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Short communication

Host Epac1 is required for cAMP-mediated invasion by *Trypanosoma cruzi*

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ABSTRACT

Mechanistic details of the modulation by cAMP of *Trypanosoma cruzi* host cell invasion remain ill-defined. Here we report that activation of host's Epac1 stimulated invasion, whereas specific pharmacological inhibition or maneuvers that alter Epac1 subcellular localization significantly reduced invasion. Furthermore, while specific activation of host cell PKA showed no effect, its inhibition resulted in an increased invasion, revealing a crosstalk between the PKA and Epac signaling pathways during the process of invasion. Therefore, our data suggests that subcellular localization of Epac might be playing an important role during invasion and that specific activation of the host cell cAMP/Epac1 pathway is required for cAMP-mediated invasion.

Trypanosoma cruzi, a protozoan parasite transmitted by blood-sucking triatomine insects from the Reduviidae family, is the etiological agent of Chagas disease, a serious health threat among people living in poor rural populations of Central and South America. Estimations indicate 6 to 8 million infected people and 65 million individuals at risk of contracting the disease, an annual incidence of 28.000 cases and 12.000 deaths (http://www.paho.org). Additionally, human migration from endemic areas of Latin America to Europe, North America, and the Western Pacific, significantly increased the prevalence of *T. cruzi* infection outside of Latin America, resulting in a global economic burden of $7.19 billion [1].

T. cruzi, has a complex life cycle including a bug-vector and a vertebrate host. In the mammalian host, *T. cruzi* is an obligate intracellular parasite that posses the ability of infecting different kind of tissues. To ensure successful cell invasion the parasite has developed multiple mechanisms of internalization, including the recruitment and fusion of lysosomes to the entry site [2]. In this process, attachment of trypomastigotes to the cellular host is accompanied by an elevation of intracellular cAMP levels. It has been shown that cAMP is able to potentiate the Ca^{2+}-dependent exocytosis of lysosomes and lysosome-mediated cell invasion by *T. cruzi* [3]. Accordingly, pharmacologic intervention of the cAMP pathway is able to modulate parasite invasion [3]. However, cAMP effectors involved in *T. cruzi* invasion remain unknown. In mammalian cells, both cAMP effector pathways, i.e. Protein Kinase A (PKA) and Exchange protein activated directly by cAMP (Epac), are involved in Ca^{2+}-triggered exocytic events (i.e. secretion) [4]. Moreover, members of the cAMP signaling pathway were localized to late endosomes/lysosomes [5], and Epac-mediated Rap activation is involved in regulated exocytosis in human sperm [6], insulin secretion [7] and pancreatic amylase release [8]. Therefore, we hypothesized that the cAMP/Epac pathway might play a role during *T. cruzi* invasion of the host cell. In this work, using a set of pharmacological tools, we demonstrated that Epac1-mediated signaling represents the main mechanism for cAMP-mediated host cell invasion.

In order to evaluate our hypothesis, we first demonstrated the ability of tissue-cultured trypomastigotes of the CL Brener strain to induce an increase in the intracellular levels of cAMP

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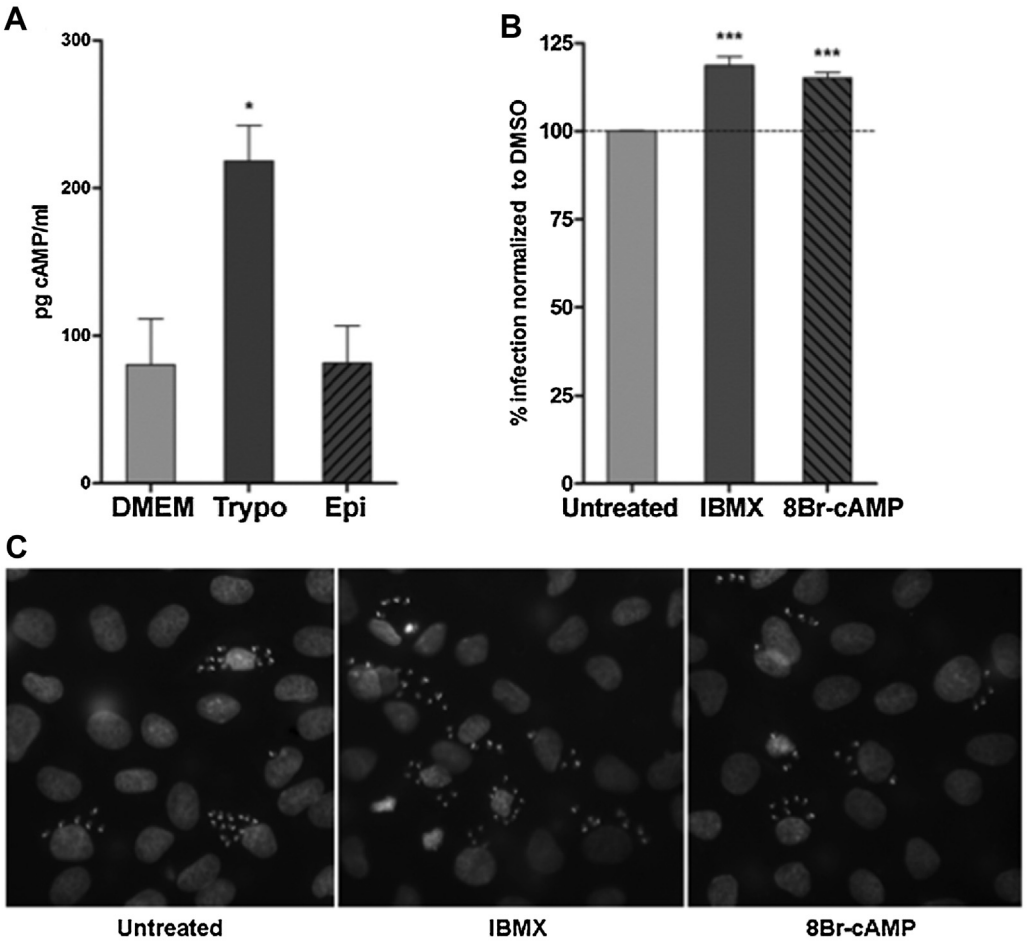

Fig. 1. A) *T. cruzi* trypomastigotes trigger elevation in host cell cAMP intracellular levels. NRK cells were incubated with trypomastigotes or epimastigotes of the CL Brener strain (100:1 parasite to cell ratio) or vehicle. After 30 min incubation cells were lysed and cAMP intracellular concentration measured with the cAMP Screen System Kit, following instructions by supplier (Applied Biosystem). Data represent the mean ± SD of three independent experiments. * $p < 0.05$, with unpaired Student's *t*-test. B) *T. cruzi* modulates host's cAMP-mediated signaling to promote invasion. Pretreated NRK cells (30 min at 300 μM IBMX or 300 μM 8-Br-cAMP) were infected with trypomastigotes of the *T. cruzi* CL Brener strain. 48 hs post-infection cells were fixed, stained with DAPI and percentage of invasion ((#Infected cells/3000 cells)*100) was calculated by fluorescence microscopy. Invasion of untreated cells was considered as basal invasion. Data represent the mean ± SD of three independent experiments. * $p < 0.05$ and *** $p < 0.001$, ANOVA with posttest comparisons. C) Representative images of DAPI staining of infected cells pretreated with the indicated inhibitor/activator.

in NRK cells (Normal Rat Kidney cell-line) (Fig. 1A). In contrast, epimastigotes from the same strain failed to promote the production of cAMP by the host cell (Fig. 1A). To confirm the positive modulation of cAMP on *T. cruzi* invasion of NRK cells, elevation of the intracellular cyclic nucleotide was attained by pretreatment of host cells with the Phosphodiesterase (PDE) inhibitor IBMX (3-Isobutyl-1-methylxanthine) (Sigma) or the non-hydrolysable cAMP analog 8-Br-cAMP (8-Bromoadenosine-3′,5′-cyclic monophosphate) (Biolog). In both cases, treatment led to an increased in the total number of infected cells, as reflected in an increased percentage of invasion (Fig. 1B and C).

The specific roles of cAMP effectors, PKA and Epac, during host invasion by *T. cruzi*, were evaluated using a set of pharmacologic tools to selectively activate or inhibit these effectors. As shown in Fig. 2, pretreatment of host cells with cAMP analogs that triggered differential activation of PKA, such as 6-Bnz-cAMP (N^6-Benzoyladenosine-3′,5′-cyclic monophosphate) (Biolog) or Sp-cAMP (Adenosine- 3′, 5′- cyclic monophosphorothioate, Sp-isomer) (Biolog), had no significant effect on *T. cruzi* invasion. On the other hand, the percentage of invasion significantly increased when host cells were pretreated with a cAMP analog that exclusively activates Epac (8-(4- Methoxyphenylthio)-2′-O-methyladenosine-3′, 5′ cyclic monophosphate) (Biolog) (Fig. 2). Consistent with this observation, differential inhibition of Epac by the recently discovered antagonist ESI-09 (Sigma) [9], showed a significant inhibition in invasion (Fig. 2). These results clearly showed the requirement of Epac activation for the positive modulation by cAMP of invasion. In addition, an increase in invasion was also observed when cells were pretreated with the PKA inhibitor H89 (Sigma) (Fig. 2). However, H89, a competitive inhibitor that blocks PKA activity through displacement of ATP from the catalytic site, could also inhibit at least 8 other kinases [10]. In order to avoid potential effects on other kinases, inhibition was achieved by using Rp-cAMP (Adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer) (Biolog), a permeable PDE-resistant cAMP analog shown to be an specific inhibitor of PKA. As for H89, pretreatment with Rp-cAMP had also a positive effect on *T. cruzi* invasion (Fig. 2). The increase in invasion observed as a result of PKA inhibition, could be translated in a PKA-dependent antagonistic effect over cAMP-mediated invasion. If inhibition of PKA increased invasion, it could then be expected that activation of PKA would have the opposite effect. Therefore, under physiological conditions, PKA-mediated phosphorylation would be negatively regulating the Epac pathway. This inhibition could be achieved, at least, in two different ways: A) through direct phosphorylation of Epac or B) At the level of Rap1, a downstream effector of Epac, and potential target for PKA-mediated phosphorylation [11]. Supporting this idea, Low and Stork

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