Leishmania donovani: Thionins, plant antimicrobial peptides with leishmanicidal activity

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Abstract

The leishmanicidal activity of plant antibiotic peptides (PAPs) from the principal families, such as wheat thionins, a barley lipid transfer protein and potato defensins and snakins, were tested in vitro against Leishmania donovani. Only thionins and defensins were active against this human pathogen at a low micromolar range of concentrations. Thionins resulted as the most active peptides tested until now. They collapsed ionic and pH gradients across the parasite plasma membrane together with a rapid depletion of intracellular ATP without affecting mitochondrial potential. Hence, the lethal effect of thionins was mostly associated to permealization of the plasma membrane leading to an immediate death of the parasite. The present work is the first evidence for leishmanicidal activity in plant peptides. Future prospects for their development as new antiparasite agents on human diseases are considered.

1. Introduction

Plants and animals produce antimicrobial peptides against their pathogens, either expressed constitutively or induced after pathogen contact (Jenssen et al., 2006). Most of these peptides, polycationic and amphipathic, cause cell lysis upon interaction with the phospholipid matrix of the plasma membrane (Shai, 2002). Animal linear antimicrobial peptides have been extensively studied on prokaryotes using chemically synthesized analogs (Jenssen et al., 2006). Peptide specificity on prokaryotes is fairly understood and mainly based on the exposure of acidic phospholipids at the outer leaflet of their plasma membrane (Shai, 2002). However, most PAPs that are active on phytopathogens such as thionins, defensins, lipid transfer proteins or snakins (Castro and Fontes, 2005; Berrocal-Lobo et al., 2002) are not linear, but possess multiple disulfide bonds which impose a more rigid structure and afford a higher stability of the peptide in the plant (Giudici et al., 2006). Recently a Pyrularia pubera thionin was synthesized chemically showing identical bactericidal activity in vitro as the native peptide (Vila-Perello et al., 2003). Approaches like that open new possibilities to the production of plant peptides to treat human infections. Surprisingly, the in vitro activity of PAPs has not been tested until now against Protozoa. For that reason the principal objective of present work was to test the activity of selected PAPs on Leishmania donovani promastigotes, the causative agent of human visceral Leishmaniasis. Leishmania promastigotes imposes important restrictions to the peptide activity. Their surface shows a high metalloproteinase activity, a thick anionic glycocalix with lipophosphoglycan, and a restrictive membrane repairing mechanism confined to the flagellar pocket (Handman, 1999). Different isoforms of thionins from wheat (Triticum aestivum), a barley (Hordeum vulgare) LTP2 (lipid transfer protein), a potato (Solanum tuberosum) defensin PTH1 and a potato snakin1, were tested against Leishmania promastigotes in vitro. In a further step, the mechanism of action of thionins, as the peptides with highest leishmanicidal activity, was studied. Some parameters related to the bioenergetic of the parasite and the membrane permeabilization, such as pH, ATP levels, the mitochondrial membrane potential and the H+/OH- gradients were measured. Our work shows that PAPs previously active against phytopathogens are also effective against...
the human pathogen Leishmania. These results may foster the use of plant antimicrobial compounds as a new source of leishmanicidal agents.

2. Materials and methods

Promastigotes from L. donovani strain (MHOM/SD/00/15-2D) were grown at 25 °C in RPMI at pH 7.2 as previously described (Luque-Ortega et al., 2003). A mixture of equimolar concentrations of the α-1, α-2 and β type I thionins from wheat (Triticum aestivum) isoforms, a barley (Hordeum vulgare) LTP2 (lipid transfer protein) α-1 isoform, and a PTH1 defensin and snakin1 from potato (Solanum tuberosum), were purified by HPLC as previously described (Caaveiro et al., 1997). Reagents were obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO). Antimicrobial activity was measured by inhibition on MTT reduction by L. donovani promastigotes as described [8]. Briefly, parasites at 2 × 10^7 cells/ml were incubated at 24 °C with different peptide concentrations for 2 h. Afterwards, parasites were washed, resuspended in 100 µl Hanks’s Glc containing MTT (0.5 mg/mL), and incubated for 2 h at 24 °C. The resulting precipitated formazan was solubilized by SDS addition and read in a 450 Bio-Rad ELISA microtiter plate with a 600 nm filter as previously described (Luque-Ortega et al., 2003). For proliferation experiments, promastigotes (2 × 10^7 cells/ml) were incubated 2 h with the peptides, washed and resuspended (2 × 10^6 cells/ml) in their culture medium. Proliferation was then estimated after 48 h by MTT reduction as previously shown (Luque-Ortega et al., 2003). Experiments were done at least twice and samples were run in triplicate. LC50 and IC50 values were calculated by Litchfield and Wilcoxon procedure as previously described (Luque-Ortega et al., 2003). Effect of thionins on membrane permeation and intracellular pH changes were reached using a pH-sensitive fluorescence dye BCECF-AM, Leishmania promastigotes were preloaded with the BCECF-AM at 25 °C in the dark and later washed. Drugs were added after fluorescence stabilization. Afterwards fluorescence was measured in a spectrofluorometer at 500-nm (excitation) and 525-nm (emission) as previously described (Luque-Ortega et al., 2003). Full permeabilization was reached with Triton X-100. The decrease in intracellular ATP drop caused by thionins was assessed by luminance in promastigotes transfected with rhodamine 123 accumulation after thionin treatment. Traces correspond to the peptide concentrations are continuous. The luminance of untreated parasites is shown as dotted trace, (d) Mitochondrial membrane potential of L. donovani promastigotes measured by thionin treatment. Traces: [A] Uncoupled parasites with 7.5 µM FCCP; [B] Untreated parasites: [C-E] Intracellular ATP decrease after thionin addition assessed by luminescence in vivo on L. donovani promastigotes. Traces correspond to the peptide concentrations are continuous. The luminance of untreated parasites is shown as dotted trace. (d) Mitochondrial membrane potential of L. donovani measured by rhodamine 123 accumulation after thionin treatment. Traces: [A] Uncoupled parasites with 7.5 µM FCCP; [B] Untreated parasites; [C-E] 0.5, 1.0 and 2.5 µM thionins, respectively.

3. Results and discussion

Thionins showed the highest activity among the different set of PAPs assayed showing IC50 = 0.2 (0.1-0.5) µM and an LC50 = 1.1 (0.9-1.3) µM for parasite proliferation (Fig. 1a). Activity of thionin α-1 isoform was almost identical to that of the type I mixture (data not shown). Amastigotes, the intracellular form of the parasite, were more resistant to thionins, with LC50 = 46.3 (36.6-59.2) µM, than promastigotes. As the expression of LPG and Gp63 is close to those in amastigotes, the change in phospholipid composition rather than peptide structure, likely underlie this difference between those Leishmania stages. In terms of concentration the leishmanicidal activity of thionins is very similar to those described for a broad spectrum of bacteria, fungi or eukaryotic cells (Castro and Fontes, 2005). Defensin (PTH1) kills Leishmania at higher concentrations than thionins with IC50 = 33.4 (21.5-52.4) µM and LC50 = 5.9 (3.2-10.8) µM (Fig. 1a). The fungal activity of radish defensin involved a specific membrane receptor, Ca2+ influx, and G protein activity (Castro and Fontes, 2005). Whereas these or similar features exist in Leishmania, this would require further study. LTP2 and snakin1 were virtually inactive at the concentrations tested; showing LC50 higher than 100 µM (Fig. 1a).

Due to their highest activity, thionins were selected for a detailed study. This activity was dependent on temperature and pH, decreasing dramatically below 25 °C and pH 7, respectively, and was inhibited by Ca2+ (94% inhibition at 1 mM), heparin and serum albumin (Table 1), both known quenchers of hydrophobic compounds. Nevertheless, these conditions are far to be physiological for cell survival. However, thionin activity increased with ionic strength which, in some way, may promote higher hydrophobic interactions with parasitic membrane (Table 1). Depolarization by valinomycin only increased modestly the parasite survival, discarding membrane potential as an essential factor for thionin effectiveness (Table 1). At 1 µM thionins dissipated H+/OH- gradients across the plasma membrane of Leishmania promastigotes, inducing a rapid equilibration between internal and external pH. Total permeation was reached with Triton X-100 (right arrow at Fig. 1b). Furthermore, this permeation lead to drop in free intracellular ATP levels with 80% of their effect reached in just 2 min even at concentrations lower than their corresponding IC50 or LC50 (Fig. 1c). This quick kinetics supports the membrane permeability as an essential step in the lethal mechanism of thionins on Leish-
manco as reported for other membrane-active compounds (Luque-Ortega et al., 2003). Additionally, it makes unlikely the establishment of mixed disulfides bounds between key metabolic enzymes and thionins previously described in vitro as essential for lethality [10]. Furthermore the maintenance of electrochemical potential in mitochondria, at least for a time range where the drop in free ATP takes place, ruled out the inhibition of thionins on mitochondrial ATP synthesis. as described for histatin 5 in Leishmania (Luque-Ortega et al., 2008) (Fig. 1d). Cytotoxicity of Pyruiaria (Luque-Ortega et al., 2008) (Fig. 1d). Cytotoxicity of Pyruiaria thionin: a single-residue mutation enhances activity against Gram-negative bacteria. FEBS Letters 536, 230-236.

Plant thionins are among the highest efficient antimicrobial peptides tested against L. donovani promastigotes so far. This work and recent works related to thionin activity against other human pathogens (Vila-Perello et al., 2003; Stec, 2006) open new therapeutic possibilities to PAPs on human infectious diseases. Including that thionin activity, although lower, was still preserved on amastigotes, the effect on promastigote might be enough to stop the infection before happen. Further studies addressing the pharmacological applications of thionins and the therapeutic potential of their shorter synthetic analogs are currently in progress.

Acknowledgments

We thank Dr. Juan Román Luque-Ortega and Dra. Katrina Ramonell for critical reading of the manuscript. Mutated luciferase gene was kindly provided by Toni Aebischer.

This work was supported by research grants from the Consejo Superior de Investigaciones Científicas (CSIC; P1F 80F171), Fondo de Investigación Sanitaria (FIS PI06115 and RD 06/0221/0006) to LR, BIO2006-07198 to J.-P., BIO2006-07198, BIO2003-4424 and BIO2006-04488 to A.M.

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