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*Tc*PDE4, a novel membrane-associated cAMP-specific phosphodiesterase from *Trypanosoma cruzi*☆

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Abstract

Cyclic nucleotide phosphodiesterases constitute the only known mechanism to inactivate regulatory signals involving cAMP or cGMP. In our laboratory a cAMP-specific phosphodiesterase associated to the flagellar apparatus, named *Tc*PDE1, was identified in *Trypanosoma cruzi*. By using the catalytic domain sequence of *Tc*PDE1 to screen a *Trypanosoma cruzi* genomic data base, a novel *T. cruzi* phosphodiesterase sequence was found and characterized. *Tc*PDE4 encodes a 924-amino acid protein and shows homology with the PDE4 vertebrate subfamily. The sequence shows three conserved domains, FYVE, phosphohydrolase and PDEaseI. The FYVE zinc-finger domain is characteristic of proteins recruited to phosphatidylinosytol 3-phosphate-containing membranes, whereas the two others are characteristic of phosphohydrolases and members of the cyclic nucleotide phosphodiesterases. Sequence analysis shows all characteristic domains present at the type-4 phosphodiesterases specific for cAMP. Moreover, *Tc*PDE4 shows the inhibition profile characteristic for PDE4 subfamily, with an IC50 of 10.46 μM for rolipram and 1.3 μM for etazolate. *Tc*PDE4 is able to complement a heat-shock-sensitive yeast mutant deficient in phosphodiesterase genes. The enzyme is specific for cAMP, Mg^{2+}-dependent and its activity is not affected by cGMP or Ca^{2+}. The association of *Tc*PDE4 with membranes was studied by subcellular fractionation of recombinant yeast and extraction in several conditions. Most of the enzyme remained associated to the membrane fraction after treatment with high salt concentration, detergent, or chaotropic agents. This support previous hypotheses that in this parasite cAMP phosphodiesterases, and consequently cAMP levels, are compartimentalized.

Keywords: cAMP; FYVE domain; Rolipram; Phosphatidylinositol 3-phosphate

1. Introduction

Cyclic nucleotide phosphodiesterases (PDEs) catalyze the degradation of the second messengers cAMP and cGMP and constitute the only known mechanism for the rapid down regulation of cyclic nucleotide signals. In consequence, these enzymes play a pivotal role in the regulation of the biological action of cyclic nucleotides.

PDEs are members of a superfamily comprising 11 different families classified according to their sequence identity, biochemical and pharmacological properties, regulation and substrate specificity. The fact that each family is composed of several genes and that many isoforms are generated by alternative splicing indicate the high complexity of this superfamily [1,2].

Although in trypanosomatids, cAMP has been involved in the control of growth and differentiation as well as in osmoregulation [3–9], very little is known about cAMP phosphodiesterases. The activity of these enzymes has been characterized in *Trypanosoma cruzi* [10], *Trypanosoma brucei* [11], *Trypanosoma gambiense* [12], and the related organism *Leishmania mexicana* [13]. However, the genes that code for these proteins have been mainly identified in *T. brucei* [14–17], where two types of genes have been reported, *TbPDE1* [14] and those of the *TbPDE2* family [15–17]. *TbPDE1* is a single copy gene

Abbreviations: PDE, phosphodiesterase; PtdIns(3)P, phosphatidylinosytol 3-phosphate; IBMX, 3-isobutyl-1-methylxanthine; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride; ADH, alcohol dehydrogenase; PKA, protein kinase A; ON, over-night; NS, non-selective; NA, not affected activity

☆ *Note*: Nucleotide sequence data reported in this paper are available in the GenBank™ databases under the accession number DQ008164.

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that is not related to the mammalian PDEs and is expressed at very low levels, whereas *TbPDE2* is a small family of at least five genes, which shows a considerable similarity to all 11 mammalian PDE families. *TbPDE1* knockout experiments have shown that the gene is not necessary for the survival of *T. brucei* in culture or for the infection of tsetse flies [14]. On the contrary, compounds that inhibit *Tb*PDE2A are lethal for *T. brucei* in culture [15]. In addition, RNAi experiments have indicated that *Tb*PDE2C is an essential enzyme for this parasite [16].

In *Trypanosoma cruzi*, the etiological agent of Chagas' disease in Latin America, our laboratory has described a calcium-stimulated adenylyl cyclase [18] and a novel membrane-bound cAMP-specific PDE, designated *Tc*PDE1, which is strongly associated to the flagellum and shows significant homology with *T. brucei Tb*PDE2 family [19].

The past decade has revealed that many cytosolic proteins are recruited to different cellular membranes to form protein–protein and lipid–protein interactions during cell signaling and membrane trafficking [20]. Recruitment of these peripheral proteins is mediated by modular membrane-targeting domains, such as the FYVE zinc finger binding domain, which recognizes PtdIns(3)P in the membranes. In most cases, proteins with FYVE fingers domains are recruited to PtdIns(3)P-containing endosomal vesicles [21].

On the other hand, taking into consideration the success of phosphodiesterase inhibitors as chemotherapeutics in a variety of physiological and pathological processes, it is important to explore the trypanosomatid PDEs as possible targets for family-specific PDE inhibitors.

This paper reports the cloning and characterization of a novel PDE in *Trypanosoma cruzi*, *Tc*PDE4, which shows strong homology with PDE4 family members. This enzyme is membrane associated, presents a high affinity and specificity for cAMP, and is strongly inhibited by rolipram and etazolate. Some of these properties point to a putative role of *Tc*PDE4 in the regulation of localized levels of this second messenger.

2. Materials and methods

2.1. Materials

All radiochemicals used in this work were purchased from Dupont NEN Life Science Products Inc., Boston, MA and restriction endonucleases were from New England Biolabs Inc., Beverly, MA. Bacto-tryptose, yeast nitrogen base and liver infusion were from Difco Laboratories, Detroit, MI. All other reagents were purchased from SIGMA Chemical Co., St. Louis, MO.

2.2. Cell cultures

T. cruzi epimastigote forms (CL Brenner strain) were cultured at 28 °C for 7 days in LIT medium (5 g/l liver infusion, 5 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) calf serum, 10 units/ml penicillin and 10 mg/l streptomycin. Cell viability was assessed by direct microscopic examination.

S. cerevisiae strain, J106 (*MATa leu2 his3 ura3 trp1 ade8 can1 pde1::URA3 pde2::HIS3*) was kindly gifted by Dr. Thevelein (see Ref. [22]). These strains were grown in YPD medium at 30 °C before transformation. Transformants were selected in minimal medium containing 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulphate), 0.5% (w/v) ammonium sulphate and 2% (w/v) glucose, supplemented with the corresponding amino acid mixture.

2.3. Preparation of T. cruzi DNA and RNA

Genomic DNA was purified as described by Pereira et al. [23]. Total cellular RNA was isolated from 10^8 epimastigote cells in exponential growth phase using TRIzol reagents (Gibco BRL, Life Technologies, Rockville, MD) as described by manufacturers.

2.4. Cloning of TcPDE4 gene

The gene sequence corresponding to the catalytic site of *T. cruzi TcPDE1* phosphodiesterase (AAP49573) was used to screen *T. cruzi* sequence databases using the WU-Blast2 algorithm. Five sequences that do not overlap with each other were identified. Two oligonucleotides carrying hemi-restriction sites (PDE_703_FW_pET-Bam: 5′-GGATCCAATGTCGG-AGGACGCTGGGCTT-3′ and PDE_703_RV_Xho: 5′-CTCGAGGCACTGCGTCAACAGAGTGG-3′) were designed from the identified sequence. 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 1–2 units of Taq DNA polymerase (Promega, Madison, WI). A band of 2772 bp was obtained and after its sequencing, we confirm that it carried the full-length gene sequence of *TcPDE4*.

2.5. Northern and Southern blots

For Northern blot analysis, 15 μg of total RNA was electrophoresed on a 1.5% (w/v) formaldehyde-agarose gel, transferred to a Hybond N+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, USA) and hybridized at 65 °C in Church's buffer (1% (w/v) BSA, 7% (w/v) SDS, 1 mM EDTA, pH 8, 0.5% (w/v) Na_2HPO_4) with a specific 1070 bp *Tc*PDE4 probe obtained by digestion of the 2772 bp fragment with *Bam*HI and *Sac*II. Blots were subjected to sequential stringent washes at 65 °C and exposed to AGFA CP-BU NEW films (AGFAGevaert N.V., Belgium). Southern blot analysis was performed with 5 μg of genomic DNA previously digested with the indicated restriction endonucleases. The products were resolved on 0.8% (w/v) agarose gels, transferred and hybridized as described for Northern blots.

2.6. RT-PCR analysis

The RT-PCR reaction was carried out as follows: 1 μg of total RNA from epimastigote cells was reverse transcribed with 50 U

of SuperScript II (Life Technologies) using random hexamers as primers. cDNA synthesis was performed according to the manufacturer's instructions for 50 min at 50 °C. Thereafter, the 5′ end of *TcPDE4* mRNA was amplified using an oligonucleotide (SL sense) derived from the *T. cruzi* spliced leader (SL) sequence as primer: 5′-AACGCTATTATTGATACAGTTTC-3′, and another one corresponding to the *TcPDE4* coding region that is positioned 668 bp downstream to the *Tc*PDE4 initiation codon: 5′-ACTGACTCAATGCGAAGATGAG-3′. The cycling profile was as follows: 94 °C for 15 s, 58 °C for 30 s, and 72 °C for 60 s, and was repeated 35 times. Finally, the amplified band was subcloned into pGEM-T Easy® vector and sequenced.

2.7. Labeling DNA probes

All probes were labeled with [α-P^{32}]-dCTP using the Prime-a-Gene kit (Promega, Madison, WI) following the manufacturer's instructions.

2.8. Complementation assay

Full-length *TcPDE4* gene was amplified by PCR using the oligonucleotides PDE_703_FW_Bam: 5′-GGATCCATGTCGGAGGACGCTGGGCT-3′ and PDE_703_RV_STOP-Xho: 5′-CTCGAGTCAGCACTGCGTCAACAGAGT-3′. The PCR product was cloned into pGEM-T Easy® vector, and subcloned into pADNS yeast expression vector [24] using the *Not* I restriction sites. The J106 yeast strain, lacking the PDE genes, was transformed with the empty vector, the *TcPDE1* gene [19], or the vector carrying the *TcPDE4* gene, using the lithium acetate procedure [25]. Transformant cells were selected in minimal medium (without leucine) at 30 °C. For heat shock complementation assays, transformants were grown in minimal medium to OD_{600} = 1.8–2 and further incubated at 55 °C for 30 min. After treatment, cells were plated in YPD at different dilutions and incubated at 30 °C for 2 days.

2.9. Soluble extracts and membrane preparation

Yeast transformants were grown in a minimal medium without leucine to OD_{600} = 0.8–1.5. Cells were harvested, washed twice with cold TE buffer, resuspended in lysis buffer (20 mM Tris–HCl, pH 7.5, 20% (v/v) glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonylfluoride, 25 units/ml aprotitinin, 0.5 mM tosyl-lysine chloromethyl ketone) and lysed by 10 cycles of 1 min vortexing in the presence of glass beads (425–600 μm) and cooling on ice. Cell debris was discarded by centrifugation at 2500 × *g* at 4 °C. The supernatants were further centrifuged for 1 h at 100,000 × *g*. The pellet was resuspended in 20 mM Tris–HCl, pH 7.5, 5% (v/v) glycerol plus the anti-protease mixture and used as membrane fraction.

For *Tc*PDE4 membrane extraction, membranes were sequentially washed with lysis buffer containing 1 M NaCl, 0.1% (w/v) sodium cholate, 0.5% (w/v) sodium cholate, or 8 M urea at 4 °C for 30 min. After each wash, membranes were centrifuged for 40 min at 100,000 × *g* and washed with lysis buffer.

2.10. cAMP phosphodiesterase assays

PDE activity was determined as described by Thompson and Appleman [26] with the modifications introduced by Londesborough [27]. The reactions were performed in the presence of 20 mM Tris–HCl, pH 7.5, 5 mM Mg^{2+} and 1 or 50 μM [^{3}H] cAMP. Incubations were carried out at 30 °C for 10 min in a total volume of 100 μl. For the determination of kinetic parameters, several independent assays were performed with 10 μg of protein and 0.1–300 μM cAMP. Ca^{2+}-calmodulin response and ion dependence was analyzed in the same conditions adding 1 mM EDTA or 1 mM EGTA as chelator. The different ions were used to a final concentration of 5 mM in the assay, and calmodulin was tested using 0.05 units.

For inhibition studies, assays were performed in the presence of 10 μg of proteins and a different concentration range of each compound according to the inhibitor used.

2.11. Expression of recombinant TcPDE4 in E. coli

Full-length *TcPDE4* gene was amplified using the following primers carrying hemi-restriction sites: PDE_703_FW_pET-Bam: 5′-GGATCCAATGTCGGAGGACGCTGGGCTT-3′ and PDE_703_RV_Xho: 5′-CTCGAGGCACTGCGTCAACAGAGTGG-3′, cloned into pGEM-T Easy® plasmid (Promega, Madison, WI), and subcloned into pET22b(+)® expression vector (Novagene, Inc.) in fusion with a C-terminal His-Tag. Expression of the recombinant protein was performed in the *BL21*(*DE3*)*pLysS* host (*E. coli B*, *F*, *dem*, *ompT*, *hsdS*, (rb^-, m_B^-), *galλ*(*DE3*), [*pLysS*, cam^r]). Bacterial cells carrying the fusion *Tc*PDE4-6*x*His were grown to OD_{600} = 0.4–0.6 and induced with 500 μM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 2 h.

2.12. Antibody preparation and Western blot analysis

*Tc*PDE4 antiserum was obtained using a female *BALB/c* strain of mice immunized by intraperitoneal injection of 10 μg of recombinant protein expressed in *E. coli* plus 0.1 ml of Freund's adjuvant followed by two more subsequent injections every 15 days with incomplete adjuvant. Mice were bled by exposing the ocular cavity. Antibodies were tested to determine titer and cross reactivity using the recombinant protein and *T. cruzi* extracts. No cross reactivity was observed at the dilutions used for Western blot analysis. Proteins were resolved in 8% (w/v) SDS-polyacrilamide gel electrophoresis as described by Laemmli [28] 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 overnight incubation with 1:1000 dilution of the mouse anti-*Tc*PDE4 antiserum, detection was carried out by incubating with a 1:5000 dilution of a goat anti-mouse IgG labeled with peroxidase (KPL Inc., Gaithersburg, MA). The latter was developed with the ECL Plus™ Western Blotting Detection System (NEN Life Science Products Inc., Boston, MA).

2.13. Sequence analysis

The search in a *T. cruzi* and *T. brucei* genome databases (http://www.tigr.org/tdb/e2k1/tca1/ and http://www.tigr.org/tdb/e2k1/tba1/ respectively) was performed with Wu-Blast2. Sequence identity was analyzed with the BLASTP (URL http://www.ncbi.nlm.nih.gov/blast/index.html) and Clustal W (URL http://www.ebi.ac.uk/clustalw/) Multiple Sequence

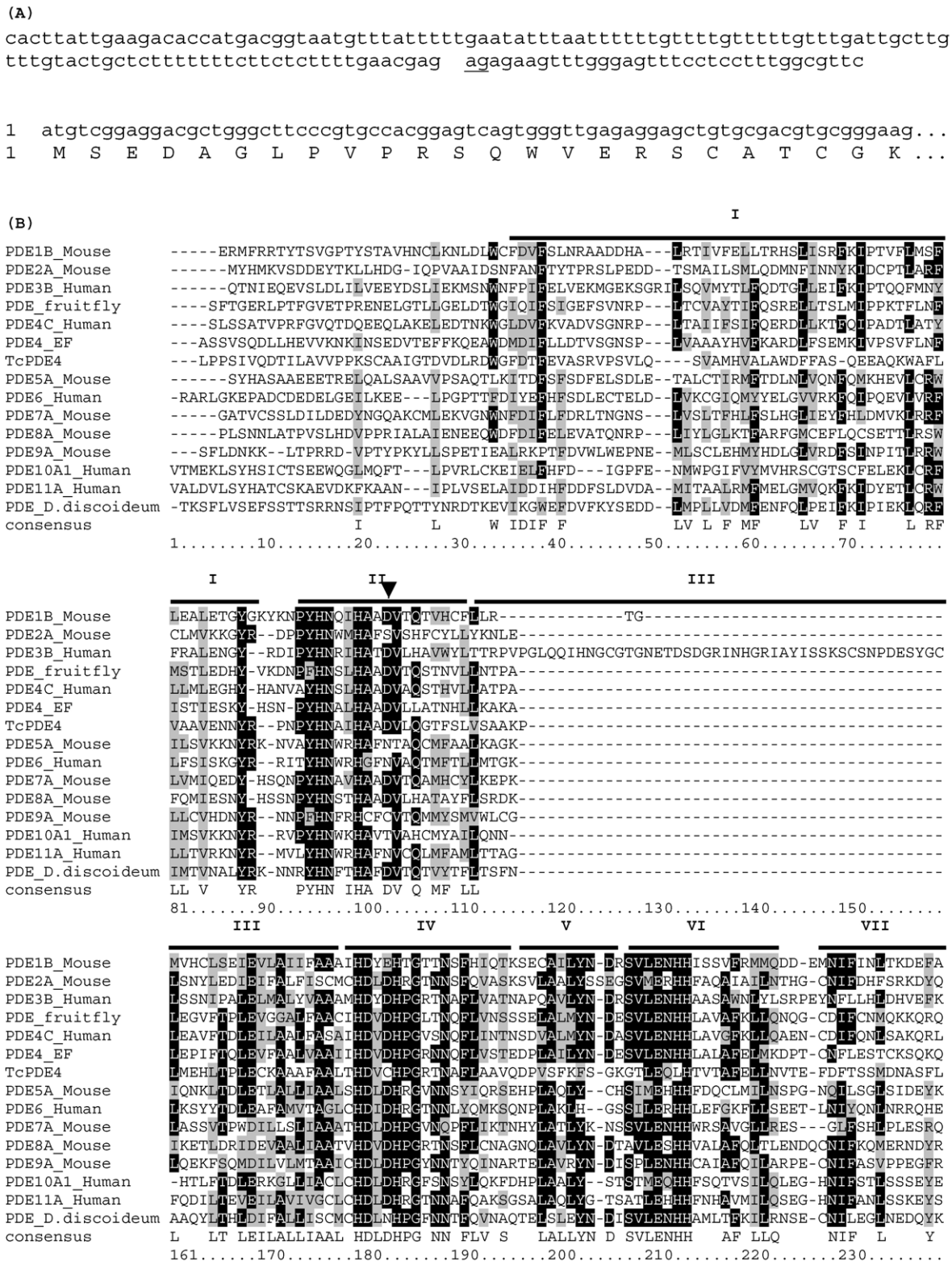

Fig. 1. (*Continued*).

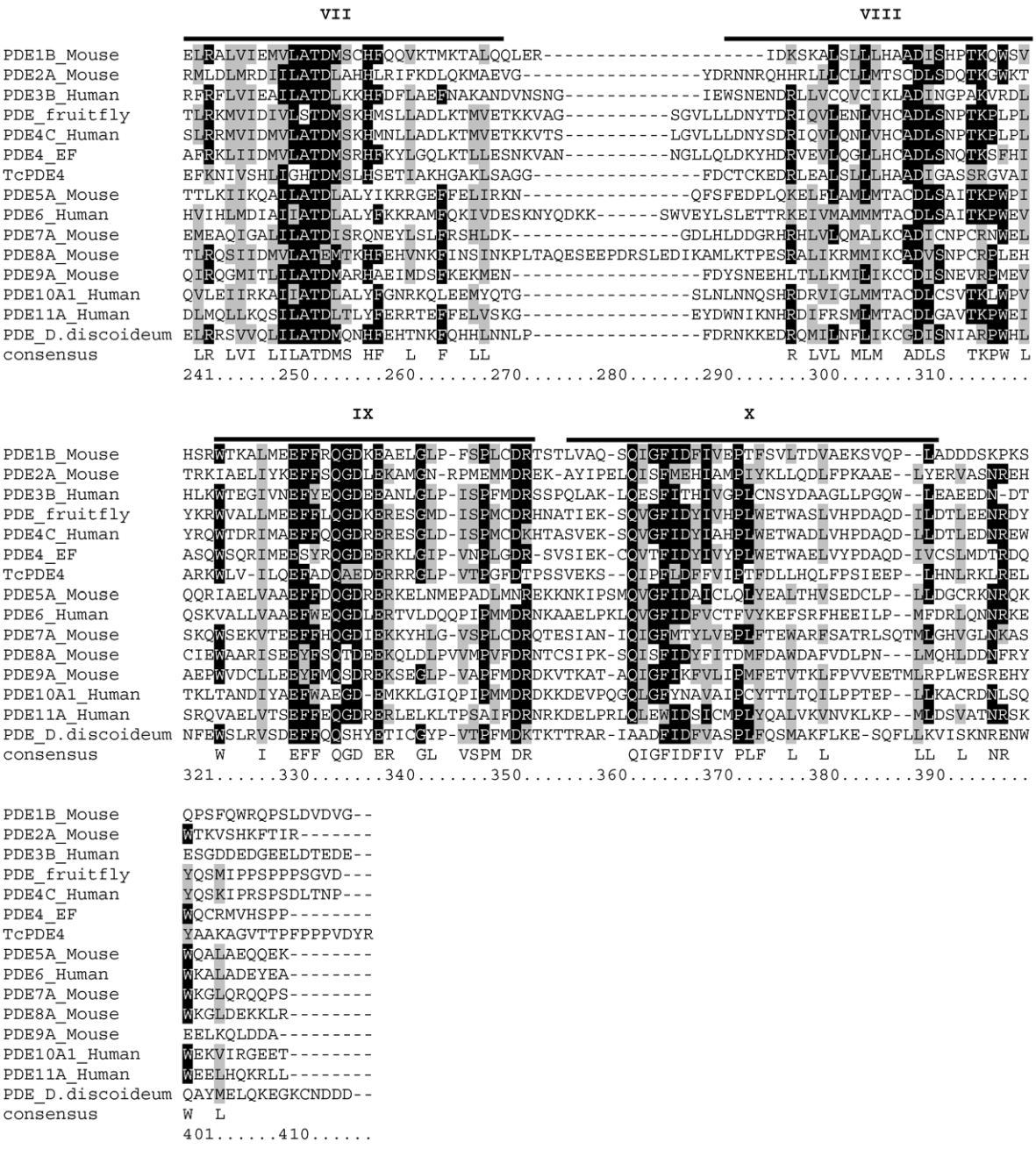

Fig. 1. Study of *Tc*PDE4 5′ non-coding neighbor and alignment of the catalytic regions of different families and species PDEs. Panel A shows *Tc*PDE4 5′ non-coding neighbor. Upstream the coding sequence is present the trans-splicing acceptor site (*ag*), and the polypyrimidine tract. Panel B present an Aligment of the catalytic regions of PDEs from different families and species. The putative subdomains common to all PDEs are denoted by roman numerals and solid lines over the sequence. (I) Subdomain present in PDE6 involved in interaction with the γ subunit. (II) Subdomain with putative zinc binding motif [H*xxx*H(*x*)25–30E]. Aspartic acid of the HAADV motif (arrow head) is conserved in all cAMP specific PDEs. On the other hand, in the cGMP specific PDEs an asparragine is observed. (III) Subdomain interrupted by the 44 amino acid insertion in PDE3. (IV) Subdomain with putative Zn binding motif. Prosite PDE signature PDOC00116 is present at this subdomain. (V) Subdomain with some homology to the RII subunit of PKA. (VI) Subdomain containing two adjacent conserved Histidines. Mutations in the second Histidine of the EXHH motif decrease or obliterate catalysis but do not affect rolipram binding in PDE4A. (VII) TD motif critical for the catalysis mainly in PDE4. (VIII) Subdomain with a highly conserved D critical for the catalytic activity of PDE5. (IX) Subdomain containing QGD motif with residues homologous to the cGMP binding in PDE5. (X) Subdomain with a conserved region poorly characterized. Sequences were aligned using the ClustalW program and edited using BOXSHADE (3.33c) software. Amino acids are colored as follows: white for different residues, black for identical residues, gray for similar and conserved residues. *Tc*PDE4 (DQ008164), PDE1B_Mouse (Q01065), PDE2A_Mouse (Q922S4), PDE3B_Human (BAA09306), PDE4_EF (BAA34310), PDE_fruitfly (S65543), PDE4C_Human (NP_000914), PDE5A_Mouse (Q8CG03), PDE6_Human (CAA46932), PDE7A_Mouse (NP_032828), PDE8A_Mouse (NP_032829), PDE9A_Mouse (AAC24344), PDE10A1_Human (AAD32595), PDE11A_Human (NP_058649), PDE_D.discoideum (AAB03508).

Alignment with default parameters setting was used to generate the alignments. Protein domains were determined using SMART (URL http://smart.embl-heidelberg.de/), and PROSITE (URL http://us.expasy.org/prosite/) software. BOXSHADE (3.33c) software was used to generate backgrounds (white for different residues, black for identical residues, gray for similar and conserved residues).

3. Results

3.1. Cloning and characterization of TcPDE4 gene

The catalytic domain of *Trypanosoma cruzi Tc*PDE1 (bases corresponding to amino acids 669–840; see Fig. 1 in Ref. [19]) was used to screen TIGR *T. cruzi* sequence databases. Two sequences matching with the catalytic center were identified. One of them coding for a 924 amino acid protein, was selected for the studies here described.

Two oligonucleotides (PDE_703_FW_pET-Bam and PDE_703_RV_Xho) designed from this sequence were used for PCR amplification of the full coding sequence from *T. cruzi* genomic DNA. A DNA fragment of the expected size (2775 bp) was obtained. After sequencing this fragment showed homology with vertebrate PDE4 subfamily members by Blast analysis. This phosphodiesterase was named *Tc*PDE4.

*Tc*PDE4 is specific for cAMP hydrolysis and sensitive to the inhibitors rolipram and etazolate (see below). Since these are two of the most relevant characteristics of mammalian PDE4 family, we decided to correlate these kinetic properties with the presence in *Tc*PDE4 of some characteristic sequence domains described in the mammalian family. A simple search for sequence homologies, at the level of the catalytic domain (amino acid position 281–623), using SMART and PROSITE, revealed a HD domain (amino acid positions 365–535), which is found in a superfamily of enzymes with phosphohydrolase activity. Also the PDEASE_I domain, characteristic of class I 3′5′-cyclic nucleotide phosphodiesterases was recognized. This domain has the signature (H-D-[LIVMFY]-*x*-H-*x*-[AG]-*x*-*x*-[NQ]-*x*-[LIVMFY]) (amino acid positions 409–420). In addition, both the sequence of the 5′ non-coding region and the trans-splicing acceptor site (ag) were identified in the RT-PCR product (Fig. 1A).

Analysis with BLASTp of *Tc*PDE4 sequence revealed homologies with members of the PDE4 family. On the other hand, SMART and PROSITE analysis indicated that there is a sequence similar to a FYVE zinc-finger domain close to the N-terminal (amino acid positions 8–74) [29]. It is interesting to point out that this zinc-finger is characteristic in proteins recruited to phosphatidylinosytol 3-phosphate-containing membranes. Three elements in these sequences are relevant: the first one, is the presence of eight cysteine residues for coordination of two Zn^{2+} atoms; the second one, is a characteristic motif: R(R/K)HHCRXCG; and the last is an arginine close to the C-terminus. In the *T. cruzi* protein some differences were evident: in the motif, the sequence was similar but not identical: AKSNCPCCG, and we found a lysine instead of an arginine close to the C-terminus.

Fig. 1B shows the alignment of the catalytic domains of PDEs from different species. The putative 10 subdomains common to all PDE catalytic domains (see Ref. [30] for details) are denoted by roman numerals and solid lines. An important element found in all PDEs is the presence of a putative zinc binding motif of the type HxxxH(x)$_{25–30}$E in subdomain II. It is known that replacement of the two histidines from this motif reduces the catalytic activity and rolipram binding [31]. In vertebrate PDEs, another interesting motif in subdomain II is that with the sequence HAADV, where replacement of D (arrow) abolishes rolipram binding [32].

3.2. Southern and Northern blot analysis

T. cruzi genomic DNA was digested with four endonucleases that cut outside the gene and three that cut inside the gene; the blot was hybridized with a probe corresponding to the first 1070 nucleotides of the *TcPDE4* coding region. Results suggest that *Tc*PDE4 is encoded by a single-copy gene (Fig. 2A). On the other hand, Northern blot analysis of *T. cruzi* total RNA, using the same fragment as a probe, revealed only one hybridization band (Fig. 2B).

3.3. Yeast phosphodiesterase-deficient strain complementation assay

To analyze whether the *TcPDE4* gene encodes a functional phosphodiesterase, the *TcPDE4* coding region was amplified by PCR and subcloned in the yeast expression vector pADNS. This construction was used to transform J106 yeast strain which lacks the endogenous PDEs genes and is sensitive to heat shock at 55 °C. Both *TcPDE4*-transformed yeasts (TcPDE4) and the *TcPDE1*-transformed yeasts (TcPDE1), which was used as a control, were resistant to a 30 min heat shock at 55 °C (Fig. 3). On the other hand, untransformed yeast (J106) or the ones transformed with the empty vector (pADNS) were not able to rescue the sensitive phenotype.

3.4. Membrane localization of recombinant TcPDE4

The presence of *Tc*PDE4 in membranes of the recombinant yeasts was studied by Western blot analysis. A band of expected molecular weight (103 kDa) was detected in yeast extracts using a polyclonal antiserum raised against the recombinant *Tc*PDE4 expressed in *E. coli*. The recognized band was present in the membrane fraction, and was not detected in the supernatant fluid (Fig. 3C), confirming the association of the enzyme to membrane fractions.

To investigate whether *Tc*PDE4 represented a peripheral or an integral membrane protein, membrane extracts from recombinant yeast were subjected to sequential washes with 1 M NaCl, 0.1% sodium cholate, 0.5% sodium cholate and 8 M urea for 30 min. As shown in Fig. 3C, small amounts of *Tc*PDE4 were released after treatment with 0.5% sodium cholate and 8 M urea, but most of the protein remained associated to membranes.

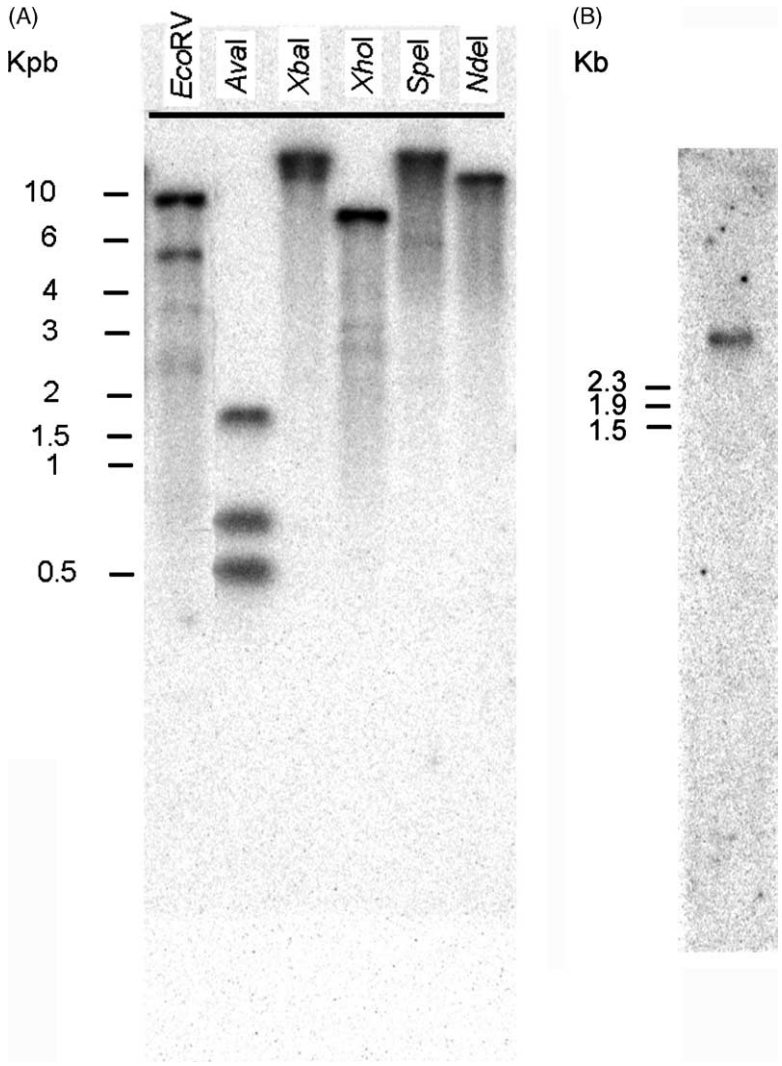

Fig. 2. Southern and Northern blot analysis of *Tc*PDE4 gene and mRNA. (A) Southern blot: 5 μg of *T. cruzi* genomic DNA digested with the indicated endonucleases, was electrophorezed, blotted and hybridized with a probe corresponding to the first 1070 nucleotides of the coding region. *Xba*I, *Xho*I, *Spe*I, and *Nde*I do not cut within the *TcPDE4* gene whereas; *Eco*RV cut at base position 833, and *Ava*I at positions 338, 782 and 2408. (B) Northern blot: 30 μg of total RNA from epimastigotes of *T. cruzi* was electrophorezed in agarose-formaldehyde gels, blotted and hybridized with the same probe described above. Ribosomal RNA molecular masses are indicated.

Table 1
Effect of PDE-specific inhibitors on *Tc*PDE4

Inhibitor	Family PDE specificity	Mammalian IC50 (μM)	IC50 *Tc*PDE4 (μM)	Inhibitor range
Vipocetine	1	20	>500	1–500 μM
cGMP	3	–	NA	20 μM–2 mM
Trequinsin	3	8	1.958	100 nM–500 μM
Milrinone	3	39	>500	1–500 μM
Imazodan	3	192	>500	1–500 μM
Etazolate	4	13.9	1.3	10 nM–50 μM
Rolipram	4	2	10.46	500 nM–1 mM
Zaprinast	5, 6, 9	0.45, 0.15, 35	728	500 nM–750 μM
Dipyridamole	5, 6, 8, 10	0.9, 0.38, 4.5, 1.1	0.146	10 nM–10 μM
Papaverine	NS	5, 25	12.88	500 nM–1 mM
IBMX	NS	2, 50	126.2	1 μM–1 mM
Theophylline	NS	–	>500	1–500 μM
EHNA	NS	0.8	>500	1–500 μM

IC50 values for each compound were determined using 10 μg of recombinant *Tc*PDE4, 50 μM cAMP and at the indicated concentration range of each compound. NS: non-selective and NA: not affected activity.

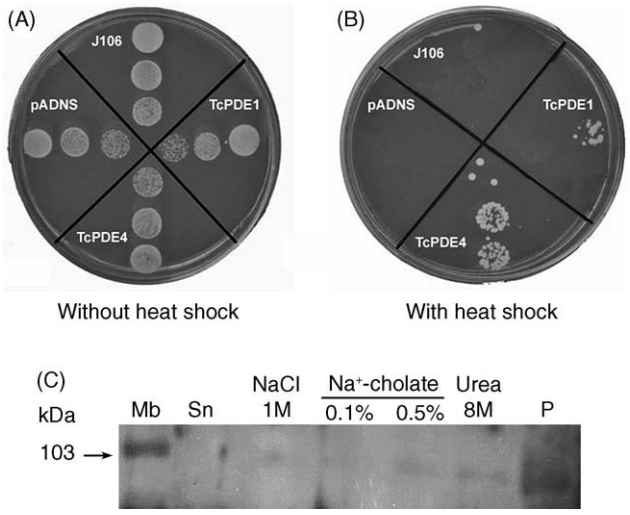

Fig. 3. Complementation of a yeast PDE-deficient strain by *Tc*PDEs and membrane association of recombinant *Tc*PDE4. The heat shock sensitive yeast strain J106, deficient in the PDE activity, was transformed (pADNS) or not (J106) with the pADNS empty vector or carrying the full-length *TcPDE4* or *TcPDE1* gene. Transformed cells were grown to OD_{600} = 1.8–2, and subjected (B) or not (A) to a heat-shock for 30 min at 55 °C. Different dilutions were plated in YPD medium. Panel C, shows the membrane association of recombinant *Tc*PDE4. The association of *Tc*PDE4 with membranes was analyzed by Western blot assay. Proteins of soluble (Sn) or membrane (Mb) extracts (30 μg) were resolved by SDS-PAGE (8% gels), electrotransferred on to Hybond C membranes and revealed with *Tc*PDE4-specific antiserum. Furthermore, yeast recombinant membranes were extracted to release *Tc*PDE4 by sequential washes with 1 M NaCl, 0.1 or 0.5% (w/v) sodium cholate, or 8 M urea. P, represent the post-extracted pellet.

3.5. Kinetic characterization of TcPDE4

The activity of *Tc*PDE4 was further characterized in recombinant yeast membranes. Recombinant *Tc*PDE4 showed a specific activity of about 4.4 nmol 5′AMP/min per mg protein, with a saturable dependence on and a high affinity for cAMP. Km value for this substrate was about 20 μM (Fig. 4A). The enzyme required Mg^{2+} for full activity and was completely blocked by EDTA. Mn^{2+} was less efficient than Mg^{2+} and Ca^{2+} was totally inactive (Fig. 4B).

Table 1 shows the effect of a variety of compounds on *Tc*PDE4. In a wide range of concentrations (20 μM to 2 mM), cGMP did not affect enzyme activity. This is indicative that this cyclic nucleotide is neither a substrate competing with cAMP nor an enzyme activator. In addition, two compounds, rolipram and etazolate, which are highly selective blockers of vertebrate PDE4 subfamily members, inhibited *Tc*PDE4. On the other hand, trequinsin, which is specific for PDE3 subfamily members, and dipyridamole, which has a wider range of selectivity, also inhibited of *Tc*PDE4. Other known selective PDE inhibitors did not affected *Tc*PDE4 activity.

The presence of soluble Ca^{2+}-calmodulin-stimulated cAMP phosphodiesterase activity has been reported in *T. cruzi* [10]. Both Ca^{2+} and Ca^{2+}-calmodulin complex were not able to stimulated *Tc*PDE4 activity in membranes (Fig. 4B). In addition calmodulin blocker chlorpromazine was not able to inhibit it (data no shown). This is in agreement with the fact that *Tc*PDE4 lacks domains involved in the interaction with this complex.

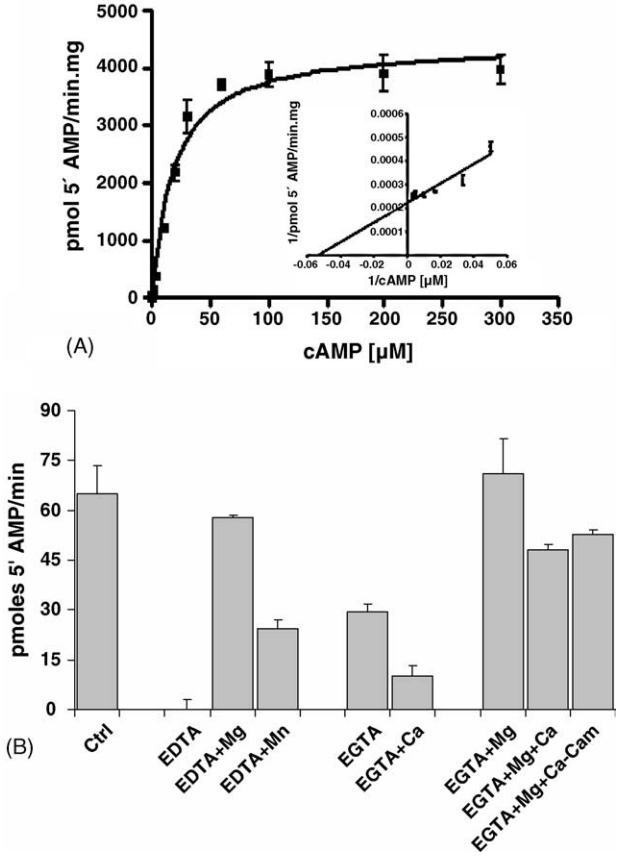

Fig. 4. Kinetic parameters and ion dependence of the recombinant *Tc*PDE4. cAMP-PDE assays were performed with membranes purified from *Tc*PDE4 recombinant yeasts using 10 μg of protein. Panel A, shows *Tc*PDE4 activity as a function of cAMP concentration. Inset in panel A, shows lineweaver burk plot. The calculated kinetic parameters were: K_m about 20 μM and V_{max} 4456 pmol 5′AMP/min mg. Panel B, present the ion dependence on *Tc*PDE4. cAMP-PDE assays were performed with membranes purified from *Tc*PDE4 recombinant yeasts using 10 μg of protein. Chelators were used at a final concentration of 1 mM and the different ions were added to reach a final concentration of 5 mM. The effect of Ca^{2+} on *Tc*PDE4 activity was tested in the absent or present of 0.05 units of calmodulin. Standard assay conditions were used as a control.

4. Discussion

In this work, we identified and characterized *Tc*PDE4, a membrane-associated cAMP-specific phosphodiesterase of *Trypanosoma cruzi*.

We found that *Tc*PDE4 is a single copy gene and that its sequence resembles mammalian type 4 phosphodiesterases. In addition, we recognized three conserved domains in *Tc*PDE4: FYVE, phosphohydrolase and PDEaseI. The FYVE zinc finger domain is a unique and relevant characteristic of this sequence. It is a conserved domain characterized by its ability to bind specifically and with high affinity to phosphatidilinositol 3-phosphate [33], a phosphoinositide that is known to be enriched in early endosome membranes [29]. Different membrane treatments, followed by Western blot analysis, confirmed that *Tc*PDE4 is strongly associated with membranes. The other phosphodiesterase characterized in *T. cruzi* by our laboratory is localized in the plasma membrane in association with the flagellar apparatus [19].

Yeast strains lacking the phosphodiesterases genes PDE1 and PDE2 have been widely used for the identification and characterization of many cAMP phosphodiesterases [34]. This system allows the study of a unique PDE isoform independently of other isoforms that could be present in the cell. Complementation assays in yeast PDE-deficient strains indicated that *Tc*PDE4 is a functional phosphodiesterase.

Interestingly, *Tc*PDE4 presented all the sequence characteristics of a cAMP-specific, rolipram-sensitive phosphodiesterase. In addition, the biochemical characterization of *Tc*PDE4 supported our sequence analysis data, revealing a specific-cAMP phosphodiesterase with a Km value of about 20 μM, which is slightly higher than that of high affinity phosphodiesterases (Km values from 0.5 to 10 μM). Yeast recombinant *Tc*PDE4 showed two-fold more activity in the presence of Mg^{2+} than in the presence of Mn^{2+}. Furthermore, Ca^{2+}, calmodulin and the phenotiazinic inhibitor chlorpromazine had no effect on *Tc*PDE4 activity, thus demonstrating that *Tc*PDE4 is not regulated by these compounds.

Recently, the complete genome sequence of *Trypanosoma cruzi* was published [35]. It is interesting that only six open reading frames were identified as phosphodiesterases in *T. cruzi* [35; Supporting Online Material Table S5].

The use of phosphodiesterase-specific inhibitors as therapeutic agents for the treatment of many diseases has long been known [36]. In *T. brucei*, some of these compounds prevent cell proliferation in culture [15]. For this reason, the search for inhibitors that could block the activity of a *T. cruzi* cAMP phosphodiesterase is of great importance because they could eventually be used as a novel therapy for the treatment of Chagas' disease. The characterization of the individual isoforms present in the parasite should be an essential requirement in this approach. The fact that *Tc*PDE4 shows specific and high affinity inhibitors, such as rolipram and etazolate, may open interesting therapeutic possibilities.

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