Hydrolysis products of cAMP analogs cause transformation of *Trypanosoma brucei* from slender to stumpy-like forms

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African sleeping sickness is a disease caused by *Trypanosoma brucei*. *T. brucei* proliferate rapidly in the mammalian bloodstream as long, slender forms, but at higher population densities they transform into nondividing, short, stumpy forms. This is thought to be a mechanism adopted by *T. brucei* to establish a stable host-parasite relationship and to allow a transition into the insect stage of its life cycle. Earlier studies have suggested a role for cAMP in mediating this transformation. In this study, using membrane-permeable nucleotide analogs, we show that it is not the cAMP analogs themselves but rather the hydrolyzed products of membrane-permeable cAMP analogs that prevent proliferation of *T. brucei*. The metabolic products are more potent than the cAMP analogs, and hydrolysis-resistant cAMP analogs are not antiproliferative. We further show that the antiproliferative effect of these membrane-permeable adenosine analogs is caused by transformation into forms resembling short, stumpy bloodstream forms. These data suggest that the slender-to-stumpy transformation of *T. brucei* may not be mediated directly by cAMP and also raise the possibility of using such adenosine analogs as antitrypanosomal drugs.

adenosine | EPACs | phosphodiesterases | trypanosomes

T*rypanosoma brucei* are parasitic protozoans that cause African sleeping sickness, a neglected tropical disease with an estimated 0.5 million patients (1) but poor available methods of treatment and a need for new drugs (2). *T. brucei* has a complex life cycle in both its insect host (tsetse flies) and mammalian hosts (humans and other mammals). In the mammalian bloodstream, *T. brucei* proliferate rapidly as long, slender forms. As parasitemia increases, many of these slender forms transform into nonproliferating short, stumpy forms. This increase in stumpy forms is thought to lengthen the survival of the mammalian host by decreasing parasite numbers, thereby increasing the possibility of transmission of the disease from this infected individual. Secondly, when ingested into the insect host, the stumpy-form parasites undergo changes that allow them to adapt to and survive in the tsetse fly midgut, thereby ensuring their transmission (3–6).

It currently is postulated that cAMP plays an important role in the transformation from slender forms to stumpy forms, although much of the data for this belief is circumstantial. For example, in the closely related *Trypanosoma lewisi* as well as in *T. brucei*, different levels of cAMP have been measured during various life cycle stages (7, 8). In addition, two distinct peaks of adenylyl cyclase activity were observed during differentiation of *T. brucei* from bloodstream forms to procyclic (insect) forms. However, a requirement for cAMP or cyclase activity in this transformation or in the subsequent release of the variable surface glycoprotein coat was not apparent (9, 10). Other studies have used high concentrations of dibutyryl cAMP and found no effect on *in vitro* differentiation of bloodstream forms to stumpy forms (11).

The strongest suggestion for a role of cAMP in slender-to-stumpy transformation came from a study done by Vassella *et al.* (12). While characterizing stumpy-inducing factor (SIF), which can transform slender forms of *T. brucei* to stumpy forms, these investigators used a cell-permeable cAMP analog (8-pCPT-cAMP) to induce slender-to-stumpy transformation in pleomorphic bloodstream-form *T. brucei* (12). The phenotype caused by this analog was further characterized to confirm the stumpy form (13). As a result, it commonly has been accepted that increased cAMP could cause the slender-to-stumpy transformation. However, an unambiguous interpretation has been clouded by the fact that no cAMP-dependent effector protein (such as a PKA or EPAC) has been conclusively identified in trypanosomes (reviewed in refs. 14 and 15).

Recently, a cAMP analog, 8-pCPT-2'-O-Me-cAMP, was identified as a modest inhibitor of the *T. brucei* PDEB family (16, 17) of cAMP phosphodiesterases (PDEs). This analog originally was designed as a specific agonist of the mammalian guanine nucleotide exchange factor, EPAC (18, 19), and does not activate mammalian PKAs. However, EPACs appear to be absent in the recently completed *T. brucei* genome. Nevertheless, 8-pCPT-2'-O-Me-cAMP was found to be potentially antiproliferative. We recently have found that this analog is a reasonable inhibitor of several mammalian PDEs as well. However, its potency as an antiproliferative agent in *T. brucei* was greater than its potency as an *in vitro* inhibitor of recombinant PDEs, prompting studies to determine its mechanism of action and the role of cAMP in trypanosome transformation.

In this study, we report that the products of hydrolysis of these membrane-permeable cAMP analogs show potent antiproliferative properties, transforming *T. brucei* into stumpy-like forms. Hydrolysis-resistant, membrane-permeable cAMP analogs do not inhibit proliferation or cause this effect. The results suggest that transformation of *T. brucei* from slender to stumpy-like forms is not caused directly by membrane-permeable cAMP analogs but rather by metabolic products derived from it, and it may not be mediated directly by cAMP. The data also suggest a potential use of similar analogs as antitrypanosomal agents.

Results

8-pCPT-2'-O-Me-cAMP Inhibits *T. brucei* PDE Activity. Although most mammalian PDE inhibitors poorly inhibit the TbrPDEB family (14), it seemed possible that some cNMP analogs (which alter the

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Abbreviations: PDE, phosphodiesterase; LC, liquid chromatography; ISTD, internal standard.

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Fig. 1. 8-pCPT-2'-O-Me-cAMP inhibits *T. brucei* PDE activity and causes a small increase in cAMP. (A) Inhibition of recombinant TbrPDEB1 holoenzyme (open triangles) or TbrPDEB2 holoenzyme (filled triangles) by 8-pCPT-2'-O-Me-cAMP. (B) Inhibition of bloodstream-form *T. brucei* lysate PDE activity by 8-pCPT-2'-O-Me-cAMP (1 μ M). (C) Fold change in cAMP levels in bloodstream-form *T. brucei* after incubation for 90 min with 25 μ M 8-pCPT-2'-O-Me-cAMP or 50 μ M dipyr. (D) Inhibition of proliferation of bloodstream-form *T. brucei* by 8-pCPT-2'-O-Me-cAMP (circles), in the presence of 1 mM inosine (triangles, broken line) or 1 mM adenine (squares)

activity of other cNMP-regulated proteins) could inhibit the TbrPDEBs. Therefore, small groups of PKA, PKG, or EPAC modulators were screened for their ability to inhibit TbrPDEB activity. One compound, 8-pCPT-2'-O-Me-cAMP, was found to inhibit the PDE activity of a recombinant TbrPDEB1 catalytic domain [supporting information (SI) Table 1]. 8-pCPT-2'-O-Me-cAMP is widely used as a selective activator of EPACs as the 2'-O-methyl group makes the analog selective against PKA activation (18, 19). It also does not activate mammalian cNMP gated channels. 8-pCPT-2'-O-Me-cAMP subsequently was tested as a competitive inhibitor for TbrPDEB1 and TbrPDEB2 (Fig. 1A, SI Fig. 6) and was found to inhibit TbrPDEB2 with a K_i of $6.8 \pm 0.6 \mu$ M ($n = 6$) and TbrPDEB1 with a K_i of $4.3 \pm 0.6 \mu$ M ($n = 6$) (Fig. 1A). The compound also inhibited total PDE activity of bloodstream-form trypanosome cell lysates with an IC_{50} of $7.8 \pm 2 \mu$ M ($n = 6$) (Fig. 1B).

To test the effects of this compound on cAMP levels in *T. brucei*, proliferating *T. brucei* bloodstream forms were incubated with this compound or dipyr. [a known inhibitor of TbrPDEBs (16, 17)] for 90 min. Treated parasites then were recovered, and cAMP levels were measured. Treatment with either 8-pCPT-2'-O-Me-cAMP or dipyr. resulted in a small (<2-fold) increase in cAMP levels ($P = 0.05$, $n = 6$) (Fig. 1C). This small increase in cAMP potentially could have been caused by increased adenylyl cyclase activity. To test this possibility, adenylyl cyclase activity in isolated *T. brucei* membrane fractions was measured in the presence or absence of 8-pCPT-2'-O-Me-cAMP or dipyr. No changes in adenylyl cyclase activity could be observed (SI Fig. 6). Therefore, we assumed that the small effect on cAMP may be caused by inhibition of trypanosome PDEs.

8-pCPT-2'-O-Me-cAMP Shows Potent Antiproliferative Properties in Bloodstream-Form *T. brucei*. The effect of 8-pCPT-2'-O-Me-cAMP on the proliferation of bloodstream forms of *T. brucei* was

Fig. 2. cAMP analogs show potent antiproliferative effects in bloodstream-form *T. brucei*, but hydrolysis-resistant cAMP analogs do not. (A) Inhibition of proliferation of bloodstream-form *T. brucei* (over 48 h) by 8-pCPT-2'-O-Me-cAMP or Sp-8-pCPT-2'-O-Me-cAMPs. (B) Inhibition of proliferation of bloodstream-form *T. brucei* by 8-pCPT-cAMP (squares), Sp-8-pCPT-cAMPs (triangles), or Sp-5,6-DCI-cBIMPS (circles).

measured over 48-h proliferation assays. A dose-response curve showed an EC_{50} (effective concentration of inhibition of proliferation) of $1.3 \pm 0.1 \mu$ M ($n = 16$) (Fig. 1D). This EC_{50} value was unexpectedly lower than the K_i s observed for the *in vitro* inhibition of TbrPDEBs. However, the compound still potentially could have been causing its effect because of PDE inhibition, because it is not known how much inhibition of PDE might be necessary to cause an increase in cAMP. Moreover, it was possible that high intracellular concentrations of the analog might be accumulated by active transport of the compound into the trypanosome. *T. brucei* has two well studied adenosine transporters (P1 and P2) (20, 21). Although selective inhibitors of these transporters are not available, other studies have used saturating concentrations of adenine or inosine to block the P2 or P1 transporter, respectively, to implicate active transport of drugs into *T. brucei* (21–23). Therefore, the effect of 8-pCPT-2'-O-Me-cAMP on bloodstream-form *T. brucei* proliferation (over the same 48-h time period) was measured in the presence of 1 mM inosine or adenine in the growth media. An EC_{50} value of $1.6 \pm 0.2 \mu$ M or $1.3 \pm 0.1 \mu$ M ($n = 8$) was obtained for the antiproliferative effect of 8-pCPT-2'-O-Me-cAMP for *T. brucei* grown with adenine or inosine, respectively (Fig. 1D), very similar to that observed in control samples. This finding suggested that the effect measured likely was caused by entry of the cell-permeable analog into the cells, primarily by passive means, and not by active transport of the P1/P2 purine transporters (although transporter mutants or other transporters cannot be ruled out).

Hydrolysis-Resistant Cell-Permeable cAMP Analogs Do Not Show Potent Antiproliferative Effects. 8-pCPT-2'-O-Me-cAMP potentially can be hydrolyzed by some PDEs. Thus, we decided to test the effect of the hydrolysis-resistant analog variant of this analog, Sp-8-pCPT-2'-O-Me-cAMPs, on both PDE inhibition and proliferation. Sp-8-pCPT-2'-O-Me-cAMPs was found to inhibit the catalytic domain of TbrPDEB2, with a K_i of 570 ± 3 nM ($n = 4$), more potently than 8-pCPT-2'-O-Me-cAMP did. This compound is more membrane-permeable than 8-pCPT-2'-O-Me-cAMP (24). If the inhibition of proliferation observed was attributable to PDE inhibition, it would be expected that this compound would show similar effects as 8-pCPT-2'-O-Me-cAMP. However, we surprisingly saw little effect of this compound on *T. brucei* proliferation at concentrations up to 250 μ M ($n = 8$) (Fig. 2A). These data suggested that the effects of 8-pCPT-2'-O-Me-cAMP observed on proliferation may not be mediated by increased cAMP (because of PDE inhibition or direct action of the analog).

Therefore, we decided to reinvestigate older published data (which used 8-pCPT-cAMP) that has been interpreted to suggest

Fig. 3. Etazolate shows weak inhibition of *T. brucei* PDE activity but potent antiproliferative activity. Inhibition of PDE activity in bloodstream-form *T. brucei* lysates [$1 \mu\text{M}$ (29)] (triangles, left y axis) and inhibition of proliferation of bloodstream-form *T. brucei* by etazolate (squares, right y axis).

that increases in cAMP can transform slender forms into stumpy forms (12). 8-pCPT-cAMP does not have a 2'-O-methyl group and thus activates both PKA as well as EPACs in mammals. As reported previously, 8-pCPT-cAMP was found to have potent antiproliferative effects, with an EC_{50} of $6.5 \pm 0.2 \mu\text{M}$ and an EC_{90} of $110 \mu\text{M}$ ($n = 8$). However, the hydrolysis-resistant version of this analog, Sp-8-pCPT-cAMPS, did not significantly affect proliferation at concentrations up to $250 \mu\text{M}$ ($n = 6$) (Fig. 2B). The effect on proliferation of a different, more membrane-permeable and hydrolysis-resistant cAMP analog, Sp-5,6-DCL-cBIMPS (25), also was tested. Sp-5,6-DCL-cBIMPS also did not significantly affect bloodstream-form *T. brucei* proliferation at $250 \mu\text{M}$ ($n = 8$), further suggesting a possible noncyclic phosphate-mediated effect (Fig. 3B).

Proliferation of bloodstream-form *T. brucei* also was tested in the presence of 8-pCPT-cGMP, which (as shown in ref. 12) had little effect on proliferation (SI Fig. 7). Finally, the effects on proliferation of another cAMP analog, (the less cell-permeable) 8-Br-cAMP, and of a more hydrolysis-resistant form, Rp-8-Br-cAMPS (an antagonist of mammalian PKAs), were tested. The trend was again similar, and 8-Br-cAMP inhibited bloodstream-form *T. brucei* proliferation with an EC_{50} of $152 \pm 0.6 \mu\text{M}$, whereas the hydrolysis-resistant Rp-8-Br-cAMPS was less effective, with an EC_{50} of $3.8 \pm 0.2 \text{ mM}$ ($n = 8$) (SI Fig. 7).

Etazolate Poorly Inhibits *T. brucei* PDEs but Is Potently Antiproliferative. Etazolate inhibits mammalian PDE4s (26) and is reported to inhibit *T. brucei* PDEs. In earlier studies, etazolate showed antiproliferative effects, causing slender-to-stumpy transformation at concentrations of 1–2 μM (12). However, recent work characterizing *T. brucei* PDE families (16, 17, 27, 28) showed that etazolate only modestly inhibits most *T. brucei* PDEs. Inhibition of total PDE activity in *T. brucei* whole-cell lysates in the presence of etazolate was measured, and etazolate was found to inhibit PDE activity with an IC_{50} of $810 \pm 10 \mu\text{M}$ [at $1 \mu\text{M}$ cAMP (29), $n = 3$] (Fig. 3). However, etazolate was potently antiproliferative, inhibiting bloodstream-form *T. brucei* proliferation with an EC_{50} of $16.3 \pm 3 \mu\text{M}$ ($n = 6$) (Fig. 3). Etazolate (an adenine/adenosine analog) is known to antagonize adenosine receptors or interact with channels (30) and could be effecting other targets that cause slender-to-stumpy transformation in *T. brucei*. Nevertheless, these data suggested that the antiproliferative effect and transformation caused by etazolate in bloodstream-form *T. brucei* may not be attributable to PDE inhibition.

Membrane-Permeable cAMP Analogs Are Hydrolyzed, and Their Products Show Bloodstream-Form-Specific Antiproliferative Effects. To this point, the data suggested that the antiproliferative effects seen may not be directly caused by the cAMP analogs but could be caused by downstream products, if the cAMP analogs were hydrolyzed either by a PDE activity in serum (present in culture

Fig. 4. 8-pCPT-2'-O-Me-cAMP is hydrolyzed by trypanosomes in media, and hydrolysis products show potent, bloodstream-form-specific antiproliferative effects. (A) Relative amount of 8-pCPT-2'-O-Me-cAMP present in medium plus *T. brucei* over time, measured by using LC/MS (y axis cAMP = 8-pCPT-2'-O-Me-cAMP). (B) Relative amount of 8-pCPT-2'-O-Me-cAMP present in lysed *T. brucei* over time, measured by using LC/MS (y axis cAMP = 8-pCPT-2'-O-Me-cAMP). (C) Inhibition of proliferation of bloodstream-form *T. brucei* by 8-pCPT-2'-O-Me-5'-AMP (squares) or 8-pCPT-2'-O-Me-ado (triangles). (D) Inhibition of proliferation of bloodstream-form (strain 427) *T. brucei* (squares), *T. evansi* (triangles, broken line), or procyclic IsTar 1.7 *T. brucei* by 8-pCPT-2'-O-Me-5'-AMP (circles) or by 8-pCPT-2'-O-Me-cAMP (diamonds).

medium) (31, 32) or by *T. brucei*. To investigate this possibility, $10 \mu\text{M}$ 8-pCPT-2'-O-Me-cAMP was incubated with $\approx 5 \times 10^5$ bloodstream-form *T. brucei* in 10 ml of culture media (in standard culture conditions). Samples were taken periodically and quantitatively analyzed by using liquid chromatography (LC)/MS. Over 48 h, 8-pCPT-2'-O-Me-cAMP levels steadily decreased (Fig. 4A) ($n = 3$), with a proportional accumulation of 8-pCPT-2'-O-Me-ado (data not shown). ATP, ADP, or adenine forms of the analog could not be detected, at least at this level of sensitivity of mass spectrometry. Lysed *T. brucei* (5×10^5 cells in $100 \mu\text{l}$) alone in the absence of media also caused similar reductions in levels of 8-pCPT-2'-O-Me-cAMP (Fig. 4B) ($n = 3$). This finding suggested that, under our trypanosome culture conditions, 8-pCPT-2'-O-Me-cAMP could be hydrolyzed into 5'-AMP/adenosine mainly by the trypanosomes themselves.

The effect of the AMP analog (8-pCPT-2'-O-Me-5'-AMP) on the proliferation of bloodstream-form *T. brucei* was tested, and it was found to inhibit proliferation with a higher potency than the cAMP analog did, with an EC_{50} of $125 \pm 3 \text{ nM}$ ($n = 8$) (Fig. 4C). The adenosine variant of this analog (8-pCPT-2'-O-Me-ado) had a more potent antiproliferative effect, with an EC_{50} of $84 \pm 4 \text{ nM}$ ($n = 7$) (Fig. 4C). The 5'-AMP analog is rapidly dephosphorylated to form the corresponding adenosine analog in HMI-9 medium alone at 37°C (SI Fig. 8) ($n = 3$). Because the 5'-AMP analog has two negative charges and poor cell permeability (33), it is highly likely that the adenosine analog enters the cell more readily. Together, these data suggest that it is the 8-pCPT-2'-O-Me adenosine that causes this effect, although the current data do not rule out 5'-AMP analog effects.

The adenosine/5'-AMP analogs affected proliferation in bloodstream forms only and not in procyclic forms of *T. brucei* (Fig. 4D), suggesting a possible transformation into stumpy-like forms and not gross growth abnormalities caused by the inhibition of common biological pathways. Certain dyskinetoplastic

Fig. 5. 8-pCPT-2'-O-Me-5'-AMP converts monomorphic bloodstream-form *T. brucei* to stumpy-like forms. (A and B) Bright-field image of Giemsa-stained *T. brucei* either untreated (A) or treated with 20 μ M 8-pCPT-2'-O-Me-5'-AMP for 48 h (B). (C) Diaphorase staining of *T. brucei* treated with 20 μ M 8-pCPT-2'-O-Me-5'-AMP for 48 h observed with phase-contrast microscopy. (D) Survival and proliferation over time (hours) of untreated *T. brucei* (squares) or *T. brucei* pretreated for 48 h with 8-pCPT-cAMP (triangles), 8-pCPT-2'-O-Me-cAMP (inverted triangles), or 8-pCPT-2'-O-Me-5'-AMP (diamonds) in SDM-79 medium containing 3 mM citrate/cis-aconitate at 27°C. (E) Expression of procyclin during *in vitro* differentiation of 8-pCPT-2'-O-Me-5'-AMP-treated *T. brucei* (48-h treatment), detected by using an antiprocyclin antibody (49). PC, procyclic *T. brucei*; BF, untreated bloodstream-form *T. brucei*. A total of 1×10^6 cells were loaded in each lane. Data shown are representative of three separate experiments.

trypanosomatids do not transform into procyclic forms (34) but exist solely as bloodstream forms. These trypanosomatids can transform into stumpy forms (35). 8-pCPT-2'-O-Me-5'-AMP potentially inhibited proliferation in the dyskinetoplastic *Trypanosoma evansi* with an EC₅₀ of 78 ± 3 nM (Fig. 4D). High concentrations of extracellular cAMP, 5'-AMP, or adenosine did not significantly affect proliferation of *T. brucei* (SI Fig. 8), suggesting that the antiproliferative effect caused by the nucleotide analogs was mediated by an intracellular "receptor."

5'-AMP/Adenosine Analogs Cause Cell-Cycle Arrest and Transformation of Slender Forms into Stumpy-Like Forms. Treatment of proliferating bloodstream-form *T. brucei* for 48 h with 10 μ M 8-pCPT-2'-O-Me-5'-AMP, and subsequent removal of drug pressure, resulted in complete cessation of proliferation (SI Table 2). Untreated parasites stained with Giemsa showed typical slender morphology (Fig. 5A), whereas parasites treated with 8-pCPT-2'-O-Me-5'-AMP for 48 h showed stumpy morphology (Fig. 5B). After 48 h of treatment with 8-pCPT-2'-O-Me-5'-AMP, NADH diaphorase activity, a marker of intermediate or stumpy forms (13, 35, 36), was detected (observed by the appearance of dark precipitates in the trypanosome body) (Fig. 5C).

DNA synthesis and cell proliferation were measured by thymidine incorporation, and 48 h of treatment with 8-pCPT-2'-O-Me-5'-AMP resulted in an 89% reduction in DNA synthesis ($n = 3$) (SI Fig. 9), suggesting cell-cycle arrest. Finally, parasites were treated with 8-pCPT-2'-O-Me-cAMP (15 μ M), 8-pCPT-cAMP (100 μ M), or 8-pCPT-2'-O-Me-5'-AMP (15 μ M) for 48 h and transferred to procyclic trypanosome growth and transformation conditions. Untreated parasites showed delayed proliferation, whereas treated stumpy-like parasites showed extended survival and proliferation under these growth conditions (Fig. 5D), with the 5'-AMP analog mimicking the cAMP analog effects. The parasites treated with the 5'-AMP analog also showed early expression of the major procyclic surface glyco-

protein and marker for procyclic form, procyclin/procyclic acidic repetitive protein (PARP), when transferred to SDM-79 plus 3 mM citrate/cis-aconitate (Fig. 5E, SI Fig. 9), similar to earlier results with the cAMP analog (12, 13). However, analog treated parasites did not give rise to fully established procyclic forms, because growth stopped after ≈ 1 week.

Together, these data suggest that the 5'-AMP/adenosine analogs convert proliferating bloodstream-form *T. brucei* into stumpy-like forms, mimicking the actions of the cAMP analogs.

Discussion

The stumpy form of *T. brucei* is believed to prolong the survival of the mammalian host and to allow transition into the procyclic stage (3–6). Yet, numerous monomorphic lines of trypanosomes (such as the strain 427) commonly used by research groups have lost the ability to respond to stumpy-inducing factor (SIF) or to become stumpy forms *in vivo* (because of serial passage between culture and rodents) (12, 37). Many monomorphic strains, however, can differentiate into procyclic forms under suitable *in vitro* conditions, leading to some suggestion that stumpy cells are not required for differentiation to procyclic forms (11). However, more recent work has reinforced the concept of the stumpy form as critical in the life cycle of *T. brucei* (5).

The specific signaling pathways in this and other differentiation processes in *T. brucei* are unclear. Observations of altered cyclase activity or cAMP levels in different life cycle stages (7–9) have supported the idea that cAMP may mediate these differentiation processes. A strong initial suggestion for a role of intracellular cAMP in mediating the transformation from slender forms to stumpy forms came with the use of membrane-permeable cAMP analogs to induce this transformation (12). However, no direct role or requirement for cAMP in these processes has been demonstrated. Moreover, older studies in trypanosomes assumed that modulators of mammalian cyclases or PDEs also were effective on their counterparts in trypanosomes (14, 15, 38). We now know that most of these pharmacological agents do not affect trypanosome PDEs or cyclases. There is, therefore, a need for a reevaluation of many of the earlier speculations for the roles of cAMP.

An important role for cAMP in these parasites is suggested by RNAi-mediated knockdown of the Tbr PDEB family in bloodstream-form *T. brucei*, which results in the death of the parasite and causes a ≈ 20 fold increase in cAMP levels (16). However, the same study also showed that the PDE knockdown did not result in stumpy-form trypanosomes but rather in oddly shaped, multinucleated cells, suggesting other possible roles for cAMP. It is therefore important to develop activators or inhibitors of trypanosome adenylyl cyclases or PDEs to pharmacologically study the roles of cAMP signaling in these parasites. Additionally, no direct effector proteins of cAMP (particularly a PKA or EPACs) have been identified in trypanosomes. To understand the specific roles of cAMP in trypanosomes, it will be essential to identify and characterize the proteins that synthesize or degrade cAMP, as well as target effector proteins.

The data here show that membrane-permeable cAMP analogs cause a transformation to stumpy-like forms, but hydrolysis-resistant analogs do not do so. Importantly, downstream products of hydrolyzed cAMP analogs caused the same effect with higher potencies. Although it currently is not possible to completely rule out any role for cAMP in this differentiation process, the present data throw into question whether the slender-to-stumpy conversion is initiated by cAMP at all.

The molecular mechanisms by which different adenosine analogs alter differentiation and proliferation are more difficult to interpret because adenosine analogs are known to cause such effects as triggering of cell-cycle arrest (39), inhibition of glycolysis (40, 41), and alteration of purine transport or salvage (42). Similarly, AMP kinases are known targets in mammals but are

as-yet-unknown in trypanosomes (43). The adenosine analogs also could potentially target cyclin-dependent kinases (44) or mitogen-activated protein kinases. A mitogen-activated protein kinase important in the differentiation of bloodstream forms to stumpy forms has been identified (45). More work will be needed to firmly identify the downstream molecular target(s) of 8-pCPT-2'-O-Me-adenosine and similar analogs.

The results of the studies reported in this article also may have implications for regulation of signal transduction pathways in mammalian systems. In mammalian cell culture, 8-pCPT-2'-O-Me-cAMP has been widely used to demonstrate involvement of EPAC in a pathway. However, the data here show that PDEs can hydrolyze this analog, and downstream metabolic products have biological effects, at least in trypanosomes. Some mammalian PDEs do hydrolyze this analog (T. R. Hinds, unpublished data). It is likely, therefore, that 8-pCPT-2'-O-Me-cAMP also is hydrolyzed to its adenosine/AMP analog in mammalian cells, and the metabolite may mediate some of the biological activity. Therefore, interpretations of some studies done with this or other cyclic nucleotide analogs may need to be revisited with this knowledge.

Finally, these analogs caused transformation to stumpy-like forms incapable of proliferation but not to fully committed procyclic forms, which appear to require a kinetoplast-dependent event (35) for differentiation. Given their potency, it should, therefore, be possible to develop drugs based on these types of analogs as antitrypanosomal agents.

Materials and Methods

Primers used to amplify PDE sequences, cloning vectors, protein expression methods, and adenyl cyclase assay methods are provided in *SI Methods and Materials*.

Analog. Nucleotide analogs were synthesized and obtained from BIOLOG Life Science Institute (Bremen, Germany). Dipyridamole and etazolate were purchased from Sigma (St. Louis, MO). Structures of the nucleotide analogs are provided in SI Fig. 10, as are analog abbreviations.

PDE/Inhibitor Assays. Traditional PDE assays were performed with 1 μ M cAMP and increasing amounts of inhibitors, as described in ref. 17. Calculations were performed with Microsoft Excel (Redmond, WA), plotted in Prism 4.0 (GraphPad Software, San Diego, CA), and presented as the average of at least three experiments.

Measurement of cAMP Levels in Trypanosomes. cAMP was extracted from bloodstream-form *T. brucei* treated with analogs or inhibitors by using ethanol:1 M HCl (99:1). The samples were evaporated, resuspended in 0.2 M NaH₂PO₄/8 mM tetrabutylammonium hydrogen sulfate (pH 6), and separated on an RP-18 column by HPLC, as described in ref. 46. cAMP fractions were collected and dried down, and levels were measured by using a cAMP EIA kit (American Qualex, San Clemente, CA).

Trypanosome Culture. Monomorphic 427 strain *T. brucei brucei* bloodstream forms were cultured in HMI-9 medium plus 10% FBS and penicillin/streptomycin at 37°C with 5% CO₂. Cell lysates were prepared as described in ref. 47. The procyclic IsTar 1.7 strain of *T. brucei* was cultured in SDM-79 medium at 27°C.

Proliferation Measurements. The effects of compounds on the proliferation of *T. brucei* were tested by using Alamar Blue (TREK Diagnostic Systems, Cleveland, OH) as a metabolic indicator (48) in 48-h proliferation assays. Exponentially growing bloodstream forms were seeded in 96-well cell culture plates (5 $\times$ 10⁴ cells per ml) with a specified amount of inhibitor or buffer control and were grown in standard culture conditions. After 48 h, 20 μ l of Alamar Blue was added per well, incubated at 37°C for 2–4 h, and read on a BioTek (Winooski, VT) fluorescence plate reader at 570 and 600 nm. Data analysis was performed by using Prism 4.0 software.

LC/MS for Detection and Quantification of Analogs. Thirty-microliter samples of media per cell were collected at respective time intervals, and analogs were extracted by using 50 μ l of acetonitrile containing 100 pmol of BMS-214662 (Bristol Meyers Squibb, New York, NY) as an internal standard (ISTD). Extracts were spun down, and the supernatant was removed, dried down, and resuspended in 25 μ l. Then, 10 μ l of this extract was analyzed by LC/MS by using an Agilent HP 1100 chromatograph and an Esquire-LC (Bruker, Billerica, MA) electrospray ion-trap mass spectrometer. Samples were separated on an RP-18 column (Zorbax SB-C18, 2.1 $\times$ 100 mm, 3.6-Å particles; Agilent Technologies, Santa Clara, CA) with water/5% acetonitrile, 1% acetic acid-acetonitrile, 1% acetic acid gradient. Quantification was performed by comparing the area under the appropriate peak in the extracted ion chromatogram for the analyte and corresponding ISTD in each sample, by using Bruker QuantAnalysis software. Relative levels were expressed as a ratio of sample to ISTD. A known amount of analyte with ISTD was used to establish the retention time and response factor.

Microscopic Methods/Imaging. Staining for diaphorase activity or Giemsa staining was performed as described in ref. 12 and 13. Cells were visualized by using phase-contrast microscopy (diaphorase activity) with a $\times$ 100 phase contrast lens or bright-field microscopy (Giemsa staining) with a $\times$ 100 objective, under oil immersion with a Nikon E600 microscope.

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