* epimastigotes

Alba Marina Gimenez[a,*], María Celeste Gesumaría[a], Alejandra C. Schoijet[b,c], Guillermo D. Alonso[b,c], Mirtha M. Flawiá[b,c], Graciela E. Racagni[a], Estela E. Machado[a]

[a] *Departamento de Biología Molecular, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto X5800, Córdoba, Argentina*
[b] *Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Científicas y Técnicas, Vuelta de Obligado 2490, C1428ADN Ciudad Autónoma de Buenos Aires, Argentina*
[c] *Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 10 April 2015
Received in revised form 9 October 2015
Accepted 13 October 2015
Available online 19 October 2015

Keywords:
Phospholipid signaling
Phosphoinositide kinase
Kinase activity
Phosphatidylinositol-3 kinase
TcPI3K
T. cruzi epimastigotes

ABSTRACT

Phosphatidylinositol (PtdIns) metabolism through phosphatidylinositol kinase (PIKs) activities plays a central role in different signaling pathways. In *Trypanosoma cruzi*, causative agent of Chagas disease, PIKs have been proposed as target for drug design in order to combat this pathogen. In this work, we studied the classes of PI4K, PIPK and PI3K that could participate in signaling pathways in *T. cruzi* epimastigote forms. For this reason, we analyzed their enzymatic parameters and detailed responses to avowed kinase inhibitors (adenosine, sodium deoxycholate, wortmannin and LY294002) and activators (Ca^{2+}, phosphatidic acid, spermine and heparin). Our results suggest the presence and activity of a class III PI4K, a class I PIPK, a class III PI3K previously described (TcVps34) and a class I PI3K. Class I PI3K enzyme, here named TcPI3K, was cloned and expressed in a bacterial system, and their product was tested for kinase activity. The possible participation of TcPI3K in central cellular events of the parasite is also discussed.
© 2015 Elsevier B.V. All rights reserved.

1. Introduction

The flagellate protozoan *Trypanosoma cruzi* is the agent responsible for Chagas disease [1], a serious endemic illness and major public health problem in Latin America. According to the World Health Organization, about 6–7 million people are estimated to be infected worldwide, mostly in Latin America, where Chagas disease is endemic [2]. This parasite, transmitted to mammalian hosts by triatomine hematophagous insects, has a complex life cycle involving several morphologically and functionally different stages [1]. The ability of *T. cruzi* to receive signals from different environments and to initiate appropriate changes in cell activity is crucial for its pathogenic activity [3].

Previous evidence demonstrated that epimastigotes, *T. cruzi* replicative forms present in triatomines, are able to respond to different agonists by activation of a phosphatidylinositol 4,5 bisphosphate specific phospholipase C (PtdIns-PLC) [4,5], enzyme subsequently cloned [6,7]. Additionally, this response was accompanied with phosphatidylinositol (PtdIns) pathway activation [8,9]. In this pathway, phosphatidylinositol kinases (PIKs) catalyze the addition of phosphates to specific positions on the inositol ring of PtdIns present in membranes. In consequence, PtdIns is sequentially converted to phosphatidylinositol 4 phosphate (PtdIns4P) and phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) by PI4K and PI4P5K respectively, which generates the PtdIns-PLC substrate. Another kinase which phosphorylates PtdIns – among other phosphoinositides – is PI3K. The products of PI3Ks-phosphatidylinositol 3 phosphate (PtdIns3P), phosphatidylinositol 3,4 bisphosphate (PtdIns(3,4)P_2), and phosphatidylinositol 3,4,5 trisphosphate (PtdIns(3,4,5)P_3) have received special attention due to their importance in signal transduction, inflammatory processes, membrane trafficking, proliferation, cytoskeletal regulation and apoptosis in several cells [10].

The PIK pathway has been known as a target for drug discovery to combat trypanosome parasites [11–14]; moreover, it has been proposed the TryPIKinome, attempting to identify candidates for the design of specific inhibitors [15,16]. In a detailed bioinformatic analysis, trypanosomal genes encoding for PIKs typical or related proteins were classified into five groups (Models 1–5), according to the presence of conserved domains. Models 1 and 2 represent class

* Corresponding author. Fax: +54 3584676232.
E-mail address: lbmrngmz@gmail.com (A.M. Gimenez).

http://dx.doi.org/10.1016/j.molbiopara.2015.10.002
0166-6851/© 2015 Elsevier B.V. All rights reserved.

III and I of PI3K, respectively. Models 3 and 4 represent PI4Ks (Model 3 is the orthologous of Pik1 and Model 4 is the orthologous of Stt4 in yeast); Model 5 includes PIK-related proteins [15]. PIPK enzymes do not share sequence homology with other PIKs; therefore, they are not included in this classification.

Recently, the genomic organization PIKs family in *T. cruzi* has been reported, showing a high degree of polymorphism at genomic localization level [17]. Despite the probable importance of these enzymes as drug targets, the current knowledge about the functionality of typical PIK genes in this parasite is limited to model 1, corresponding to class III PI3K (TcVps34) which is involved in osmoregulation and receptor-mediated endocytosis processes in epimastigotes [18]. Model 2, represented by class I PI3K frequently related to signaling pathways, remains unproved in trypanosomes, and it was suggested that these parasites would not possess a PI3K-dependent signaling system [11]. With regards to Models 3 and 4, Rodgers et al. demonstrated in *Trypanosoma brucei* the presence and functionality of a class III PI4K, essential for normal growth and morphology in procyclic forms [19]. In *T. cruzi*, our laboratory determined by means of enzymatic activity the participation of PIKs enzymes and PtdIns-PLC pathway in the response to several external stimuli such as Carbachol [8], 1–40 peptide [9] and hyperosmotic stress [20], suggesting a mayor role for lipid kinases in events leading to parasite differentiation [21]. However, no functional analyses of these enzymes have been done, nor has their relation to TryPIKinome been studied.

Therefore, in this work, we determined the biochemical properties and inhibitor responses of PI3Ks, PI4K, and PI4P5K from *T. cruzi* epimastigotes, supported by bioinformatic search and analysis of phylogenetic localization. Finally, we report the molecular cloning and functional characterization of TcPI3K, the first class I PI3K described in parasitic protozoa, and discuss the possible involvement of this enzyme in signaling pathways of *T. cruzi*.

2. Materials and methods

2.1. Organism and growth conditions

For biochemical studies, *T. cruzi* Tulahuen strain was used. Epimastigote forms were grown at 28 °C in a modified Warren medium [22], consisting of 33 g/l Brain Heart Infusion, 3 g/l trytose, 3.2 g/l Na_2PO_4H, 0.4 g/l KCl and 0.3 g/l glucose, supplemented with 0.1% hemin, 10% fetal bovine serum, 100,000 units/l penicillin, and 100 mg/l streptomycin. Cells in logarithmic phase (day 6) were harvested by centrifugation at 1500 × *g* for 10 min, and washed twice with 25 mM Tris–HCl pH 7.2; 1.2 mM $MgSO_4$; 2.6 mM $CaCl_2$; 4.8 mM KCl; 120 mM NaCl and 100 mM glucose [Krebs–Ringer–Tris (KRT) buffer].

For molecular cloning, *T. cruzi* of sequenced strain CL Brener [23] was used. Epimastigote forms were cultured at 28 °C for 7 days in LIT medium [5 g/l liver infusion, g/l bacto-tryptose, 68 mM NaCl, 5.3 mM KCl, 22 mM Na_2PO_4, 0.2% (w/v) glucose, 0.002% (w/v) hemin], supplemented with 10% (v/v) newborn calf serum, 100,000 units/l penicillin, and 100 mg/l streptomycin.

2.2. Treatment with PI3K inhibitors during growth

Cultures were initiated with a cell density of 2×10^6 epimastigotes per ml. PI3K inhibitors used were 50–100 nM wortmannin and 20–50 μM LY294002, both dissolved in 0.1% DMSO. Drugs or DMSO were added after 24 h and growth was followed by counting the number of motile cells per ml of culture medium in a Neubauer chamber.

2.3. Treatment in vivo with enzyme effectors

For measuring PI3K inhibition in vivo, cells were harvested as described above and incubated with the inhibitors (50–100 nM wortmannin and 20–50 μM LY294002) or 0.1% DMSO during 20 min before membrane preparation. To measure the effects of PKA activators/inhibitors on PI3K activity, epimastigotes were incubated with 25 μM dibutiryl-adenil-monophosphate cyclic (dBAMPc), 25 nM KT5720 or the carrier 0.1% DMSO during 60 min before membrane preparation described below. The following procedure was identical to control cells.

2.4. Preparation of membranes from epimastigotes

Washed cells were resuspended in five volumes of 50 mM HEPES (pH 7.4) containing 0.25 M sucrose, 5 mM KCl, 1 mM EDTA and 4 μg/ml leupeptin. The suspension was frozen at −180 °C in liquid nitrogen and thawed; this freeze/thaw cycle was performed three times. Membrane fractions were prepared as described previously [8]. Briefly, the homogenate was centrifuged at 100 × *g* for 15 min to remove cell debris, and supernatant was centrifuged at 1000 × *g* for 15 min and 105,000 × *g* for 45 min. Pellets were washed, resuspended with 50 mM HEPES (pH 7.4) and used as a source of enzymatic activities. Supernatant of the 105,000 × *g* centrifugation was used as soluble fraction.

2.5. Preparation of membranes from yeast

Yeast membrane was obtained as described by Stack et al. [24]. Briefly, yeast spheroplasts were resuspended in 15 mM HEPES, 0.1 M KCl, 3 mM EGTA, 10% glycerol and protease inhibitors. Lysates were centrifuged at 750 × *g* during 5 min and the supernatant was centrifuged at 100,000 × *g* during 30 min at 4 °C to generate Yp (*pellet*) and Ys (*supernatant*) fractions, which were frozen at –20 °C until use.

2.6. Lipid kinase assay

PI3K, PI4K and PI4P5K were assayed simultaneously as described [9] using endogenous lipids as substrates, unless otherwise stated. The membrane fraction isolated (30–80 μg protein), was added to thermally equilibrated 50 mM HEPES buffer (pH 7.4) containing 0.1 mM EDTA, 0.5 mM DTE, 10 mM $MgCl_2$, 0.1 mM sodium *o*-vanadate [inhibitor of phosphoinositide phosphatases [25,26]] and [γ-^{32}P]ATP (500 cpm/pmol). Endogenous lipid phosphorylation was allowed to proceed for 10 min at 30 °C in a total volume of 100 μL. For kinetic assays, exogenous PtdIns was added as substrate in liposome form (PtdIns/phosphatidylcholine, 1:1) and exogenous ATP was used in the form of ATP-Mg^{2+}. The incubation mixture was subsequently quenched with 1.5 ml of chloroform/methanol (1:2, v/v). Yeast membrane fractions Yp and Ys were used as control for PI3K activity, according to Stack et al. [24].

The protein content of membrane samples was determined by the Bradford method [27], with bovine serum albumin as the standard.

2.7. Treatment in vitro with enzyme inhibitors and activators

To analyze inhibition or activation on kinase activities, 0.1–2 mM adenosine, 0.5–2 mM sodium deoxycholate, 0.01–1% Triton X-100, 0.5–2.5 mM $CaCl_2$, 150 μM phosphatidic acid, 200 μM spermine, 50 μg/ml heparin, 50–100 nM wortmannin and 20–50 μM LY294002 were used. Effectors or carriers were added to kinase assay mixture just before adding [γ-^{32}P]ATP.

2.8. Phospholipid extraction and separation

The lipids were extracted from membranes according to Stubbs et al. [28]. Briefly, 0.5 ml of 2.4 N HCl and 0.5 ml of chloroform were added to test tubes. After vortex, the lower (organic) phases were extracted and the upper phases were washed with 1 ml chloroform. Both lower phases were mixed with 2 ml methanol/HCl 1 M (1:1, v/v). The organic phases obtained were dried under N_2 atmosphere and resuspended in 40 μl chloroform/methanol (9:1, v/v).

For measuring the formation of isolated PtdIns(4,5)P_2 and thus determinate PIPK activity, phospholipids were separated by TLC in an acid solvent system [29]. Briefly, the samples were spotted on silica gel plates impregnated O.N. with 1% potassium oxalate solution and heated at 110 °C during 60 min just before use. Chromatoplates were developed in chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v). For the determination of PI3K and PI4K activities, a boric acid system [30] was used. Silica gel plates were impregnated with 1,2-cyclohexylenedinitrilotetraacetic acid (CTDA) solution [1.52 g CTDA dissolved in 166 ml H_2O/10 N NaOH/ethanol (55:1:110, v/v)] O.N. and heated at 110 °C during 60 min just before use. The boric acid system was composed by 4 g boric acid and 0.125 g of 2,6-ditertbutyl-4-methyl phenol (BHT) dissolved in methanol/chloroform/H_2O/formic acid (50:40:5:2, v/v) plus 25 μl ethoxyquin. In both systems, the position of radiolabeled lipids was determined by autoradiography on Kodak film. Spots were scraped off the plates, and the amount of labeled phospholipids was determined by scintillation counting.

2.9. Cloning of TcPI3K gene

The gene sequence corresponding to the human p110-PI3K (AAB29081) was used to screen *T. cruzi* sequences in the GeneDB data base using the WU-Blast2 algorithm. Two oligonucleotides carrying hemi-restriction sites (PI3K-pET-Fw-BamHI 5′-GGATCCGTCTTCCCCCAGCGAATTGTT-3′ and PI3K-pET-Rv-HindIII 5′-AAGCTTATGTGTCATAATATGAATATAGTCA-3′) were designed from the identified sequence (Tc00.1047053510167.10). PCR amplifications were carried out using 600–800 ng of *T. cruzi* genomic DNA, 100 ng of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 2 units of Pfu Ultra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA). Three PCR products were sequenced and compared with the original sequence found at GeneDB.

2.10. Expression and purification of recombinant TcPI3K

Full-length TcPI3K gene was amplified using the following primers carrying hemi-restriction sites: PI3K-pET-Fw-BamHI 5′-GGATCCGTCTTCCCCCAGCGAATTGTT-3′ and PI3K-pET-Rv-HindIII 5′-AAGCTTATGTGTCATAATATGAATATAGTCA-3′, cloned into pGEM-T Easy plasmid (Promega, Madison, WI). To express the N-terminal His-tagged TcPI3K in *Escherichia coli*, the coding sequence was transferred from pGEM-T Easy into the pET-22b(+) expression vector (Invitrogen). The construct [pET-22b(+)-TcPI3K] was transformed into BL21(DE3) pLysS host (*E. coli* B, F^-, dem, ompT, hsdS, (rb^-, mB^-), gal^- (DE3), [pLysS, camr]), and the recombinant protein was induced with 300–500 μM isopropyl-1-thio-D-galactopyranoside (IPTG) at 25 °C, 30 °C or 37 °C for 3–16 h. Cells were harvested by centrifugation and resuspended in lysis buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100) containing a protease inhibitor mixture. The protein extract was prepared by sonication and centrifuged at 10,000 × *g*; the obtained pellet (P10 fraction) and supernatant (S10 fraction) were stored at −20 °C. The S10 fraction was used for purification using a nickel–nitrilotriacetic acid–agarose resin (Invitrogen) and eluted with lysis buffer containing 20–200 mM imidazole.

2.11. Determination of specific activity of TcPI3K

Class I PI3K activity was determined by measuring the formation of PtdIns(3,4,5)P_3 using PI3-Kinase Activity ELISA: Pico (Echelon Biosciences), following the manufacturer's instructions. Briefly, endogenous membranes from *T. cruzi* epimastigotes and P10 and S10 fraction of *E. coli* carrying or not pET-22b(+)-TcPI3K were obtained as described above and used as source of enzyme for PI3K activity reaction. The enzymatic reaction was allowed to proceed during 30 min; products of the reaction were incubated with a PtdIns(3,4,5)P_3-detector protein and added to an ELISA 96-wells plate pre-coated with PtdIns(3,4,5)P_3 to perform the competitive assay. Absorbance was measured at 450 nm in an ELISA plate reader.

2.12. Northern and western blot analyses

Total cellular RNA was isolated from 10^8 epimastigotes in the exponential growth phase using TRIzol reagents, as described by the manufacturer (Invitrogen). Northern blot analyses were performed as described by Alonso et al. [31]. The products were revealed with a specific 1850-bp TcPI3K probe obtained by digestion of the 3146-bp fragment with BamHI and EcoRV. All probes were labeled with [α-^{32}P]dCTP using the Prime-a-Gene kit (Promega, Madison, WI) following the manufacturer's instructions. For western blot analysis, proteins were solved in 8% (w/v) SDS-polyacrylamide gel electrophoresis, as described by Laemmli [32], and electrotransferred to Hybond- C membranes (Amersham Pharmacia Biotech, Piscataway, USA). The membranes were blocked with 5% (w/v) non-fat milk suspension in TBS-Tween for 2 h. After 1 h incubation with 1:1000 dilution of mouse anti-His antibody (Sigma, St. Louis, USA), detection was carried out by incubating with a 1:5000 dilution of anti-mouse IgG labeled with peroxidase (PerkinElmer, Boston, USA). The latter was developed with the ECL Plus™ Western Blotting Detection System (NEN Life Science Products Inc., Boston, MA).

2.13. Bioinformatics

Sequences with high homology to PIKs were identified in *T. cruzi* by BLASTp analysis by screening mammalian and yeast PIKs protein sequences against the *T. cruzi* database. Sequences and data were searched from NCBI (http://www.ncbi.nlm.nih.gov/), ExPASy Molecular Biology Server (http://us.expasy.org/) and Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). The *T. cruzi* genome database at GeneDB (http://www.genedb.org/genedb/tcruzi/) was searched using Wu-Blast2. Sequence identity was analyzed using BlastP (http://www.ncbi.nlm.nih.gov/blast/). Sequences were initially aligned using ClustalW (http://www.ebi.ac.uk/clustalw/) with BioEdit Sequence Alignment Editor 4.8.8, and the alignment was then visually refined. Transmembrane segments were determined using TMPred (http://www.ch.embnet.org/software/TMPRED_form.html/). Phylogenetic analysis was performed using the neighbor-joining method.

2.14. Statistical analysis

The results are shown as mean ± standard error of the mean (SEM) for three or more independent experiments, unless otherwise stated. Graphical and statistical analysis of the data was performed using OriginPro 8.5 and SigmaPlot 11.0 softwares.

Table 1
Effect of inhibitors/activators on PI4K activity.

DOC		Triton X-100		Adenosine		$CaCl_2$	
C (mM)	% *A*	*C* (%)	% *A*	*C* (mM)	% *A*	*C* (mM)	% *A*
0.0	100	0.00	100	0.0	100	0.0	100
0.5	19 ± 6*	0.01	160 ± 10*	0.1	98 ± 5	1.0	65 ± 7*
1.0	16 ± 5*	0.05	110 ± 8	0.2	93 ± 8	1.5	48 ± 10*
1.5	21 ± 4*	0.10	100 ± 12	0.5	96 ± 11	2.0	36 ± 9*
2.0	13 ± 4*	1.00	15 ± 5*	2.0	20 ± 12*	2.5	35 ± 5*

DOC: sodium deoxycholate, *C*: concentration, % *A*: percentage of PI4K activity related to control untreated (100%).
* $p < 0.05$, $n = 3$.

3. Results

3.1. Phosphatidylinositol 4 kinase (PI4K)

Phosphatidylinositol 4 kinases could be classified in class II or III depending on their sequences, enzymatic parameters and inhibitor profiles. Class II PI4Ks are peripheral proteins of approx. 55 kDa, activated by Triton X-100 and inhibited by adenosine; while class III PI4Ks are larger proteins of 90–270 kDa, Triton X-100 activated but adenosine insensitive. Furthermore, Km values for PtdIns and ATP-Mg^{2+} are 3-7-fold lower for class II than for class III PI4Ks and their cellular functions are distinctive [33].

In order to contribute to elucidate the biological functions of PI4K in trypanosomatids, we analyzed biochemical properties of this enzyme in *T. cruzi* epimastigotes and we attempt to determine the class of PI4K present in *T. cruzi* membrane extract, by means of enzymatic parameters (Km, Vmax) and sensitiveness to adenosine. There were no significant differences when 1000 g or 105,000 g membrane fractions were used as source of enzyme; therefore, here we describe results obtained with 1000 g membrane fraction.

Km value for ATP was determined by varying concentration of ATP in presence of 3 mM Mg^{2+} (Fig. 1A). The enzyme activity showed typical saturation kinetics: the apparent Km value was 425 ± 38 μM and the apparent Vmax value was 64.5 ± 22 pmol/min/mg protein (Fig. 1C). When exogenous PtdIns was added, a progressive and concentration-dependent increment in the rate of PtdIns4P was observed (Fig. 1B); the apparent Km and Vmax values for PtdIns were 2.0 ± 0.33 μM and 17.2 ± 4 pmol/min /mg protein, respectively (Fig. 1D).

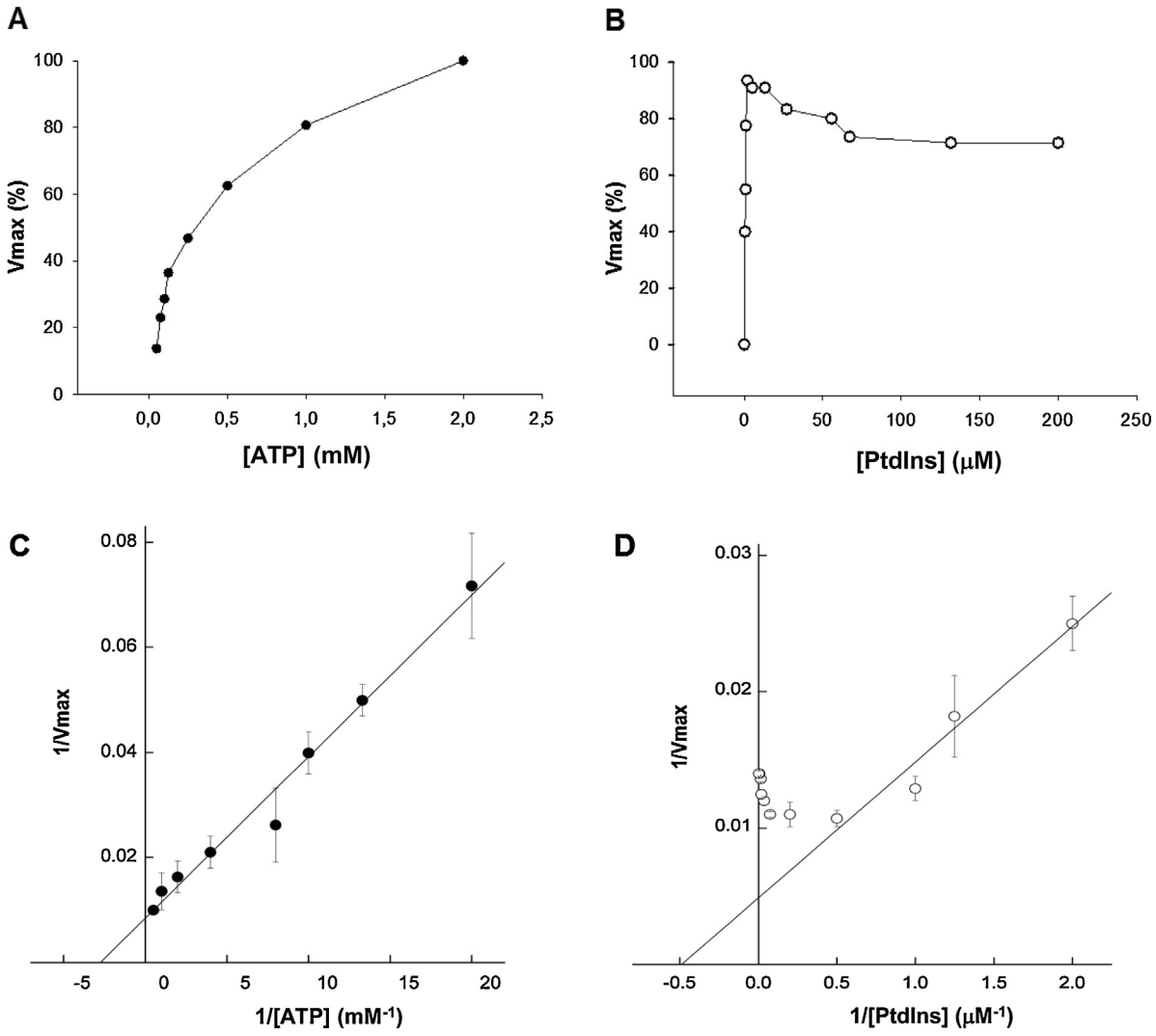


Fig. 1. Determination of enzymatic parameters Vmax and Km for PI4K.
Plots were constructed by varying [ATP] (A) or [PtdIns] (B) added exogenously and measuring PI4K activity at each point. 1000 g epimastigote membrane fractions were used as source of enzyme and kinase reaction were carried out with [γ-^{32}P]ATP, as described in Section 2. Lineweaver–Burk plots shows mean ± standard error of the mean (SEM) from three independent experiments. Km-ATP = 425 ± 38 μM, Vmax-ATP = 64.5 ± 22 pmol/min/mg protein (C). Km-PtdIns = 2.0 ± 0.33 μM, Vmax-PtdIns = 17.2 ± 4 pmol/min/mg protein (D).

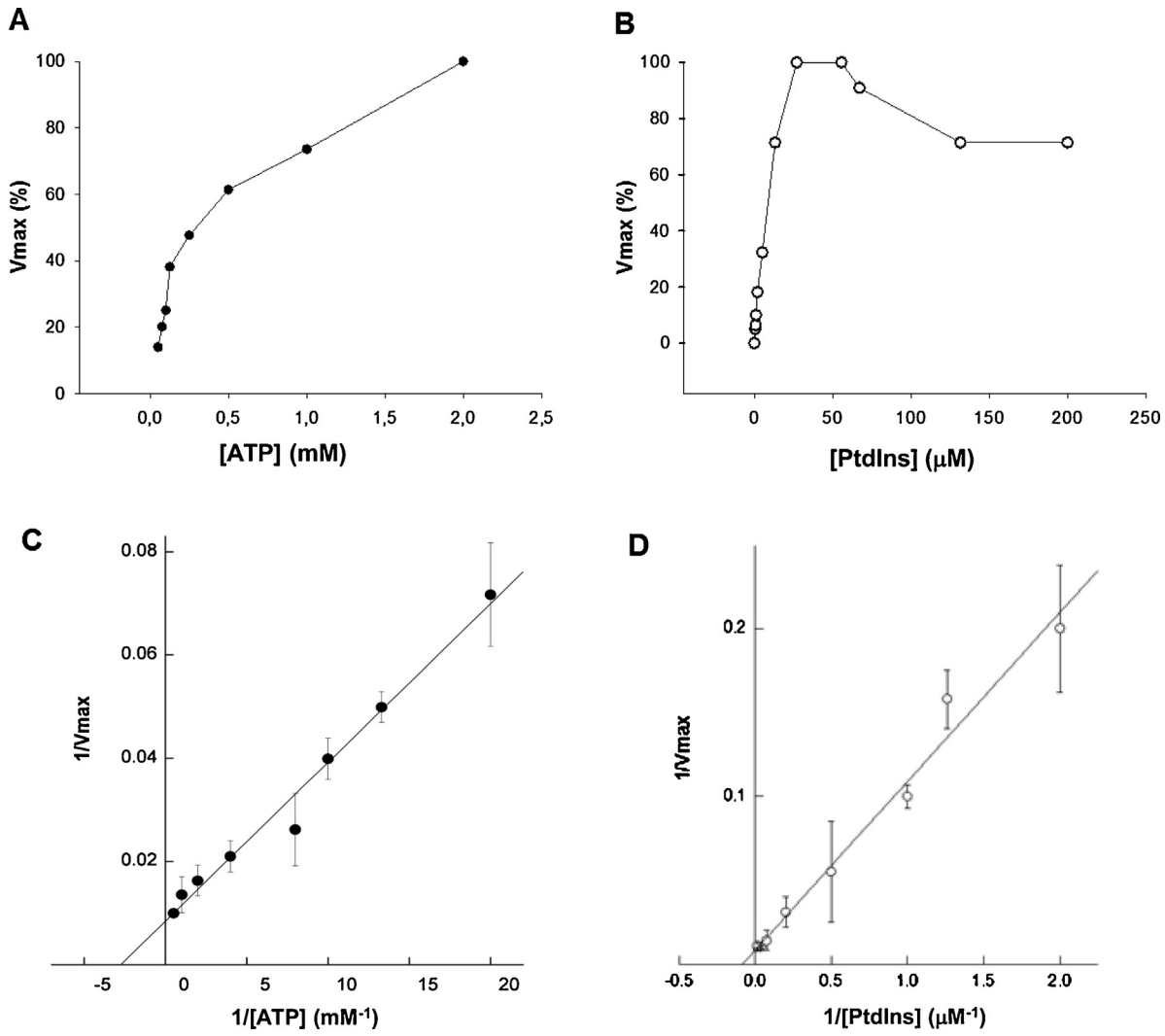


Fig. 2. Determination of enzymatic parameters Vmax and Km for PIPK.
The enzymatic activity of phosphatidylinositol phosphate kinase (PIPK) was determined based on [γ-^{32}P]ATP phosphorylation of parasite 1000 g membrane fraction, obtained as described in Section 2 (A and B). Lineweaver–Burk plots were constructed by varying [ATP] (C) or [PtdIns] (D) added exogenously and measuring PIPK activity at each point. Values are mean ± standard error of the mean (SEM) from three independent experiments. Km-ATP = 370 ± 38 μM, Vmax-ATP = 41.5 ± 3 pmol/min/mg protein (C). Km-PtdIns = 13.0 ± 0.42 μM, Vmax-PtdIns = 9.0 ± 1 pmol/min/mg protein (D).

The effects of various concentrations of sodium deoxycholate (DOC), Triton X-100, Mg^{2+}, Ca^{2+} and adenosine on PI4K activity were examined (Table 1). DOC markedly inhibited the activity at all concentrations tested (0.5–2 mM), while the addition of Triton X-100 to the assay system increased the enzymatic activity (60 ± 10%) with respect to the untreated control (100%). The optimal concentration of Mg^{2+} for PI4K was 3 mM; concentrations higher than 5 mM were inhibitory. Similarly, this enzyme resulted unaffected by Ca^{2+} at micromolar concentrations (not shown) but inhibited at millimolar concentrations. Adenosine did not significantly affect PI4K activity at micromolar concentration.

3.2. Phosphatidylinositol phosphate kinase (PIPK)

Enzymatic parameters of the enzyme responsible of generating PtdIns(4,5)P_2 in *T. cruzi* epimastigotes membrane extract, PIPK, were also analyzed. Km value for ATP was determined from Lineweaver–Burk plot by varying concentration of ATP in presence of 3 mM Mg^{2+}. The apparent Km and Vmax values were 370 ± 38 μM and 41.5 ± 3 pmol/min/mg protein, respectively (Fig. 2A–C). This enzyme also increased its activity with exogenous PtdIns in a concentration-dependent manner (Fig. 2B); Km and Vmax values were 13.0 ± 0.42 μM and 9.0 ± 1 pmol/min/mg protein, respectively (Fig. 2D).

In mammals, there are two classes of PIPKs: class I, which preferentially uses PtdIns4P as substrate to form PtdIns(4,5)P_2 and also phosphorylate PtdIns3P to generate both PtdIns(3,4)P_2 or PtdIns(3,5)P_2, and class II, which prefers PtdIns5P over PtdIns4P as substrate generating PtdIns(4,5)P_2 in both cases [34]. To determine whether the PIPK activity detected in *T. cruzi* was class I or II, we investigated the effect of phosphatidic acid (PtdOH), spermine and heparin on the kinase activity, all of them recognized as class I PIPK-activators. PIPK activity was increased by 2-fold when PtdOH (150 μM) was added to the assay system. In addition, both spermine (200 μM) and heparin (50 μg/ml) increased the specific activity 2 and 2.5-fold with respect to the untreated control, respectively. The effect of different free Ca^{2+} concentration (0.5–2.5 mM) on the enzymatic activity was also studied. PIPK activity was increased 3-fold at 2 mM of Ca^{2+} with respect to the control without free Ca^{2+} added.

3.3. Phosphatidylinositol 3 kinases (PI3Ks)

In a previous work, we report the identification of the first phosphatidylinositol 3 kinase in *T. cruzi*, TcVps34 [18]. We observed that the cloned enzyme specifically phosphorylates PtdIns to produce PtdIns3P, thus confirming that it belongs to class III PI3K family. Here we first analyze the endogenous production of PtdIns3P in *T. cruzi* epimastigotes.

Fig. 3 shows a profile of [γ-^{32}P]ATP-phosphorylation products of 1000 g membrane extract from epimastigotes, when separated by TLC using pyridine/borate solvent system. When soluble fraction was used as source of enzyme, no significant phosphoinositide kinase activity was identified (data not shown). Yp and Ys fractions from yeast were used as control for differential migration of PtdInsP isomers [24]. Thus, in the first place we analyzed the relative proportion of PtdInsP isomers, i.e., PtdIns3P/PtdIns4P ratio. PtdIns3P formation was 0.53 ± 0.04 pmol/min/mg protein vs. 2.55 ± 0.02 pmol/min/mg protein for PtdIns4P. Thus, PtdIns3P/PtdIns4P ratio was approximately 0.2.

Our next goal was to examine whether inhibition of PI3K in epimastigotes of *T. cruzi* was affecting their normal growth. The inhibitors used were wortmannin and LY294002; parasite growth was monitored by counting living cells in Neubauer chamber. Fig. 4(A and B) shows a typical curve of epimastigotes growth until day 6 of culture, and observed effects by adding 50 nM or 100 nM wortmannin (Fig. 4A) and 20 μM or 50 μM LY294002 (Fig. 4B).

Treatment with both drugs produced a significant decrease in cell growth, indicating the possible vital importance of the enzyme inhibited. Then we verified that the delay observed in parasite growth rate was actually produced by specific inhibition of PI3K. We evaluated the effect of inhibitors at the same concentrations employed, on PI3K activity in vivo, on parasites incubated for 60 min, and in vitro, adding inhibitors to the kinase reaction mix. Fig. 4C shows the inhibitory effect of 50 nM wortmannin and 20 μM LY294002 on PI3K activity determined in epimastigotes membranes. The decrease of PI3K activity ranged 25–40% compared to the control (carrier 0.1% DMSO). There were not significative differences when 100 nM wortmannin and 50 μM LY294002 were used, resulting in the same inhibition of PI3K activity than lower concentrations.

PI3K proteins have three isoforms in mammals: class I, generally composed of a p110 catalytic subunit and a p85 accessory subunit (class Ia); class II, of which, to date, only one catalytic subunit is known, p170; and class III or Vps34 yeast homologous, usually associated with an accessory protein p150 or Vps15 yeast homologous [10]. The database of *T. cruzi* sequenced genome strain (CL Brener) contains several sequences that encode hypothetical proteins with predicted PIKs domains. Of these, two sequences showed high homology to the conserved catalytic domain of PI3Ks:

(1) Tc00.1047053511903.160; encoding TcVps34 [18],
(2) Tc00.1047053510167.10; encoding a hypothetical protein of 1055 amino acids (accession number XP_805828) with predicted molecular weight of 120 kDa. This protein sequence has essential conserved domains of p110-catalytic subunit of class I PI3K: a Ras-binding domain (RBD) and a C2 domain at the carboxyl terminus (residues 177–248 and 291–423, respectively), an accessory domain of class I PI3K (residues 535–702), and a class I PI3K catalytic domain (residues 796–1055). Based on the similarity to the characteristic structure of PI3Ks of class I, here we refer to the hypothetical protein XP_805828 as TcPI3K.

TcVps34 and TcPI3K sequences were compared with PI3Ks sequences from different organisms (mammals, plants, yeasts and protozoa) using ClustalW and BLASTp programs. We compared both the complete protein sequences and the isolated catalytic domains. Supplementary Table 1 shows the statistical parameters resulting from comparing TcVps34 with other class III PI3Ks (A) and TcPI3K with other class I PI3Ks (B). It was observed that the catalytic domain of TcVps34 shares 84–71% identity (identical amino acids) and 92–84% similarity (amino acids with similar function) with the catalytic domains of PI3Ks from *T. brucei* (TbVps34) and *Leishmania major* (putative), respectively. On the other hand, the catalytic domain of TcPI3K has 49% identity and 69% similarity compared with the catalytic domain of class I PI3K from *Homo sapiens*, these being significant values considering the evolutionary distance.

Using the catalytic domains of each protein a phylogenetic tree was performed (Supplementary Fig. 1). TcVps34 forms a cluster with their counterparts from kinetoplastids *T. brucei* and *Leishmania* spp. In fact, these isoforms are more related to class III PI3Ks belonging to other protozoa (*Giardia lamblia*, *Entamoeba histolytica*, *Plasmodium falciparum*, *Tetrahymena thermophila*) than vegetal proteins (*Nicotiana tabacum*, *Brassica napus*), yeast (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*), fungi (*Aspergillus fumigatus*), invertebrates (*Caenorhabditis elegans*, *Drosophila melanogaster*) and mammals (*H. sapiens*, *Mus musculus*). While class II PI3K is present only in higher eukaryotic organisms, the class I TcPI3K shares a common origin with its putative homologue from *Leishmania infantum*, distant in evolutionary terms from isoforms corresponding to higher eukaryotes.

3.4. Class I phosphatidylinositol 3 kinase (TcPI3K)

Based on the nucleotide sequence of the gene corresponding to TcPI3K (Tc00.1047053510167.10), specific oligonucleotides were designed for their amplification from parasite genomic DNA. An amplification product of ~3.15 Kbp was obtained; this apparent molecular weight is in agreement with that expected for TcPI3K coding region (3146 bp). The amplification product was cloned into pGEM-T Easy vector and subcloned into the expression vector pET-22b(+) in order to produce IPTG-inducible expression of His-Tagged TcPI3K. A Northern blot assay, using a DNA-radioactive probe corresponding to the first 1850-bp of the TcPI3K coding region, revealed a major hybridization band (Fig. 5A), indicating that this gene is expressed in epimastigotes.

After trying several conditions, the target protein was successfully obtained in the soluble cytosolic fraction (S10) of 16 h induction at 25 °C. Further purification was performed by passing S10 fraction through Ni-NTA resin. The purified protein His-TcPI3K was recovered in the elution fraction with 100 mM imidazole (Fig. 5B). However, considerable amounts of recombinant protein were lost during the purification process; consequently for further analysis the S10 fraction was used.

The kinase activity of recombinant TcPI3K was tested using the S10 fraction as source of enzyme and exogenous PtdIns and [γ-^{32}P]ATP as substrates. After the phosphorylation assay, radioactive phospholipids were resolved by TLC and revealed by autoradiography. As shown in Fig. 5C, the recombinant protein was able to phosphorylate PtdIns giving PtdIns3P as the major product, as evidenced by the position of the standards run in a borate buffer system [30]. The detection of this product was very weak in non-induced *E. coli* extracts, indicating that bacterial proteins were not responsible for the PI3K activity.

For determination of specific activity of TcPI3K, the formation of PtdIns(3,4,5)P_3 was measured by indirect ELISA assay, using PI3-Kinase Activity ELISA: Pico (Echelon Biosciences). Class I PI3K activity was measured both in P10 and S10 fraction of *E. coli* carrying or not the plasmid pET-22b(+)-TcPI3K and induced or not with 300 μM of IPTG for 16 h at 25 °C (Table 2). The highest specific activity of the recombinant TcPI3K was 0.78 ± 0.06 pmol/min/mg protein, measured in S10 fraction of IPTG-induced *E. coli* pET-

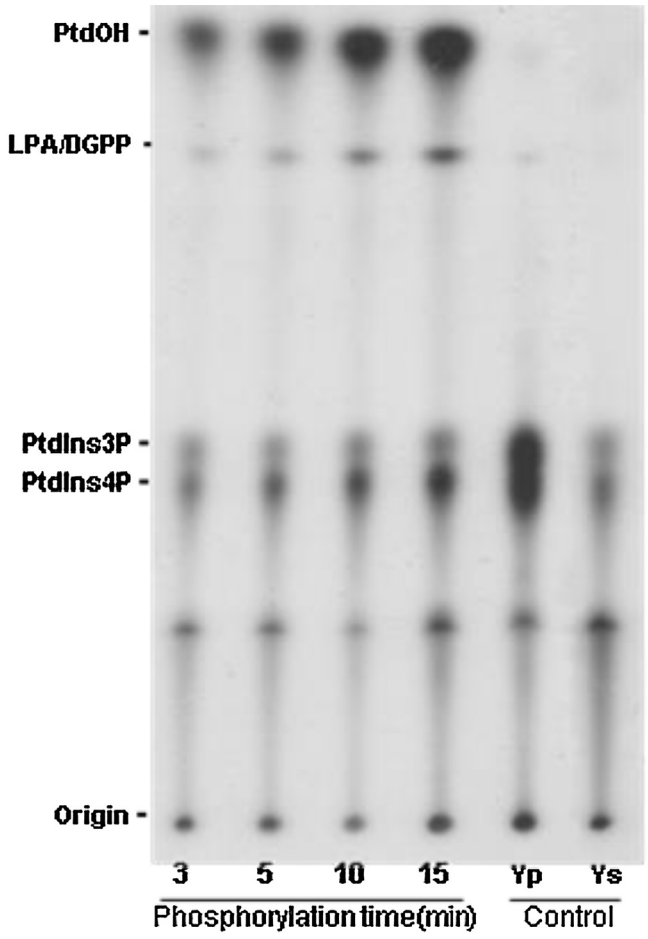


Fig. 3. Identification of PtdIns3P in *T. cruzi* epimastigote membranes.
A phosphorylation assay was performed with 30 μg of 1000 g membrane fraction as source of enzymes and [γ-^{32}P]ATP during the indicated times. Phosphoinositides were separated using boric acid TLC solvent system and labeled lipids were detected by autoradiography. Yp and Ys yeast fractions were used as controls for PtdIns3P identification. A representative experiment from three performed independently is shown. PtdOH: phosphatidic acid; LPA: lisophosphatidic acid; DGPP: diacylglycerol pyrophosphate; PtdIns4P: phosphatidylinositol 4 phosphate; PtdIns3P: phosphatidylinositol 3 phosphate.

22b(+)-TcPI3K bacteria. The levels of PI3K activity observed in non-induced bacteria fractions could be explained for the basal expression of TcPI3K even in the absence of inductor, as in wild-type *E. coli* fractions this activity was not detected.

Another mediator of signal transduction pathways that seems very likely to regulate TcPI3K activity is Protein Kinase A (PKA). TcPI3K not only has predicted sites for phosphorylation for this protein kinase but was proved to interact with the catalytic subunit of *T. cruzi* PKA (pBD-TcPKAc) through yeast two-hybrid system. Moreover, an in vitro phosphorylation analysis demonstrated that TcPKAc could phosphorylate TcPI3K, among other seven proteins important for *T. cruzi* growth, adaptation and differentiation [35]. However, so far the nature of this phosphorylation is unknown, for example, whether it activates or inhibits TcPI3K. In order to unravel this question, a PKA activator (dibutyryl-cAMP, cAMP non-degradable analog) and an inhibitor (KT5720) were used in this study (Fig. 5D). The specific activity of the native TcPI3K in endogenous membranes from *T. cruzi* epimastigotes was 0.33 ± 0.04 pmol/min/mg protein (100%), whereas membranes from epimastigotes treated with PKA activator showed a significant decrease in class I PI3K activity (66 ± 5%) with respect to the untreated control (100%). On the other hand, epimastigotes treated with PKA inhibitor increased their PtdIns(3,4,5)P_3 levels in 136 ± 10% with respect to the control.

4. Discussion

The ability of *T. cruzi* to receive signals from different environments and to initiate appropriate changes in cell activity is crucial for its pathogenic activity. Several biochemical events allow the parasite to adapt to adverse conditions, and responses of parasites to environmental changes often involve changes in the phospholipid metabolism. In this scenario, new knowledge about parasite PtdIns metabolism could contribute to the development of new tools to combat Chagas disease.

In this study, we determined enzymatic and biochemical properties of PI4K in *T. cruzi* epimastigotes. The enzyme showed an allosteric-like behavior, mainly with PtdIns as exogenous substrate; however further analyses are necessary to corroborate or refute this idea. The relatively high Km values of this enzyme for PtdIns and ATP-Mg^{2+}, their activation by Triton X-100 but inhibition by DOC, and their insensitiveness to adenosine, are parameters consistent with class III PI4K. In agreement, the predicted molecular weight of the two putative PI4Ks encoded in *T. cruzi* genome is compatible with class III PI4K [15]. In this study, we could not conclusively determinate whether both PI4KIIIa and PI4KIIIb isoforms are functional in *T. cruzi*, as their biochemical properties are similar. However, since this enzyme contributes to the PtdIns(4,5)P_2 pool which is subsequently hydrolyzed by PtdIns-PLC after several stimuli [8,9,20], and these signaling functions are likely to be exclusive of class III-a isoforms [33], we suggest that the PI4K activ-

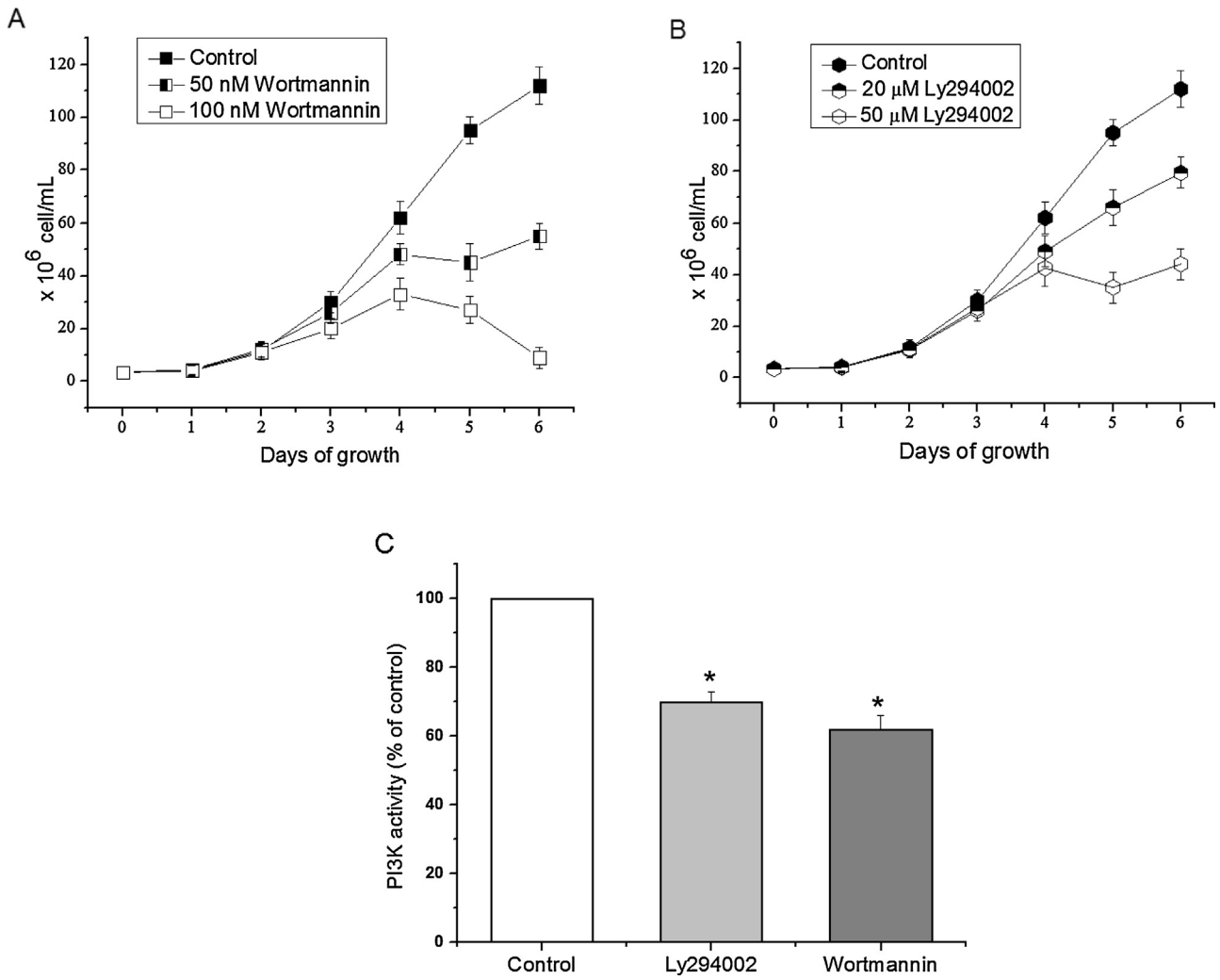


Fig. 4. Effect of wortmannin and LY294002 on epimastigotes growth and PI3K activity.
50–100 nM wortmannin (A), 20–50 μM LY294002 (B) or 0.1% DMSO (A and B control) were added to epimastigote cultures and growth was followed up by counting cell number in a Neubauer chamber for 6 days. A representative experiment from two performed independently is shown. In others experiments, membranes from epimastigotes treated or not with 50 nM wortmannin or 20 μM LY294002 were analyzed for PI3K activity (C). Values are percentages of control without inhibitor ± standard error of the mean (SEM) from three independent experiments, $p < 0.05$. Students' *t*-test was used for statistical calculations.

ity detected in this work corresponds to PI4KIIIa [i.e., Model 4 of TryPIKinome [15]].

The mechanism of signal transduction which involves the metabolism of PtdIns(4,5)P_2 is widely acknowledged as pivotal in cell signaling in most organisms. In unicellular eukaryotes, it was proposed that several agonists could interact with membrane sensors or through heterotrimeric G-protein, activating phospholipase Cδ-like enzymes and thus initiating PtdIns(4,5)P_2-dependent signaling pathways [36]. In spite of the acknowledged importance of this phospholipid and the signaling processes which initialize in lower eukaryote cells, PIPK, the main enzyme responsible for its production, so far remains poorly understood. Here we showed that *T. cruzi* PIPK activity was increased in the presence of both heparin and spermine, reported as class I PIPK activators but class II PIPK inhibitors [37]. Also, the addition of PtdOH in the phosphorylation assay resulted in increased enzymatic activity. Since PtdOH is a regulator of class I – and not class II – PIPKs (reviewed in [38]), our results indicate the presence of class I (PI4P5K) enzyme in *T. cruzi* epimastigotes.

Besides being substrate of phosphoinositide kinases, phosphatases and phospholipases, PtdIns(4,5)P_2 has a pivotal role in signaling, participating in actin cytoskeleton remodeling, membrane localization and vesicle trafficking processes [34]. Among the variety of effectors which has been proved to interact with PtdIns(4,5)P_2 is phospholipase D (PLD), acting as cofactor for enzyme activation. As occurs in higher eukaryotes, in *T. cruzi* epimastigotes this phospholipid can at least be substrate for PtdIns-PLC [8,9,20] and TcPI3K (discussed below). With respect to PLD, unpublished results indicate that increased levels of PtdIns(4,5)P_2 would activate PLD in epimastigotes as a result of hyperosmotic stress, therefore suggesting that PtdIns(4,5)P_2 also acts as cofactor for PLD activation in this parasite (Santander et al., unpublished). However, further studies are necessary to determine its participation in other cellular processes.

In higher eukaryotes, the PtdIns4P isomer is largely predominant over PtdIns3P, as their cellular functions are distinctive. Although the PtdIns3P/PtdIns4P ratio observed in *T. cruzi* (0.2) is a higher value than those reported for most organisms including mammals and plants, is still consistent with those found in yeast and *Tetrahymena* cells where PtdIns3P isomer reaches 15–20% of total PtdInsP [39]. Taking into account that PtdIns3P has a central role in *Dictyostelium discoideum* chemotaxis [40] and *G. lamblia* encystment (Gesumaría et al., unpublished), this phospholipid

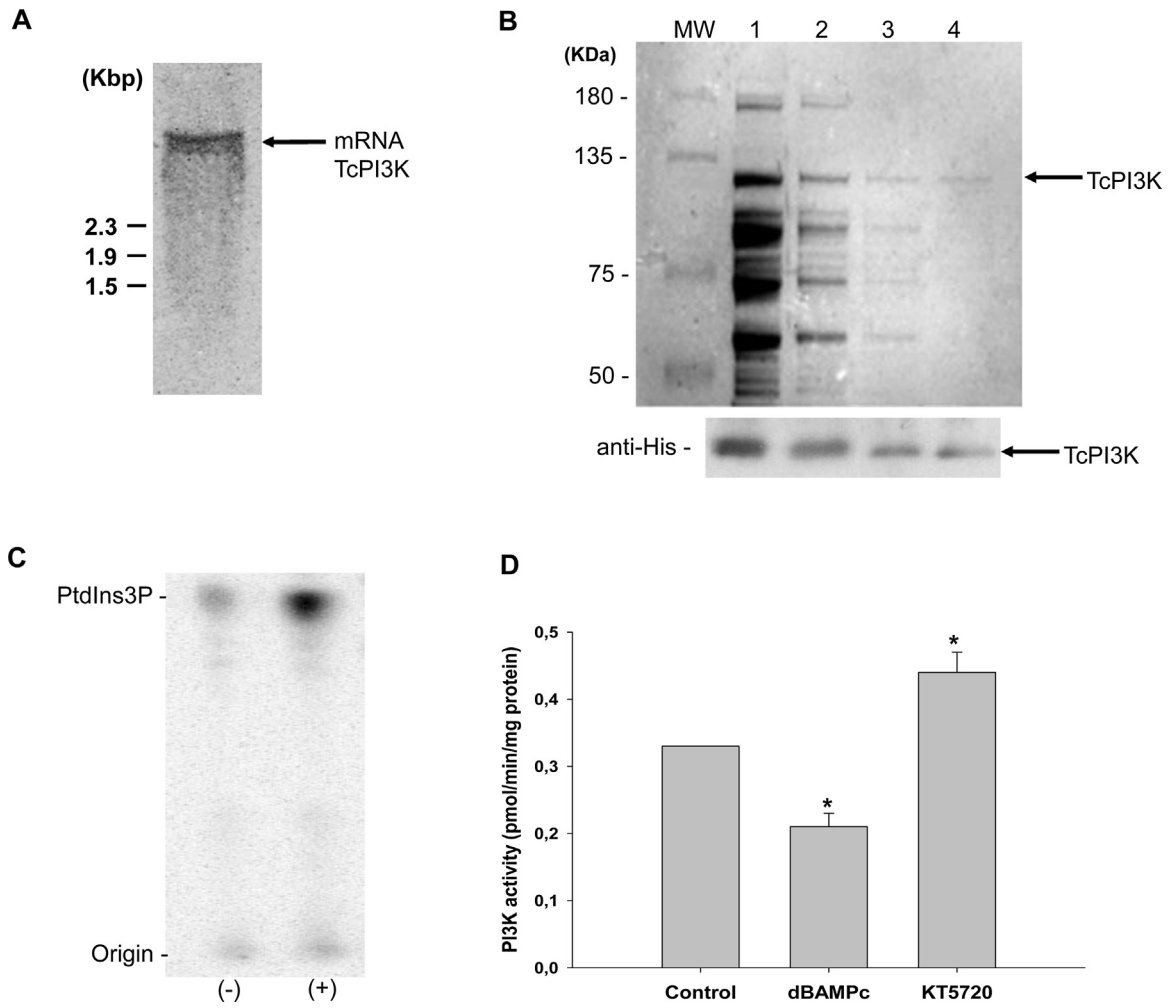


Fig. 5. Cloning and expression of TcPI3K.
Northern blot assay was performed by electrophoresis of total RNA (30 μg) from epimastigotes in agarose-formaldehyde gels, blotting and hybridization with a probe corresponding to the first 1850-bp of the TcPI3K coding region (A). His-tagged TcPI3K recombinant protein was affinity-purified from the soluble fraction of IPTG-induced *E. coli* BL21-pET28b(+)-TcPI3K bacterias using a nickel–nitrilotriacetic acid–agarose resin, and samples of induced soluble fraction (lane 1) and elutions with 20, 50, and 100 mM imidazole (lanes 2–4, respectively) were examined by SDS-PAGE (B). Western blot was performed with 50 μg of the same samples, anti-His as primary antibody and anti-IgG-HRP as secondary antibody. Reactive bands were detected by ECL/autoradiography (B, lower panel). Kinase activity of recombinant TcPI3K was assayed incubating 60 μg of total protein from S10 fractions of non-induced (−) or IPTG-induced (+) *E. coli* BL21 (DE3) with [γ-^{32}P]ATP and phosphatidylinositol. The reaction products were separated by TLC, and labeled lipids were detected by autoradiography (C). Membranes from epimastigotes treated with 0.1% DMSO (control), 25 μM dibutiryl-adenil-monophosphate cyclic (dBAMPc) or 25 nM KT5720 were analyzed for class I PI3K activity using an indirect ELISA protocol described in Section 2. Values are expressed as pmoles of PIP_3 per min per mg of protein ± standard error of the mean (SEM) from two independent experiments performed in duplicate, $p < 0.05$ (D). Students' *t*-test was used for statistical calculations.

Table 2
Recombinant TcPI3K specific activity.

E. coli strain	IPTG 300 μM	TcPI3K specific activity (pmol/min/mg protein)	
		P10 fraction	S10 fraction
BL21	–	ND	ND
	+	ND	ND
BL21-pET-22b(+)-TcPI3K	–	0.11 ± 0.02	0.22 ± 0.03
	+	0.66 ± 0.04	0.78 ± 0.06

ND: not detected, $n = 2$.

could form part of an ancient signaling pathway critical for lower eukaryotes.

Inhibition of PI3K affected the normal growth of epimastigotes, causing a delay evident from the fourth day of growth. In addition, there was a tendency to enter in stationary phase or death cell, more marked with the addition of wortmannin. It must be noted that this effect was not due to a delay in the lag phase, because the addition of inhibitors was performed after 48 h of initiating the culture, similar to that implemented by Braga and de Souza [12]. Also, a non-specific toxic effect of the inhibitors was discarded since the concentrations employed are non-toxic and specific for PI3K inhibition [41]. Moreover, these compounds have been widely used to determine the potential roles of PI3Ks from protozoa such as *T. thermophila*, *T. brucei* and *G. lamblia* [39,42–44]. The great difference observed in effectiveness as inhibitor of wortmannin (nanomolar concentrations) compared with LY294002 (micromolar concentrations) could be due to a closer shape complementarity between the PI3K active site and wortmannin, which could irreversibly modify the active site. Differently, LY294002 binds in the same ATP binding site in another orientation [45]. Since *T. cruzi* class III PI3K (TcVps34) was sensitive to micromolar concentrations of wortmannin [18], we suggest that the delay in parasite growth was due to specific inhibition of class I PI3K.

To our knowledge, this is the first evidence of a class I PI3K protein with demonstrated catalytic activity in protozoan parasites. Moreover, TcPI3K was active without any regulatory subunits

(p55/85, p84/101). In mammals these are accessory adapters which bind to catalytic subunits, maintain their stability, inactivate their basal kinase activity and facilitate the contact with lipid substrates in the membrane [10]. The lack of regulatory subunits to TcPI3K explains the basal activity measured and suggests the existence of some other type of regulation for the catalytic function, as well as a membrane-interacting domain. In this regard, the Ras-binding domain (RBD) predicted in TcPI3K sequence could serve as membrane-interacting domain through Rab proteins, which share 30% identity with Ras proteins and are able to associate to cell membranes [46]. In *T. cruzi*, several Rab proteins actively participate in parasite cell biology, such as TcRAB11, active in reservosomes [47], TcRAB7 in the Golgi complex of epimastigotes [48] and TcRAB5 with GTPase activity [49]. Therefore, RBD of TcPI3K could act both as membrane interacting and as regulatory domain for enzyme activation.

Our results suggest that TcPI3K could be negatively regulated by phosphorylation by PKA. This Ser/Thr kinase participates in cAMP-dependent pathways, considered as key to multiple processes leading to adaptation and differentiation in *T. cruzi* and other lower eukaryotes [50–52]. Experiments in progress indicate that TcPI3K activity could be associated to proliferation events in epimastigotes, which should be arrested when the parasite begins a differentiation process, since after complete differentiation, trypomastigote forms are not replicative. Accordingly, a bioinformatic analysis of microarray data deposited in the Gene Expression Omnibus (GEO data base) by Minning et al. [53] indicates that TcPI3K expression is significantly up-regulated in the proliferative (amastigote) mammalian infectious stage when compared with non-proliferative (trypomastigote) stage in the same host (summarized in Supplementary Table 2). Therefore, it is likely that TcPI3K expression/activity is positively regulated during proliferation, but negatively regulated during the differentiation process.

The main proliferative pathway in which class I PI3Ks participates is the PI3K-PKB/Akt pathway [10]. The pathway may involve binding of PtdIns(3,4,5)P_3 to the phosphoinositide-dependent protein kinase (PDK) or directly to pleckstrin homology domain (PH) of PKB/Akt, which is the effector protein in both cases. In *T. cruzi*, Pascuaccelli et al. [54] cloned a protein with characteristics and activity of PKB, but without PH domain. However, an updated bioinformatic search indicates the existence of a protein (XP_809943) with 30 extra amino acids which correspond to a PH-like domain. This difference is probably due to the data provided in the parasite complete genome sequencing [23]. As for proteins that negatively regulate the levels of PtdIns(3,4,5)P_3, *T. cruzi* genome also encodes proteins with high homology to classical inositol-3 phosphatase PTEN (XP_814678) and inositol-5 phosphatase $SHIP_2$ (XP_817971).

Taken together, our results and previous works suggest that *T. cruzi* not only is capable of PtdIns-based signal transduction but also could use PI3K-dependent signaling pathways. Since PI3K-PKB/Akt system is widely avowed as a promoting factor of growth, proliferation and survival in higher eukaryotes, TcPI3K would be an attractive target for specific inhibitors design in order to combat this parasite.

Acknowledgments

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Argentina), Secretaría de Ciencia y Técnica of Universidad Nacional de Río Cuarto (SECyT, UNRC, Río Cuarto, Córdoba, Argentina) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). The authors would like to thank to Norma Marchesini (*in memorian*), Gabriela Hernandez and Veronica Santander (UNRC, Argentina) who provided supporting discussion for this paper, to Priscila Denapoli (University of São Paulo, Brazil) for helping with bioinformatic analyses and to Ileana Martinez (UNRC, Argentina) for language revision. A.M.G. and M.C.G. were fellows of CONICET, Argentina. A.C.S., G.D.A. and M.M.F. are members of the Scientific Investigator Career of CONICET, Argentina.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2015.10.002.

References

[1] C. Chagas, Nova tripanozomiaze humana. Estudos sobre a morfolojia e o ciclo evolutivo do *Schizotrypanum cruzi* n. gen., n. sp., ajente etiolojico de nova entidade morbida do homem, Mem. Inst. Oswaldo Cruz 1 (1909) 159–218.
[2] World Health Organization (W.H.O.) web page, <http://www.who.int/mediacentre/factsheets/fs340/en/> (consulted 04.10.15).
[3] M. Parsons, L. Ruben, Pathways involved in environmental sensing in trypanosomatids, Parasitol. Today 16 (February (2)) (2000) 56–62, PubMed PMID: 10652488.
[4] E.E.M. Machado-Domenech, M. Garcia, M.N. Garrido, G. Racagni, Phospholipids of *Trypanosoma cruzi*—increase of polyphosphoinositides and phosphatidic-acid after cholinergic stimulation, Fems Microbiol. Lett. 95 (August (2–3)) (1992) 267–270, PubMed PMID: WOS: A1992JJ66600025.
[5] M.N. Garrido, M.I. Bollo, E.E. Machado-Domenech, Biphasic and dose-dependent accumulation of INSP3 in *Trypanosoma cruzi* stimulated by a synthetic peptide carrying a chicken alpha D-globin fragment, Cell. Mol. Biol. 42 (September (6)) (1996) 859–864, PubMed PMID: 8891353.
[6] T. Nozaki, A. Toh-e, M. Fujii, H. Yagisawa, M. Nakazawa, T. Takeuchi, Cloning and characterization of a gene encoding phosphatidyl inositol-specific phospholipase C from *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 102 (August (2)) (1999) 283–295, PubMed PMID: 10498184.
[7] T. Furuya, C. Kashuba, R. Docampo, S.N. Moreno, A novel phosphatidylinositol-phospholipase C of *Trypanosoma cruzi* that is lipid modified and activated during trypomastigote to amastigote differentiation, J. Biol. Chem. 275 (March (9)) (2000) 6428–6438, PubMed PMID: 10692446.
[8] N. Marchesini, M. Bollo, G. Hernandez, M.N. Garrido, E.E. Machado-Domenech, Cellular signalling in *Trypanosoma cruzi*: biphasic behaviour of inositol phosphate cycle components evoked by carbachol, Mol. Biochem. Parasitol. 120 (March (1)) (2002) 83–91, PubMed PMID: 11849708.
[9] V. Santander, M. Bollo, E. Machado-Domenech, Lipid kinases and Ca(2+) signaling in *Trypanosoma cruzi* stimulated by a synthetic peptide, Biochem. Biophys. Res. Commun. 293 (April (1)) (2002) 314–320, PubMed PMID: 12054601.
[10] B. Vanhaesebroeck, J. Guillermet-Guibert, M. Graupera, B. Bilanges, The emerging mechanisms of isoform-specific PI3K signalling, Nat. Rev. Mol. Cell Biol. 11 (May (5)) (2010) 329–341, PubMed PMID: 20379207.
[11] B.S. Hall, C. Gabernet-Castello, A. Voak, D. Goulding, S.K. Natesan, M.C. Field, TbVps34, the trypanosome orthologue of Vps34, is required for Golgi complex segregation, J. Biol. Chem. 281 (September (37)) (2006) 27600–27612, PubMed PMID: 16835237.
[12] M.V. Braga, W. de Souza, Effects of protein kinase and phosphatidylinositol-3 kinase inhibitors on growth and ultrastructure of *Trypanosoma cruzi*, Fems Microbiol. Lett. 256 (March (2)) (2006) 209–216, PubMed PMID: 16499608.
[13] R. Diaz-Gonzalez, F.M. Kuhlmann, C. Galan-Rodriguez, L. Madeira da Silva, M. Saldivia, C.E. Karver, et al., The susceptibility of trypanosomatid pathogens to PI3/mTOR kinase inhibitors affords a new opportunity for drug repurposing, PLoS Negl. Trop. Dis. 5 (August (8)) (2011) e1297, PubMed PMID: 21886855, Pubmed Central PMCID: 3160303.
[14] L. Demmel, K. Schmidt, L. Lucast, K. Havlicek, A. Zankel, T. Koestler, et al., The endocytic activity of the flagellar pocket in *Trypanosoma brucei* is regulated by an adjacent phosphatidylinositol phosphate kinase, J. Cell Sci. 127 (May (Pt. 10)) (2014) 2351–2364, PubMed PMID: 24639465, Pubmed Central PMCID: 4021478.
[15] D. Bahia, L.M. Oliveira, F.M. Lima, P. Oliveira, J.F. Silveira, R.A. Mortara, et al., The TryPIKinome of five human pathogenic trypanosomatids: *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania major*, *Leishmania braziliensis* and *Leishmania infantum*—new tools for designing specific inhibitors, Biochem. Biophys. Res. Commun. 390 (December (3)) (2009) 963–970, PubMed PMID: 19852933.
[16] F.M. Lima, P. Oliveira, R.A. Mortara, J.F. Silveira, D. Bahia, The challenge of Chagas' disease: has the human pathogen, *Trypanosoma cruzi*, learned how to modulate signaling events to subvert host cells? New Biotechnol. 27 (December (6)) (2010) 837–843, PubMed PMID: 20172059.
[17] P. Oliveira, F.M. Lima, M.C. Cruz, R.C. Ferreira, A. Sanchez-Flores, E.M. Cordero, et al., *Trypanosoma cruzi*: genome characterization of phosphatidylinositol kinase gene family (PIK and PIK-related) and identification of a novel PIK gene, Infect. Genet. Evol.: J. Mol. Epidemiol. Evolut. Genet. Infect. Dis. 25 (July) (2014) 157–165, PubMed PMID: 24727645.

[18] A.C. Schoijet, K. Miranda, W. Girard-Dias, W. de Souza, M.M. Flawia, H.N. Torres, et al., A *Trypanosoma cruzi* phosphatidylinositol 3-kinase (TcVps34) is involved in osmoregulation and receptor-mediated endocytosis, J. Biol. Chem. 283 (November (46)) (2008) 31541–31550, PubMed PMID: 18801733, Pubmed Central PMCID: 2581564.
[19] M.J. Rodgers, J.P. Albanesi, M.A. Phillips, Phosphatidylinositol 4-kinase III-beta is required for Golgi maintenance and cytokinesis in *Trypanosoma brucei*, Eukaryot. Cell 6 (July (7)) (2007) 1108–1118, PubMed PMID: 17483288, Pubmed Central PMCID: 1951100.
[20] S. Bonansea, M. Usorach, M.C. Gesumaria, V. Santander, A.M. Gimenez, M. Bollo, et al., Stress response to high osmolarity in *Trypanosoma cruzi* epimastigotes, Arch. Biochem. Biophys. 527 (November (1)) (2012) 6–15, PubMed PMID: 22884762.
[21] A.M. Gimenez, V.S. Santander, A.L. Villasuso, S.J. Pasquare, N.M. Giusto, E.E. Machado, Regulation of phosphatidic acid levels in *Trypanosoma cruzi*, Lipids 46 (October (10)) (2011) 969–979, PubMed PMID: 21667213.
[22] L.G. Warren, Metabolism of *Schizotrypanum cruzi* Chagas. I. Effect of culture age and substrate concentration on respiratory rate, J. Parasitol. 46 (October) (1960) 529–539, PubMed PMID: 13783227.
[23] N.M. El-Sayed, P.J. Myler, D.C. Bartholomeu, D. Nilsson, G. Aggarwal, A.N. Tran, et al., The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease, Science 309 (July (5733)) (2005) 409–415, PubMed PMID: 16020725.
[24] J.H. Stack, P.K. Herman, P.V. Schu, S.D. Emr, A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole, EMBO J. 12 (May (5)) (1993) 2195–2204, PubMed PMID: 8387919, Pubmed Central PMCID: 413440.
[25] L.H. Mak, R. Woscholski, Targeting PTEN using small molecule inhibitors, Methods 77–78 (May) (2015) 63–68, PubMed PMID: 25747336.
[26] L. Spinelli, Y.E. Lindsay, N.R. Leslie, PTEN inhibitors: an evaluation of current compounds, Adv. Biol. Regul. 57 (January) (2015) 102–111, PubMed PMID: 25446882.
[27] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (May) (1976) 248–254, PubMed PMID: 942051.
[28] E.B. Stubbs Jr., J.A. Kelleher, G.Y. Sun, Phosphatidylinositol kinase, phosphatidylinositol-4-phosphate kinase and diacylglycerol kinase activities in rat brain subcellular fractions, Biochim. Biophys. Acta 958 (2) (1988) 247–254, PubMed PMID: 2827783.
[29] N. Marchesini, V. Santander, E. Machado-Domenech, Diacylglycerol pyrophosphate: a novel metabolite in the *Trypanosoma cruzi* phosphatidic acid metabolism, FEBS Lett. 436 (October (3)) (1998) 377–381, PubMed PMID: 9801152.
[30] J.P. Walsh, K.K. Caldwell, P.W. Majerus, Formation of phosphatidylinositol3-phosphate by isomerization from phosphatidylinositol 4-phosphate, Proc. Natl. Acad. Sci. U. S. A. 88 (Ocyober (20)) (1991) 9184–9187, PubMed PMID: 1656463, Pubmed Central PMCID: 52677.
[31] G.D. Alonso, A.C. Schoijet, H.N. Torres, M.M. Flawia, TcrPDEA1 a cAMP-specific phosphodiesterase with atypical pharmacological properties from Trypanosoma cruzi, Mol. Biochem. Parasitol. 152 (March (1)) (2007) 72–79, PubMed PMID: 17222469.
[32] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (August (5259)) (1970) 680–685, PubMed PMID: 5432063.
[33] A. Balla, T. Balla, Phosphatidylinositol 4-kinases: old enzymes with emerging functions, Trends Cell Biol. 16 (July (7)) (2006) 351–361, PubMed PMID: 16793271.
[34] A. Toker, Phosphoinositides and signal transduction, Cellular Mol. Life Sci.: CMLS 59 (May (5)) (2002) 761–779, PubMed PMID: 12088277.
[35] Y. Bao, L.M. Weiss, V.L. Braunstein, H. Huang, Role of protein kinase A in *Trypanosoma cruzi*, Infect. Immun. 76 (October (10)) (2008) 4757–4763, PubMed PMID: 18694966, Pubmed Central PMCID: 2546855.
[36] I.V. Shemarova, Phosphoinositide signaling in unicellular eukaryotes, Crit. Rev. Microbiol. 33 (3) (2007) 141–156, PubMed PMID: 17653984.
[37] C.E. Bazenet, A.R. Ruano, J.L. Brockman, R.A. Anderson, The human erythrocyte contains two forms of phosphatidylinositol-4-phosphate 5-kinase which are differentially active toward membranes, J. Biol. Chem. 265 (October (29)) (1990) 18012–18022, PubMed PMID: 2170402.
[38] S. Cockcroft, Phosphatidic acid regulation of phosphatidylinositol 4-phosphate 5-kinases, Biochim. Biophys. Acta 1791 (September (9)) (2009) 905–912, PubMed PMID: 19298865.
[39] G. Leondaritis, A. Tiedtke, D. Galanopoulou, D-3 phosphoinositides of the ciliate *Tetrahymena*: characterization and study of their regulatory role in lysosomal enzyme secretion, Biochim. Biophys. Acta 1745 (September (3)) (2005) 330–341, PubMed PMID: 16081170.
[40] J.S. King, R.H. Insall, Chemotaxis: finding the way forward with *Dictyostelium*, Trends Cell Biol. 19 (October (10)) (2009) 523–530, PubMed PMID: 19733079.
[41] S.P. Davies, H. Reddy, M. Caivano, P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors, Biochem. J. 351 (October (Pt. 1)) (2000) 95–105, PubMed PMID: 10998351, Pubmed Central PMCID: 1221339.
[42] S.S. Cox, M. van der Giezen, S.J. Tarr, M.R. Crompton, J. Tovar, Evidence from bioinformatics, expression and inhibition studies of phosphoinositide-3 kinase signalling in *Giardia intestinalis*, BMC Microbiol. 6 (2006) 45, PubMed PMID: 16707026, Pubmed Central PMCID: 1483827.
[43] P.P.E. Kovács, Phosphatidylinositol 3-kinase-like activity in tetrahymena. Effects of Wortmannin and LY 294002, Acta Protozool. 42 (2003) 277–285.
[44] Y. Hernandez, G. Zamora, S. Ray, J. Chapoy, E. Chavez, R. Valvarde, et al., Transcriptional analysis of three major putative phosphatidylinositol kinase genes in a parasitic protozoan, *Giardia lamblia*, J. Eukaryot. Microbiol. 54 (January–February (1)) (2007) 29–32, PubMed PMID: 17300515, Pubmed Central PMCID: 3124632.
[45] E.H. Walker, M.E. Pacold, O. Perisic, L. Stephens, P.T. Hawkins, M.P. Wymann, et al., Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine, Mol. Cell 6 (October (4)) (2000) 909–919, PubMed PMID: 11090628.
[46] P. Brennwald, P. Novick, Interactions of three domains distinguishing the Ras-related GTP-binding proteins Ypt1 and Sec4, Nature 362 (April (6420)) (1993) 560–563, PubMed PMID: 8464498.
[47] S.M. Mauricio de Mendonca, J.L. Nepomuceno da Silva, N. Cunha e-Silva, W. de Souza, U. Gazos Lopes, Characterization of a Rab11 homologue in *Trypanosoma cruzi*, Gene 243 (February (1–2)) (2000) 179–185, PubMed PMID: 10675626.
[48] J.R. Araripe, N.L. Cunha e Silva, S.T. Leal, W. de Souza, E. Rondinelli, *Trypanosoma cruzi*: TcRAB7 protein is localized at the Golgi apparatus in epimastigotes, Biochem. Biophys. Res. Commun. 321 (August (2)) (2004) 397–402, PubMed PMID: 15358190.
[49] J.R. Araripe, F.P. Ramos, N.L. Cunha e Silva, T.P. Urmenyi, R. Silva, C.F. Leite Fontes, et al., Characterization of a RAB5 homologue in *Trypanosoma cruzi*, Biochem. Biophys. Res. Commun. 329 (April (2)) (2005) 638–645, PubMed PMID: 15737633.
[50] I.V. Shemarova, cAMP-dependent signal pathways in unicellular eukaryotes, Crit. Rev. Microbiol. 35 (1) (2009) 23–42, PubMed PMID: 19514907.
[51] M. Gonzales-Perdomo, P. Romero, S. Goldenberg, Cyclic AMP and adenylate cyclase activators stimulate *Trypanosoma cruzi* differentiation, Exp. Parasitol. 66 (August (2)) (1988) 205–212, PubMed PMID: 2840306.
[52] V. Jimenez, Dealing with environmental challenges: mechanisms of adaptation in *Trypanosoma cruzi*, Res. Microbiol. 165 (April (3)) (2014) 155–165, PubMed PMID: 24508488, Pubmed Central PMCID: 3997592.
[53] T.A. Minning, D.B. Weatherly, J. Atwood 3rd, R. Orlando, R.L. Tarleton, The steady-state transcriptome of the four major life-cycle stages of *Trypanosoma cruzi*, BMC Genomics 10 (2009) 370, PubMed PMID: 19664227, Pubmed Central PMCID: 2907688.
[54] V. Pascuccelli, C. Labriola, M.T. Tellez-Inon, A.J. Parodi, Molecular and biochemical characterization of a protein kinase B from *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 102 (July (1)) (1999) 21–33, PubMed PMID: 10477173.