Spermidine is essential for normal proliferation of trypanosomatid protozoa

Nélida S. González, Alejandra Huber, Israel D. Algranati*

Instituto de Investigaciones Bioquímicas 'Fundación Campomar', Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires and CONICET, A. Machado 151, 1405 Buenos Aires, Argentina

Received 8 September 2001; revised 21 October 2001; accepted 23 October 2001

First published online 2 November 2001

Edited by Pierre Jolles

Abstract Trypanosomatid parasites containing a metabolically unstable ornithine decarboxylase (ODC) are naturally resistant to high levels of α-difluoromethylornithine (DFMO) because this ODC inhibitor, though causing a drastic reduction of intracellular putrescine, elicits only a moderate decrease of the spermidine endogenous pool. In this study we have used a combination of DFMO with cyclohexylamine (CHA; bis-cyclohexylammonium sulfate), an inhibitor of spermidine synthase, to reach a more complete depletion of spermidine. Under these conditions we have observed the arrest of proliferation not only in trypanosomatids with stable ODC but also in parasites with an enzyme of high turnover rate. In all cases the reinitiation of proliferation occurred only after the addition of exogenous spermidine, and neither putrescine nor spermine were able to induce the same effect.

Key words: Spermidine; Polyamine; Spermidine synthase; Trypanosomatid proliferation; Cyclohexylamine

1. Introduction

Many studies carried out with prokaryotic and eukaryotic organisms have conclusively shown that polyamines play essential roles in cell growth and proliferation [1–3]. These cationic substances are implicated in multiple functions related to macromolecular biosyntheses [4–9]; therefore, it is not surprising that intracellular levels of polyamines are accurately regulated by different mechanisms [4,10]. In trypanosomatid protozoa, polyamines have the additional role of maintaining the endogenous redox equilibrium through the compound bis-glutathionyl spermidine (trypanothione) [11–13].

We have recently reported that *Crithidia fasciculata* and some other parasites containing a metabolically unstable ornithine decarboxylase (ODC) are able to grow continuously even at very high concentrations of α-difluoromethylornithine (DFMO) [14–16], a specific and irreversible inhibitor of the enzyme [17,18]. On the contrary, *Leishmania mexicana* and *Trypanosoma brucei*, which have a rather stable ODC with a half-life longer than 8 h [19,20], are susceptible to the drug and stop their proliferation after repeated treatments with DFMO [16,21]. The measurement of endogenous polyamine pools of various parasites cultivated for a long time in the absence and presence of the inhibitor has indicated that putrescine was almost completely depleted by DFMO in *Crithidia* as well as in *Leishmania* and *Trypanosoma* [16]. In contrast, the spermidine levels were only partially decreased by the same treatment in all cases, but they were markedly reduced to values lower than 40% of the normal concentrations in those parasites which stopped their multiplication after several passages in the presence of the drug [16,21]. These results seem to indicate that spermidine could be the essential polyamine for the maintenance of normal proliferation in trypanosomatid protozoa. This possibility was also supported by the recent construction of *Leishmania donovani* cell lines deficient in the spermidine synthase gene [22]. The resulting parasites became auxotrophic for polyamines, and this requirement could only be fulfilled by the addition of exogenous spermidine to the culture medium but not by putrescine, spermine or other polyamines.

In the present work we have used cyclohexylamine (CHA; bis-cyclohexylammonium sulfate), a well known inhibitor of spermidine synthase [23,24], to induce the depletion of spermidine intracellular pools in *C. fasciculata* and a *Trypanosoma cruzi* cell line which expresses ODC enzymatic activity after transfection with an exogenous ODC gene [25]. We were able to demonstrate the inhibition of proliferation in both trypanosomatids only when spermidine endogenous levels were reduced to one third or less of its normal values, independently of the intracellular concentrations of putrescine.

2. Materials and methods

2.1. Chemicals

Minimal essential medium, fetal calf serum and amino acids were obtained from Gibco BRL (Gaithersburg, MD, USA); vitamins, bases, hemin, polyamines, aminoguanidine hemisulfate salt, HEPES buffer, antibiotics and CHA were from Sigma (St. Louis, MO, USA). Benzoyl chloride was purchased from Merck Schuchardt (Germany) and [1,4-^{14}C]putrescine dihydrochloride (110 mCi/mmol) was obtained from NEN Life Science Products, Inc. (Boston, MA, USA). DFMO was a gift from the Merrell Dow Research Institute (Cincinnati, OH, USA).

2.2. Parasite cultures

C. fasciculata (ATCC 11745), *T. cruzi* strain Tul2 [26] and pODC-7 transformed *T. cruzi* (Tul7) [25] were cultivated with shaking at 28°C in the semi-defined medium SDM-79 [27] which contains only traces of polyamines. Hemin (20 mg/l), 10% heat-inactivated fetal calf serum and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) were

*Corresponding author. Fax: (54)-11-4865 2246.
E-mail addresses: algra@iib.uba.ar (I.D. Algranati), ialgranati@leloir.org.ar (I.D. Algranati).

Abbreviations: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; CHA, cyclohexylamine (bis-cyclohexylammonium sulfate)

0014-5793/01/$20.00
PII: S0014-5793(01)03091-5

added to the medium. Parasite growth was usually initiated at a concentration of 5–10×10^6 cells/ml and followed by cell counting. Some cultures were supplemented with 0.1 mM spermidine or spermine. In these cases 0.2 mM aminoguanidine was also added in order to inhibit polyamine oxidase activity [28].

2.3. In vivo labeling with radioactive putrescine

Exponentially growing parasites were incubated overnight in the absence and presence of 3 mM CHA. Cells were collected by centrifugation (3000×*g*, 10 min) and resuspended in fresh SDM-79 medium at a concentration of 2×10^8 parasites/ml with or without the addition of CHA. [1,4-^{14}C]Putrescine (0.3 μCi/ml) was added and after incubation for 24–60 h at 28°C, parasites were sedimented, washed with PBS and resuspended in 100 μl of 0.2 M perchloric acid. Cell extracts were neutralized with KOH and precipitates discarded after centrifugation. The supernatant fluids were analyzed by paper chromatography using a mixture of *n*-butanol, acetic acid, pyridine and water (15/3/10/12, v/v) as running solvent. The radioactive spots were detected with a radiochromatogram scanner and identified by using internal polyamine standards.

2.4. Polyamine analysis

Parasites were harvested, washed with PBS and resuspended in 10% TCA. After centrifugation, cell extracts were treated with benzoylchloride as described by Morgan [29]. The benzoyl derivatives were separated by high-performance liquid chromatography (HPLC) on a Beckman system equipped with a C_{18} reversed-phase column and analyzed spectrophotometrically.

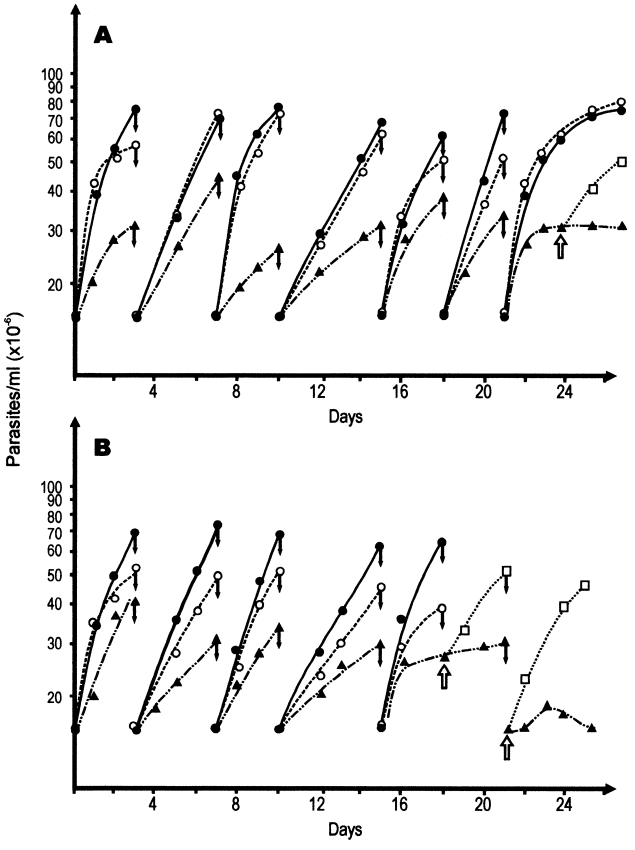

Fig. 1. Effects of DFMO and CHA on the growth of *C. fasciculata* (A) and ODC-transformed *T. cruzi* (Tul7) epimastigotes (B). (●) Control; (○) 5 mM DFMO and (▲) 5 mM DFMO+3 mM CHA. SDM-79 medium was used in all cases. At the times indicated by the black arrows, cultures were diluted with fresh medium in the absence or presence of the corresponding inhibitors. The white arrows indicate the addition of 0.1 mM spermidine to the cultures containing DFMO and CHA. (□) Growth curves after spermidine supplementation. In B, the curves corresponding to control and DFMO-containing cultures during the last two passages are not shown.

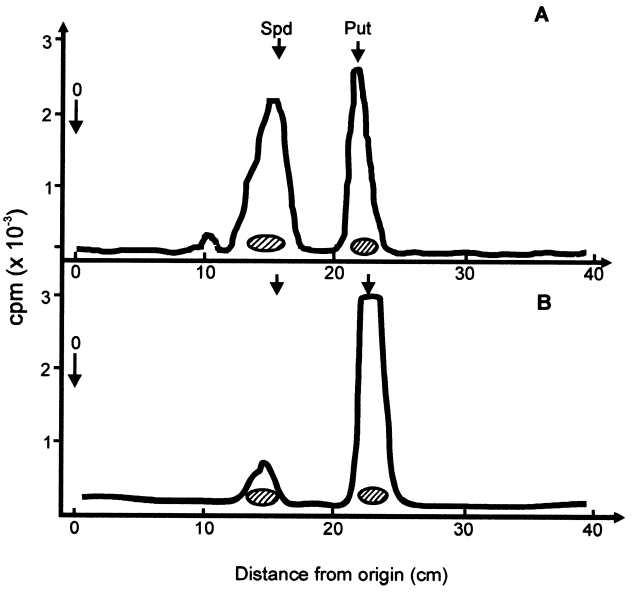

Fig. 2. Chromatographic analysis of *C. fasciculata* extracts obtained from parasites incubated with radioactive putrescine in the absence (A) or presence (B) of 3 mM CHA. All conditions as described in Section 2.3. Shaded spots show the position of standard putrescine (Put) and spermidine (Spd).

3. Results and discussion

3.1. Effect of DFMO and CHA on the growth of C. fasciculata and ODC-transformed T. cruzi

Previous studies carried out in our laboratory with *L. mexicana* promastigotes have shown that when this parasite was cultivated in a polyamine-free medium, the addition of DFMO decreased the growth rate and proliferation was completely arrested after several passages in the presence of the drug.

The ability of *Leishmania* cultures to resume proliferation after the inhibition correlated with the intracellular concentrations of spermidine, and this fact led us to assume that the parasite multiplication can not occur when spermidine decreases below a critical level of around 40% of normal values [21]. Recent results have indicated that monogenetic trypanosomatids as *C. fasciculata* were naturally resistant to DFMO even at drug concentrations of 20 or 50 mM [16]. This property was related to the high turnover of *Crithidia* ODC; the continuously synthesized new molecules of active enzyme seem to produce enough putrescine rapidly converted into spermidine, thus avoiding a too drastic depletion of the latter polyamine in the presence of the inhibitor. This possibility would also indicate that spermidine is indeed the essential polyamine needed to maintain the normal proliferation of trypanosomatids. In order to confirm this hypothesis we have now followed the proliferation of *C. fasciculata* and ODC-transformed *T. cruzi* in the presence of 5 mM DFMO with or without the addition of 3 mM CHA. Fig. 1A shows that *Crithidia* growth was not affected by DFMO, as previously reported [16], but the parasite multiplication was halted after cultivation for a few passages in the presence of both inhibitors, DFMO and CHA. A similar behavior was observed with ODC-transformed *T. cruzi* cultures (Fig. 1B), although in this case the arrest of proliferation could also be obtained with DFMO alone, as it was observed previously

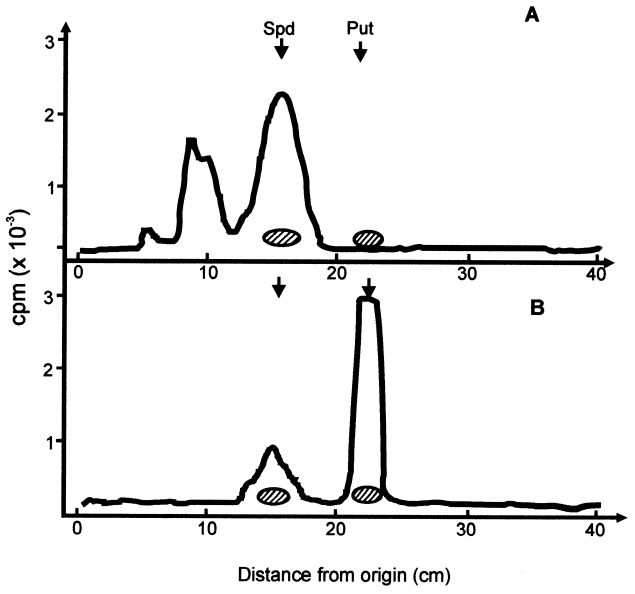

Fig. 3. Chromatographic analysis of ODC-transformed *T. cruzi* extracts prepared from parasites incubated with labelled putrescine in the absence (A) or presence (B) of 3 mM CHA. All other details are as in Fig. 2 and Section 2.3.

[25]. The inhibition of growth elicited by the mixture of DFMO and CHA could be overcome by the addition 0.1 mM spermidine (Fig. 1A,B). Neither spermine nor putrescine were able to reinitiate parasite proliferation, and several days after the addition of these polyamines to the cultures, both *Crithidia* and *T. cruzi* were lysed (results not shown). On the other hand, we have confirmed that spermidine cannot be converted to putrescine in these parasites (unpublished results).

3.2. Inhibition of in vivo conversion of putrescine into spermidine by CHA

Radioactive putrescine was added to trypanosomatid cultures in order to follow the synthesis of spermidine from putrescine. Fig. 2A shows that working with *C. fasciculata* cultivated in the absence of the spermidine synthase inhibitor, about 50% of labelled putrescine was transformed into spermidine. In addition, a small amount (about 8% of total radioactivity) of another compound not yet identified appeared in the chromatogram. When CHA was present in *Crithidia* cultures spermidine synthesis decreased markedly and the third radioactive spot was not detected (Fig. 2B). We have performed a similar experiment with several strains of wild-type and ODC-transfected *T. cruzi*. In the absence of CHA, putrescine was totally converted to spermidine and other unknown compounds which account for 30% of total radioactivity (Fig. 3A); upon the addition of the inhibitor, the formation of spermidine was drastically reduced and the other related compounds completely disappeared (Fig. 3B). The described results demonstrate that the biosynthesis of spermidine from putrescine could be strongly inhibited by CHA in *C. fasciculata* as well as in *T. cruzi*. It is worthwhile to point out that the radioactive compounds with lower mobilities than spermidine in the chromatograms depicted in Figs. 2A and 3A should presumably be spermidine derivatives, since they disappeared when spermidine synthase was inhibited by CHA.

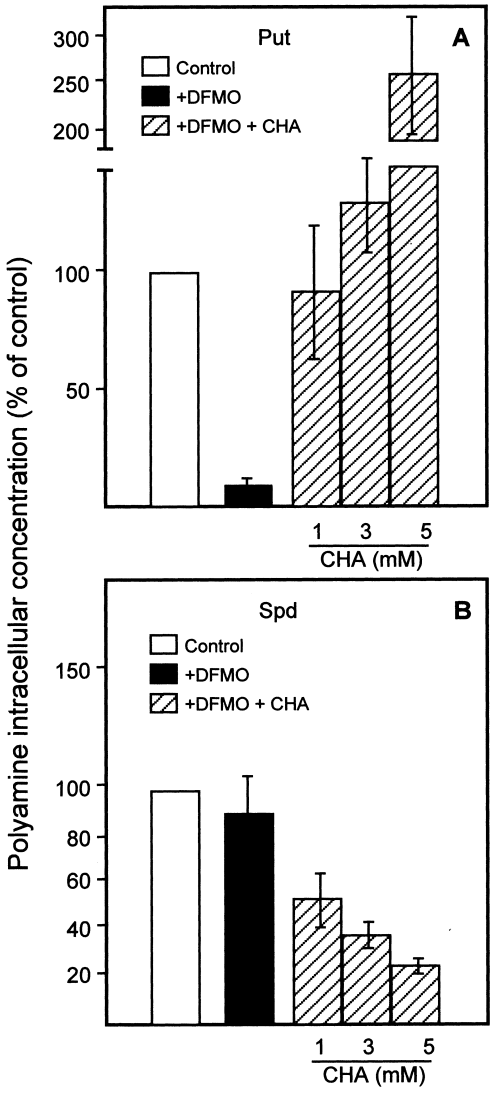

Fig. 4. Polyamine intracellular levels in *C. fasciculata* after cultivation for several passages with 5 mM DFMO in the absence and presence of different concentrations of CHA. A,B: The endogenous levels of putrescine and spermidine, respectively. All data are expressed as percentages of polyamine concentrations in *Crithidia* cultivated without any inhibitor (control). Values are the mean ± S.D. of three experiments.

3.3. Intracellular pools of polyamines

Analyses of endogenous polyamine pools in *C. fasciculata* cultivated for repeated passages under different conditions during 3–4 weeks have been performed by HPLC after benzoylation of the parasite extracts. The monogenetic trypanosomatid cultures were carried out in the absence and presence of DFMO or with a mixture of the ODC inhibitor and different levels of CHA. Only the *Crithidia* cultures containing both inhibitors at the same time stopped their proliferation, as shown in Fig. 1A. Although our data of polyamine internal pools were somewhat variable, we have consistently observed that in the presence of DFMO alone, the intracellular putrescine was depleted almost completely (Fig. 4A), while spermidine concentrations decreased only about 10–30% (Fig. 4B). Upon addition of increasing levels of CHA to cultures containing DFMO, a stepwise reduction of the internal spermidine was detected, with a concomitant accumulation of pu-

trescine which reached concentrations markedly higher than the normal values (Fig. 4A,B). *Crithidia* proliferation was partially decreased at 1 mM CHA and completely arrested at 3 mM or higher concentrations of the spermidine synthase inhibitor. Under the latter conditions, spermidine endogenous levels were reduced to one third or less of the values found in parasites cultivated in the absence of inhibitors (Fig. 4B).

The results described in the present study demonstrate that the polyamine requirement for normal proliferation of trypanosomatid protozoa can be fulfilled exclusively by spermidine, probably due to its unique role as substrate for trypanothione synthesis [11–13]. This conclusion was confirmed by the fact that *Crithidia* and ODC-transformed *T. cruzi* cultures inhibited by a mixture of DFMO and CHA can resume their normal growth and multiplication after supplementing the medium with exogenous spermidine (Fig. 1A,B). Neither spermine nor putrescine were able to replace spermidine in the restoration of normal proliferation.

In addition, we have found that after inhibition of *Crithidia* growth with a combination of DFMO and CHA, spermidine was markedly depleted with a simultaneous accumulation of putrescine. This fact strongly supports the idea previously suggested by several authors that parasites with an ODC of high turnover are tolerant to DFMO because they always contain a fraction of newly synthesized and active enzyme [30] sufficient to produce small amounts of putrescine rapidly converted into spermidine (and eventually into trypanothione), which can support protozoan proliferation.

Acknowledgements: We are grateful to Ms. Marta Bravo for HPLC determinations and Dr. S.H. Goldemberg for helpful discussions. This work was partially supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (Argentina), the National Research Council (CONICET, Argentina), the University of Buenos Aires, the Swedish Agency for Research Cooperation with Developing Countries (SAREC) and the United Nations Industrial Development Organization (UNIDO).

References

[1] Cohen, S. (1998) A Guide to the Polyamines, Oxford University Press, Oxford.
[2] Pegg, A.E. (1988) Cancer Res. 48, 759–774.
[3] Marton, L.J. and Pegg, A.E. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 55–91.
[4] Igarashi, K. and Kashiwagi, K. (2000) Biochem. Biophys. Res. Commun. 271, 559–564.
[5] Watanabe, S., Kusama-Eguchi, K., Kobayashi, H. and Igarashi, K. (1991) J. Biol. Chem. 266, 20803–20809.
[6] Pohjanpelto, P. and Hölttä, E. (1996) EMBO J. 15, 1193–1200.
[7] Ponagiotides, C.H., Artandi, S., Calame, K. and Silverstein, S.J. (1995) Nucleic Acids Res. 23, 1800–1809.
[8] Atkins, J.F., Lewis, L.B., Anderson, C.W. and Gesteland, R.F. (1975) J. Biol. Chem. 250, 5688–5695.
[9] Algranati, I.D. and Goldemberg, S.H. (1977) Trends Biochem. Sci. 2, 272–274.
[10] Igarashi, K. and Kashiwagi, K. (1999) Biochem. J. 344, 633–642.
[11] Fairlamb, A.H. and Cerami, A. (1985) Mol. Biochem. Parasitol. 14, 187–198.
[12] Fairlamb, A.H., Blackburn, P., Ulrich, P., Chait, B.T. and Cerami, A. (1985) Science 227, 1485–1487.
[13] Fairlamb, A.H. and Cerami, A. (1992) Annu. Rev. Microbiol. 46, 695–729.
[14] Ceriani, C., González, N.S. and Algranati, I.D. (1992) FEBS Lett. 301, 261–264.
[15] Ceriani, C. (1997) Ph.D. Thesis, University of Buenos Aires, Buenos Aires.
[16] Carrillo, C., Cejas, S., Cortés, M., Ceriani, C., Huber, A., González, N.S. and Algranati, I.D. (2000) Biochem. Biophys. Res. Commun. 279, 663–668.
[17] Bacchi, C.J. and Mc Cann, P.P. (1987) in: Inhibition of Polyamine Metabolism: Biological Significance and Bases for New Therapies (Mc Cann, P.P., Pegg, A.E. and Sjoerdsma, A., Eds.), pp. 317–344, Academic Press, Inc., Orlando, FL.
[18] Hunter, K.J., Strobos, C.A.M. and Fairlamb, A.H. (1991) Mol. Biochem. Parasitol. 46, 35–44.
[19] Sánchez, C.P., González, N.S. and Algranati, I.D. (1989) Biochem. Biophys. Res. Commun. 161, 754–761.
[20] Phillips, M.A., Coffino, P. and Wang, C.C. (1987) J. Biol. Chem. 262, 8721–8727.
[21] González, N.S., Sánchez, C.P., Sferco, L. and Algranati, I.D. (1991) Biochem. Biophys. Res. Commun. 180, 797–804.
[22] Roberts, S.C., Jiang, Y., Jardim, A., Carter, N.S., Heby, O. and Ullman, B. (2001) Mol. Biochem. Parasitol. 115, 217–226.
[23] Hibasami, H., Tanaka, M., Nagai, J. and Ikeda, T. (1980) FEBS Lett. 116, 99–101.
[24] Batchelor, K.W., Smith, R.A. and Watson, N.S. (1986) Biochem. J. 233, 307–308.
[25] Carrillo, C., Cejas, S., González, N.S. and Algranati, I.D. (1999) FEBS Lett. 454, 192–196.
[26] Segura, E.L., Subias, E., Esteva, M., Cabeza Meckert, P., Brozina, A. and Laguens, R.P. (1980) Medicina (Buenos Aires) 40, 97–102.
[27] Brun, R. and Schonenberger, M. (1979) Acta Trop. 36, 289–292.
[28] Kaur, K., Emmett, K., Mc Cann, P.P., Sjoerdsma, A. and Ullman, B. (1986) J. Protozool. 33, 518–521.
[29] Morgan, D.M.L. (1998) in: Polyamine Protocols (Morgan, D.M.L., Ed.), pp. 111–1118, Humana Press, Totowa, NJ.
[30] Heby, O. and Persson, L. (1990) Trends Biochem. Sci. 15, 153–158.