Characterization of TbPDE2A, a Novel Cyclic Nucleotide-specific Phosphodiesterase from the Protozoan Parasite Trypanosoma brucei*

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This study reports the identification and characterization of a cAMP-specific phosphodiesterase from the parasitic hemoflagellate Trypanosoma brucei. TbPDE2A is a class I phosphodiesterase. Its catalytic domain exhibits 30–40% sequence identity with those of all 11 mammalian phosphodiesterase (PDE) families, as well as with PDE2 from Saccharomyces cerevisiae, duance from Drosophila melanogaster, and regA from Dictyostelium discoideum. The overall structure of TbPDE2A resembles that of human PDE11A in that its N-terminal region contains a single GAF domain. This domain is very similar to those of the mammalian PDE2, -5, -6, -10, and -11, where it constitutes a potential cGMP binding site. TbPDE2A can be expressed in S. cerevisiae, and it complements an S. cerevisiae PDE deletion strain. Recombinant TbPDE2A is specific for cAMP, with a $K_m$ of $\leq 2 \mu M$. It is entirely resistant to the nonselective PDE inhibitor 3-isobutyl-1-methylxanthine, but it is sensitive to trequinsin, dipyridamole, sildenafil, and ethaverine with $IC_{50}$ values of 5.4, 5.9, 9.4, and 14.2 $\mu M$, respectively. All four compounds inhibit proliferation of bloodstream form trypanosomes in culture, indicating that TbPDE2A is an essential enzyme.

Cyclic nucleotide-specific phosphodiesterases represent a large and divergent group of enzymes. In eukaryotes, two classes of phosphodiesterases (PDEs)$^{1}$ have been recognized (1, 2). Class I includes all currently known families of mammalian PDEs (see below), as well as a number of PDEs from lower eukaryotes, such as PDE2 from the yeast Saccharomyces cerevisiae or the product of the regA gene of Dictyostelium discoideum. All class I enzymes show a considerable extent of amino acid sequence conservation within their catalytic domains. In contrast, the class II PDEs show little sequence similarity to the class I enzymes, and their $K_m$ are generally much higher than those of the class I enzymes. Class II PDEs were identified (e.g. in S. cerevisiae (1), D. discoideum (3), and Candida albicans (4)).

Class I PDEs have been studied particularly well in mammals, where 11 distinct PDE families have been identified to date, based on their substrate specificities, kinetic properties, allosteric regulators, inhibitor sensitivities, and amino acid sequences. Each family exhibits >50% amino acid sequence identity within the conserved catalytic domain of about 250 amino acids length, while sequence identity between different families is only 30–40% in the same region (5). Family members also share extensive similarity in regions outside the catalytic domain, while no significant similarity of that region can be detected between different families. Besides their amino acid sequences, the different families also display distinctive pharmacological properties, which form the basis for the development of family-specific PDE inhibitors for clinical use (5–9). Genetic defects in PDE genes have been suspected as the underlying causes of a number of diseases (10–13), although the relationship between PDE genes and disease is clearly established only between PDE6 and some forms of retinal degenerative disease (11).

PDEs have become highly attractive targets for drug development over the last few years. A growing number of family-specific and subtype-specific inhibitors have been developed despite the considerable sequence conservation between the catalytic domains of different families. Several PDE inhibitors are being used or are under exploration for ailments as diverse as autoimmune disease (14, 15), arthritis (16), asthma (17, 18), and impotency (19, 20) and as anti-inflammatory agents (21). The ongoing development of new and ever more subtype-specific inhibitors holds great promise for achieving more specific drug action with fewer side effects as well as new areas for the application of PDE inhibitors. In view of the potential of PDE inhibitors as chemotherapeutics, it is surprising how little is currently known about PDEs of parasites as possible drug targets.

The African trypanosome Trypanosoma brucei is a eukaryotic microorganism that causes the fatal human sleeping sickness (22), as well as Nagana, a devastating disease of domestic animals in large parts of sub-Saharan Africa. Chemotherapy of human trypanosomiasis is in a dismal state (23). New drugs and drug targets are urgently required, and the cyclic nucleotide-specific PDEs may constitute a class of new drug targets. cAMP signaling in trypanosomes is still largely unexplored (24, 25). cAMP is involved in the regulation of differentiation of bloodstream form trypanosomes from the proliferative “long slender” forms to the insect-preadapted, nonproliferative “short stumpy” forms (26). Several gene families for adenyl cyclases...
have been identified in *T. brucei* (24, 27, 28). Even less is known about trypanosomal phosphodiesterases. An early study demonstrated PDE activity in cell lysates of bloodstream form *T. brucei* (29), and experiments with PDE inhibitors suggested that interference with PDE activity might affect cell differentiation (26, 30). A recent report (31) described a PDE activity in cultured *Leishmania mexicana*. This enzyme is partly soluble and partly particulate, and its characteristics indicate that it may represent a class II PDE.

The current study describes the identification and characterization of a cAMP-specific PDE from *T. brucei* (ThPDE2A) with considerable amino acid sequence similarity to the class I PDEs. This classification is further supported by its low *Km* for cAMP as a substrate. In its N terminus, ThPDE2A contains a single GAF domain (32), which may represent a noncatalytic cGMP-binding site. ThPDE2A is a representative of a small, dispersed family of similar, but nonidentical genes. It can be expressed in the yeast *S. cerevisiae*, and it complements the heat-shock susceptibility phenotype of a *PDE1/PDE2* deletion strain. Inhibitor studies showed that ThPDE2A is fully resistant to most inhibitors tested, including broad spectrum inhibitors such as IBMX. However, the enzyme is sensitive to the family 5 inhibitors dipyridamole and sildenafil, the family 3 inhibitor trequinsin, and the calcium-channel blocker ethaverine.

**EXPERIMENTAL PROCEDURES**

*Materials*—Radiochemicals were purchased from PerkinElmer Life Sciences. Barium hydroxide solution was purchased from Sigma. PDE inhibitors were from the following sources: IBMX, zaprinast, ethaverine, papaverine, theophylline, milrinone, trequinsin, vinpocetine, 8-methoxymethyl-IBMX, dipyridamole, and rolipram were from Sigma; erythro-9-(2-Hydroxy-3-nonyl) adenine, HCl, zaritidine, clobamide, and Ro-20–1724 were from BioMol (Plymouth Meeting, PA); ezetilone and pentoxifylline were from Calbiochem; and sildenafil citrate was a gift from Pfizer Central Research (Sandwich, Kent, UK). DNA sequencing was outsourced to Microsynth GmbH (Balghach, Switzerland). Reactions were run with BigDye terminators (PE-Biosystems) and were analyzed on an ABI Prism 377 instrument.

*Cell Culture*—Trypanosoma brucei brucei strain 427 (derived form MiTat 15a) was grown as procyclic form at 27 °C in SDM medium (33). Monomorphic bloodstream forms of strain 221 (MiTat 1.2) were cultivated as described by Hesse et al. (34). The yeast strain 255 (MATa leu2-3 leu2-112 ura3-52 his3-52 his4 cam pde1::URA3 pde2::HIS3; Ref. 35) was a gift of John Colicelli (UCLA). Yeast transformation was done as described (36). Transfected were selected on synthetic minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose, supplemented with an amino acid mixture lacking leucine (SC-leu). Heat shock experiments were performed by replicating patches onto YPD plates prewarmed to 55 °C, and the heat shock was continued for 15 min. After cooling the plates to room temperature, they were incubated for 2–3 days at 30 °C.

**ThPDE2A Constructs**—Screening of the *T. brucei* EST data base for class I PDE homologs identified plasmid pT2928 (accession number W84978) as coding for a potential *T. brucei* PDE. The cDNA sequence of pT2928 was completely sequenced and shown to represent the catalytic domain and the complete 3′-untranslated region of a candidate phosphodiesterase. This gene was termed ThPDE2A. The cDNA of pT2928 was then used to screen a genomic library of *T. brucei* in AEMBL4 (37), and a 6.3-kilobase pair genomic DNA fragment was isolated and sequenced. The fragment contained the *ThPDE2A* gene, flanked by an unrelated gene on either side (see “Results”). Full-length ThPDE2A for recombinant expression was constructed as follows: The 3′-end of the open reading frame of ThPDE2A was amplified from the cDNA plasmid pt2928 using the forward primer pde2fjyr (5′-ATGACAATGATG-GATGGTCTTAT-3′) and the reverse primer pde2ir (5′-CTTCTCAG-GGGATCCCTATCCATAT-3′) and the reverse primer pde2ir (5′-ATGACAATGATG-GATGGTCTTAT-3′). The resulting PCR fragment (396 bp) was cloned into pGEM-T-Easy (Promega) and verified by sequencing.

For generating an N-terminally truncated form of ThPDE2A without the noncatalytic cGMP-binding domain (starting at Met124 of the full sequence), the corresponding region was amplified from genomic DNA using the forward primer pde2f1 (5′-GGAATTCCAAACATATCTGG-ATAAAGAAGCACTACATGCACTACATACATGCACACGAG-3′), containing an EcoRI site (underlined) followed by a Kozak sequence and the start codon (boldface type, underlined), and the reverse primer pde2gr (5′-TTCGACATCCAATGATGCGGAACTACATGCGAAGCAGGACGACGAGACC3′), containing an NcoI site immediately before the stop codon and introduced an NcoI site immediately before the stop codon and cloned into plasmid pTPDE23U.

The 5′-end of the open reading frame was amplified from a fragment of genomic DNA, using the forward primer pde2f (5′-GAATATCGGAAAACATATCTGG-ATAAAGAAGCACTACATGCACTACATACATGCACACGAG-3′), containing an EcoRI site (underlined) followed by a Kozak sequence and the start codon (boldface type, underlined), and the reverse primer pde2gr (5′-TTCGACATCCAATGATGCGGAACTACATGCGAAGCAGGACGACGAGACC3′), containing an NcoI site immediately before the stop codon and introduced an NcoI site immediately before the stop codon and cloned into plasmid pTPDE23U.

*For inserting a hemagglutinin tag (amino acid sequence: YPYDVP-DYAGIFPM)* at the C terminus of both constructs, two complementary oligonucleotides, Hf1 (5′-CATGGTTACCCTGACATG) and
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Cytotoxicity Determination—Cytotoxicity of PDE inhibitors was determined for bloodstream forms in culture by determining acid phosphatase activity as described (41). Exponentially growing monomorphic bloodstream forms MTTat 1.2 were transferred into colorless medium (42) (cell density 3 \times 10^7 cells/ml culture) and were seeded into microtiter wells (191-T) containing 1 ml of inhibitor or solvent control. Plates were incubated for both 20 and 40 h at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. At the end of the growth period, cells were lysed by the addition of 20 μl of lysis/substrate buffer (20 mg/ml p-nitrophenylphosphate in 1% sodium acetate, pH 5.5, 1% Triton X-100), and the incubation was continued for another 4 h at 37 °C. Production of p-nitrophenol was determined at 405 nm on a microtiter plate reader. To control for intrinsic absorbance by the inhibitors, control series containing inhibitor dilutions but no cells were run for every experiment, and the resulting absorbance values were subtracted as background from the experimental readings. All assays were run in triplicates.

Results

The TbPDE2A Locus—Upon searching the T. brucei expressed sequence tag data base for potential phosphodiesterase genes, an expressed sequence tag clone (pT2928) was identified. The corresponding plasmid was obtained (courtesy Philip Majiwa, ILRI) and sequenced. The cDNA fragment contained the 3′-part of a cDNA that unambiguously represented a phosphodiesterase gene, termed TbPDE2A according to the recently proposed rules for the nomenclature of trypanosomatid genes (43). Southern blot analysis of genomic DNA demonstrated that TbPDE2A is not a single gene but rather a member of a small gene family (Fig. 2A). This was further confirmed by screening a procyct cDNA library, which resulted in the identification of several cDNA clones that represent different PDE2 family members (unpublished results). The cDNA fragment from pT2928 was then used to screen a genomic library of T. brucei, and the TbPDE2A locus was recovered on a 6-kb pair genomic EcoRI DNA fragment. The fragment was sequenced, as were several cDNA clones for TbPDE2A. The organization of the TbPDE2A locus (Fig. 2B) demonstrates that it contains three different, closely spaced genes. The first one is a RIME element (nucleotides 376–876), a member of a family of abundant, highly transcribed, repetitive transposable elements (44). Within this element, nucleotides 868–632 on the reverse strand represent the open reading frame coding for a RIME-associated protein. The RIME element is flanked by two 12-bp direct repeats (nucleotides 364–375 and 877–888). The open reading frame for TbPDE2A extends from nucleotide 1770 to 3225 and codes for a protein of 485 amino acids. The predicted initiator methionine codon was functional, and the predicted open reading frame coded for an active protein when expressed in S. cerevisiae (see below). The coding region is followed by a long 3′-untranslated region of 1196 nucleotides, and the poly(A)-addition site is represented by nucleotide 4420.

Downstream of the TbPDE2A gene, a gene for a member of the

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3 U. Bauer, unpublished data.
NHP2/RS6 family of nuclear proteins (45) is coded for by nucleotides 4635–5062. This is the first instance where such a gene was detected in *T. brucei*, and the role of the corresponding protein remains unknown. The presence of unrelated genes upstream and downstream of *TbPDE2A* demonstrated that the members of this PDE family are not closely linked.

Expression of *TbPDE2A* was analyzed both by Northern blot hybridization and by reverse transcriptase-polymerase chain reaction. Both approaches demonstrated that *TbPDE2A* is expressed both in the bloodstream and the procyclic (insect stage) form of the trypanosome life cycle (data not shown).

**Predicted Amino Acid Sequence of *TbPDE2A***

The open reading frame of *TbPDE2A* predicts a protein of 485 amino acids, with a calculated molecular mass of 55,348 (Fig. 3). The N terminus of *TbPDE2A* contains a single GAF domain (Val^3–Val^117; Ref. 32), which may function in cGMP binding. The presence of a single GAF domain in *TbPDE2A* is reminiscent of the human PDE11A, which also has a single GAF domain, while all other mammalian PDEs with such domains (PDE2, -5, -6, and -10) contain two of them in a closely spaced arrangement. The overall sequence identity between the single GAF domain of *TbPDE2A* and either of the corresponding domains of mammalian PDE2, -5, -6, -10, and -11 varies between 30 and 50%, with several residues (Leu^59, Cys^60, Pro^62, Asn^77, Lys^78, Phe^88, and Asp^91) strongly or absolutely conserved.

For mammalian PDE5A, where cGMP-binding by the GAF domain was experimentally demonstrated, the interaction with cGMP was predicted to occur via Asn^77, Lys^78, and Asp^91, all of which are strongly conserved (46).

The catalytic domain of *TbPDE2A* is located between Phe^205 and Phe^438, as predicted by analogy with other PDEs. All class I PDEs, *TbPDE2A*, mammalian PDE1, version number U40372, PDE2 (U21101), PDE3 (M91667), PDE4 (ST5213), PDE5 (NM_0001083), PDE6 (NM_000283), PDE7 (U68171), PDE8 (AF068247), PDE9 (AF031147), PDE10 (AF127479), *D. melanogaster* dunce (P12252), *S. cerevisiae* PDE2 (M14653), and *D. discoideum* RegA (U60170). Amino acids in the gray box (His^269–Tyr^281) represent the phosphodiesterase signature motif (47).
enzyme and the truncated form fully restored heat shock resistance of the indicator strain, indicating that TbPDE2A is active in *S. cerevisiae* and that the N-terminal domain is not required for the activity of the catalytic domain. Two promoters of different strengths were used for these expression experiments (an attenuated form of CYC1 as a weak promoter and TEF2 as a strong promoter), but essentially identical results were obtained. Thus, minimal amounts of TbPDE2A are apparently sufficient to rescue the heat shock resistance phenotype of the PP5 strain.

**Characterization of TbPDE2A Activity**—For the characterization of the catalytic activity of TbPDE2A, the enzyme was expressed in the PDE-deficient yeast strain PP5, using plasmid pLT1 with the strong TEF2 promoter. TbPDE2A was expressed either as the full-length enzyme or in its N-terminally truncated form (amino acids 124–485), which lacks the GAF domain. To be able to monitor protein expression and stability, both constructs contained a hemagglutinin tag at their C termini. *In vivo* activity of all constructs was first assessed by analysis of the heat shock phenotype conferred to the host strain, and stability under assay conditions was monitored by immunoblotting with an anti-hemagglutinin antibody.

Both constructs exhibited very similar activities with cAMP as the substrate, with a $K_m$ in the range of 2 $\mu M$ and a $V_{\text{max}}$ of 1 $\mu M \times \min^{-1} \times \text{mg}$ (Table I). These $K_m$ values are well within the range of other class I PDEs. With both constructs, cAMP hydrolysis was unaffected by the presence of a 100-fold excess of cGMP in the reaction (data not shown). This observation defines the catalytic activity of TbPDE2A as that of a cAMP-specific phosphodiesterase. In addition, it indicates that cGMP either does not bind to the GAF domain or that such a binding does not directly influence the catalytic activity of the enzyme under the conditions of our assay.

**Inhibitor Profile of TbPDE2A**—Inhibitor studies were performed on lysates from PP5 expressing the full-size TbPDE2A. For the initial screening, all inhibitors were used at a concentration of 100 $\mu M$, with a substrate concentration of 1 $\mu M$ cAMP. Only a few of the inhibitors tested exhibited a significant effect on enzyme activity (trequinsin, ethaverine, sildenafil, and dipyriddamole), even at the high concentration used for the screen. Several others (ethazolate, erythro-9-(2-hydroxy-3-nonyl)adenine, zardaverine, pentoxyfylline, 8-methoxy-IBMX, and vinpocetine), showed no significant effect on enzyme activity (trequinsin, ethaverine, sildenafil, and dipyriddamole). Interestingly, the linker region between the cGMP-binding domain and the catalytic domain contains a phosphorylation site for cAMP/cGMP kinases ([Lys$^{144}$–Thr$^{147}$]). The putative nucleotide-binding domain and the catalytic domain contains a vicinal histidine residues ([Lys$^{144}$–Thr$^{147}$]), which are essential for the activity of the full-length enzyme.

**Comparison of Mr and enzyme parameters of full-size (TbPDE2A) and N-terminally truncated (TbPDE2AT) phosphodiesterase**

|            | $M_r$ | $K_m$ (M) | $V_{\text{max}}$ (1/min/µg) |
|------------|-------|-----------|-----------------------------|
| TbPDE2A    | 55,313| 2.28 ± 0.56| 1.17 ± 0.20 |
| TbPDE2AT   | 41,248| 1.18 ± 0.26| 0.81 ± 0.14 |

**Fig. 4. Heat shock resistance.** The heat shock-sensitive PDE deletion strain of *S. cerevisiae*, PP5, was transformed with plasmids containing a weak promoter (attenuated CYC1; series 1) or a strong promoter (TEF2; series 2) and expressing the following constructs: N-terminally truncated TbPDE2A containing a C-terminal hemagglutinin tag (a); full-size TbPDE2A, containing a C-terminal hemagglutinin tail (b); empty vector (c); full-size TbPDE2A containing a C-terminal Ty1-tag (d). A, control plate without heat shock; B, plate with heat shock. Two or three independent clones were tested for each construct.
potency of these inhibitors toward TbPDE2A is not correlated with their family specificity for mammalian PDEs (Table II). Trequinsin is an inhibitor of the PDE3 family; dipyridamole inhibits families 5, 6, 9, 10, and 11 (7); and sildenafil is quite specific for family 5. Ethaverine was not known so far as a PDE inhibitor.

The four compounds were further analyzed for their effects on cell growth in culture. Bloodstream form trypanosomes were grown in microtiter plates for 20 or 40 h in the presence of serial dilutions of the inhibitors (Fig. 6), and cell proliferation was determined by an acid phosphatase-based assay (41). All four compounds inhibited trypanosome growth with IC50 values that were about 10-fold higher than those determined with the soluble enzyme. The Hill slopes of the dose-response curves were close to 1 for three of the compounds (dipyridamole: 1.38 ± 0.21; sildenafil: 1.73 ± 0.69; and trequinsin: 1.09 ± 0.63), while it was 5.19 ± 1.52 for ethaverine. This indicates that the observed inhibition of cell proliferation by the first three compounds is indeed due to the inhibitory effect of the compounds on PDE activity, while the inhibition by ethaverine may be due to the combined effects of calcium channel blocking and inhibition of PDE activity. The results obtained with the first three compounds indicate that the activity of TbPDE2A, and possibly other members of this family, is essential for trypanosome proliferation in culture.

Inhibition of TbPDE2A Raises the Intracellular cAMP Concentration—Inhibiting PDE activity in vivo should raise the intracellular cAMP concentration. To verify if the inhibitors tested above do in fact inhibit trypanosomal PDEs in vivo, trypanosomes were incubated with sildenafil (200 μM), trequinsin (50 μM), dipyridamole (100 μM), or ethaverine (100 μM) for 30 and 120 min. Control cells were incubated for the same lengths of time without drug. Cell morphology and motility were normal under these conditions. Cells were collected and processed for cAMP determination as detailed under "Experimental Procedures." cAMP concentrations were normalized to the values of the control cells at the respective time points. The results given in Fig. 7 demonstrate that trequinsin, sildenafil, and dipyridamole, but not ethaverine, lead to an increase in the intracellular cAMP concentration, although the extent of this increase varies between the different inhibitors. The cAMP concentration of control cells (66 ± 14 pmol/109 trypanosomes) corresponds very well with the values obtained earlier with another assay procedure and in different trypanosome strains (26).

DISCUSSION

The current study reported the identification and characterization of a member of a small family of cAMP-specific phosphodiesterases from T. brucei. This is the first report of cloning

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**Table II**

| Inhibitor     | Mammalian PDE family inhibited | IC50 for TbPDE2A (μM) |
|---------------|-------------------------------|-----------------------|
| Trequinsin    | 3                             | 5.4                   |
| Dipyridamole  | 5 and 6                       | 5.9                   |
| Sildenafil    | 5                             | 9.4                   |
| Ethaverine    | 14                            | 14.2                  |
| Etazolate     | 4                             | 30.3                  |
| Zaprinast     | 5 and 6                       | 42.4                  |
| IBMX          | Nonselective                  | 545                   |
| Cilostamide   | 3                             | >100                  |
| Rolipram      | 4                             | >100                  |
| Theophylline  | Nonselective                  | >100                  |
| Vinpocetine   | 1                             | >100                  |
a gene for a phosphodiesterase from a parasitic protozoon. TbPDE2A is coded for by a gene that represents a small family of related but different genes. DNA sequence analysis of the locus revealed the presence of genes unrelated to phosphodiesterases upstream and downstream of the open reading frame for TbPDE2A, demonstrating that the genes of this PDE family are not clustered. The open reading frame predicts a protein consisting of 485 amino acids, with a molecular mass of 55,313. The predicted start codon is functional, as demonstrated by expression of the recombinant protein in S. cerevisiae, and no potential extension of the open reading frame upstream of this start codon is predicted from the DNA sequence. The open reading frame codes for a protein with a C-terminal catalytic domain with strong homology to class I PDEs. The extent of sequence conservation and the inhibitor profile unambiguously classify TbPDE2 as a new family of the class I PDEs.

Fig. 7. cAMP content of trypanosomes in the presence of TbPDE2A inhibitors. Intracellular cAMP was determined in HCl extracts of bloodstream trypanosomes incubated in growth medium in the presence of inhibitors for 30 or 120 min. Control cells were incubated without inhibitors for the same lengths of time. The figure presents one of two independently determined, very similar data sets. Gray boxes, 30-min incubation; black boxes, 120 min incubation; asterisks, cAMP concentration significantly different from control values (p < 0.02). T. cruzi

Phosphodiesterase from T. brucei

Analysis of recombinant TbPDE2A demonstrated that it is a cAMP-specific phosphodiesterase with a Km for cAMP in the 2 μM range. This Km is typical for many of the class I PDEs. It is also in agreement with the available estimates of the intracellular concentration of cAMP in T. brucei (1–10 μM; Ref. 26). Recombinant proteins with or without the GAF domain exhibited similar activities with cAMP as a substrate, and the activity of both constructs was not affected by the presence of excess cGMP. These observations confirm that TbPDE2A is a cAMP-specific phosphodiesterase and that cGMP either does not bind to the GAF domain or that such a binding does not directly affect its catalytic activity. Thus, the GAF domain may be involved in the interactions with other components of the cell.

TbPDE2A displays a unique pharmacology that sets it apart from all previously characterized PDE families. IBMX and theophylline, two nonselective inhibitors of most PDEs, are not effective on TbPDE2A. Three compounds that were found to inhibit TbPDE2A at the low micromolar level are specific inhibitors of different mammalian PDE families. Trequinsin (IC50 for TbPDE2A = 5.4 μM) is an inhibitor of family 3, and dipyridamole (IC50 = 5.8 μM) is an inhibitor of the mammalian families 5 and 6, as is sildenafil (IC50 = 9.4 μM). However, the IC50 values of trequinsin and sildenafil for TbPDE2A are much higher than those observed with mammalian PDEs. Furthermore, at a concentration of 5 μM, trequinsin is likely to also inhibit PDE3 and PDE4. In conjunction, the current data indicate a pharmacological profile of TbPDE2A that is unlike that of any of the mammalian PDE classes. TbPDE2A is completely insensitive to the methylxanthine inhibitors; it is inhibited by the class 3 inhibitor trequinsin, although at rather high concentrations, while it is not affected by other class 3 inhibitors such as cilostamide. In addition, TbPDE2A is sensitive to ethaverine (IC50 = 14 μM), a derivative of the nonspecific inhibitor papaverine, which exerts only marginal activity against TbPDE2A. This finding was unexpected, since ethaverine is pharmacologically known only as a calcium channel blocker (53).

A similar pattern of inhibition was observed when cytotoxicity was determined with cultured bloodstream forms. Interestingly, the dose-response curve for ethaverine showed a very steep Hill slope (5.19 ± 1.52), indicating that the effect of this compound on cell proliferation might be due to a combined effect of calcium channel blockage and PDE inhibition. In contrast to ethaverine, dipyridamole (which is not only a PDE inhibitor but also a potent inhibitor of adenosine transporters) showed a Hill slope of around 1 (1.37 ± 0.21), with no sign of cooperative inhibition of cell proliferation. This suggests that even in the presence of dipyridamole, sufficient amounts of purines can be taken up by the trypanosomes to allow unconstrained proliferation in culture. The presence of the TbPDE2A inhibitors leads to a continuous increase in intracellular cAMP concentration.

The biological role of TbPDE2A is currently not known. The identification of inhibitors of this enzyme has now provided the necessary tools for its investigation and for the experimental dissection of cAMP signaling in trypanosomes. The observation that inhibitors of TbPDE2A prevent cell proliferation in culture demonstrates that TbPDE2A, or the TbPDE2 family as a whole, may be essential for cell proliferation. This is also supported by the observation that TbPDE2 mRNA is constitutively expressed. In conjunction, these data indicate that TbPDE2A and its isoforms may represent interesting targets for the development of a new generation of trypanocidal drugs, based on phosphodiesterase inhibitors. TbPDE2A and its relatives in
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T. brucei as well as in other protozoa may offer a new class of targets for the development of urgently required, novel and effective anti-protozoal drugs.

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