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Cysteine-protease activity elicited by Ca^{2+} stimulus in *Plasmodium*

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Abstract

Bloodstage malaria parasites require proteolytic activity for key processes as invasion, hemoglobin degradation and merozoite escape from red blood cells (RBCs). We investigated by confocal microscopy the presence of cysteine-protease activity elicited by calcium stimulus in *Plasmodium chabaudi* and *Plasmodium falciparum* in free trophozoites or for the later parasite within RBC using fluorescence resonance energy transfer (FRET) peptides. Peptide probes access, to either free or intraerythrocytic parasites, was also tested by selecting a range of fluorescent peptides (653–3146 Da molecular mass) labeled with Abz or FITC. In the present work we show that Ca^{2+} stimulus elicited by treatment with either melatonin, thapsigargin, ionomicin or nigericin, promotes an increase of substrate hydrolysis, which was blocked by the specific cysteine-protease inhibitor E-64 and the intracellular Ca^{2+} chelator, BAPTA. When parasites were treated with cytoplasmic Ca^{2+} releasing compounds, a cysteine-protease was labeled in the parasite cytoplasm by the fluorescent specific irreversible inhibitor, Ethyl-Eps-Leu-Tyr-Cap-Lys(Abz)-NH_2, where Ethyl-Eps is Ethyl-(2*S*,3*S*)-oxirane-2,3-dicarboxylate. In summary, we demonstrate that *P. chabaudi* and *P. falciparum* have a cytoplasmic dependent cysteine-protease activity elicited by Ca^{2+}.

Keywords: Malaria; Calpain; Cysteine-protease; Melatonin; Calcium

1. Introduction

Malaria is one of the most important infectious diseases in the world, being responsible for more than one million deaths each year, most of them children under five (WHO, www.rbm.who.int). The cell cycle of *Plasmodium* within red blood cells (RBCs) is marked by its 24 h multiplicity [1,2] according to the host circadian rhythm. The host melatonin hormone peak during the night period [3,4] has been shown to modulate the *Plasmodium* cell cycle through a cytoplasmic Ca^{2+} increase resulting in parasite synchronization [5,6]. Calcium homeostatic control permits a series of related processes to be readily turned on and off in response to ion mobilization from the major intracellular calcium reservoirs, the endoplasmic reticulum, mitochondria and acidic compartments [7–9]. Molecular and cellular studies on Ca^{2+} signaling in *Plasmodium* parasites [10,11] have revealed the Ca^{2+} storage organelles to include the endoplasmic reticulum based in its sensitivity to Ca^{2+}-ATPase inhibitors such as thapsigargin and ciclopiazonic acid [12–19] and acidic Ca^{2+} pool [15–17,20]. In addition, mitochondria are thought to play a role in Ca^{2+} homeostatic control in these parasites [21]. Exposure to a high extracellular Ca^{2+} concentration might create appropriate conditions for the use of Ca^{2+} as a second messenger in the modulation of parasite intracellular signaling events [22].

Downstream mechanisms related to melatonin and Ca^{2+} signaling might involve molecular changes such as protein activation or switches in gene expression in the parasites. Such events usually involve remodeling proteolysis [23–26], a key-driving element able to activate or inactivate intermediate regulating factors, as it occurs with cyclins in the progression of cell cycle in mammals [27,28].

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Cysteine-proteases are involved in the progression of the intraerythrocytic life cycle, with roles in degradation of hemoglobin [26,29–31] and erythrocyte cytoskeletal proteins, such as ankyrin and band 4.1 [32], and erythrocyte rupture [33]. We report in this work the presence of a cysteine-protease activity elicited by Ca^{2+}-in malaria parasites, which was detected by hydrolysis of FRET peptides and inhibited by an irreversible fluorescent-labeled cysteine-protease inhibitor. The FRET peptides and the inhibitor Ethyl-Eps-Leu-Tyr-Cap-Lys(Abz)-NH_2 (Abz-peptidyl-epoxide) were incorporated by the parasites and the hydrolytic activity or enzyme labeling by inhibitor were observed at the same time as the cytoplasmic Ca^{2+} release. The loading of peptides into free parasites and parasites inside RBC have been described previously [34]. Here we further investigate the permeation of peptides into the *Plasmodium* developmental stages (ring, trophozoite, and schizont) using fluorescent Abz- and FITC-peptides of molecular weight ranging from 653 to 3146 Da.

2. Materials and methods

2.1. Reagents

Ionomycin, nigericin, thapsigargin (Thg), melatonin, PMSF, pepstatin A, E-64, saponin, probenecid, L-polylysine, and MOPS were purchased from Sigma-Aldrich (St. Louis, MO). BAPTA and Fluo-4 AM were from Molecular Probes, Inc. (Eugene, OR). All other reagents were analytical grade.

2.2. Plasmodium falciparum parasites

P. falciparum parasites (Palo Alto strain) were maintained in continuous in vitro culture in adult RBC [35] and synchronization was achieved by sorbitol treatment [36]. Parasitemias were determined from microscopic examination of Giemsa-stained thin films.

2.3. Plasmodium chabaudi parasites

P. chabaudi parasites were maintained by synchronous parasitemia in mice (Balb/C strain) by weekly transfer infection. Leukocytes and platelets were removed from whole blood by filtration through a powdered cellulose column (Whatman CF11). The infected RBC's were then washed twice by centrifugation at 1500 × *g* for 7 min in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, and 1 mM $CaCl_2$). Infected RBC (10^7 ml^{-1}) were lysed in PBS with 60 μg ml^{-1} saponin. RBC membranes were removed by centrifugation (9000 × *g* for 10 min at 4 °C), the parasites were washed twice in MOPS buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, 50 mM MOPS, and 1 mM $CaCl_2$, pH 7.2) and dispensed in the same buffer.

2.4. Peptide synthesis

The FRET peptides Abz-KLRSSKQ-EDDnp and Abz-AIKFFARQ-EDDnp, where the fluorescent group is Abz (*ortho*-aminobenzoic acid) and the quencher group is EDDnp (ethylene diamine-2-4-dinitrophenyl) were synthesized by the solid-phase synthesis method [37], using the Fmoc procedure with EDDnp attached to a glutamine residue. An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system; Shimadzu) was used for the synthesis. All peptides obtained were purified by semi-preparative HPLC using an Econosil C18 column. The molecular mass and purity of synthesized peptides were checked by amino acid analysis and mass spectroscopy with MALDI-TOF (matrix assisted laser-desorption ionization—time-of-flight), using a TOFSpec E instrument (Micromass, Manchester, UK). The stock solutions of EDDnp peptides were prepared in DMSO/water (1:1), and the concentrations were measured spectrophotometrically using molar absorption coefficient of 17,300 M^{-1} cm^{-1} at 365 nm. The peptides Abz-GFSPFR-NH_2, Abz-KRPPGFSPFR-NH_2, and Abz-KRPPGFSPFRSS-NH_2 were synthesized by solid-phase methodology and Abz was introduced in the last coupling as Boc-Abz. The peptides $(FITC)_2$-KRPPGFSPFR-NH_2, $(FITC)_2$-KRPPGFSPFRSS-NH_2, and $(FITC)_2$-GLMISLMKRPPGFSPFRSSIR-NH_2 were also synthesized by solid-phase methodology and fluorescein (FITC) was introduced into the peptides after the cleavage of the peptides from the resin, therefore, two molecules of FITC were attached to each peptide, one at the α-amino group of the N-terminal amino acid and the other at the ε-amino group of Lys.

2.5. Synthesis of the cysteine-protease affinity probe Ethyl-Eps-Leu-Tyr-Cap-Lys(Abz)-NH_2

Ethyl-(2*S*,3*S*)-oxirane-2,3-dicarboxylate (Ethyl-Eps) was obtained as earlier described [38]. The peptide segment was obtained by solid-phase peptide synthesis using Fmoc-Rink amide resin given the sequence: Leu-Tyr(tBut)-Cap-Lys(Boc-Abz)-Resin. Ethyl-Eps was linked at the amino group of leucine using HBTu/HOBt, NMM and DMF as solvent. The peptide was then cleaved from the resin using the solution 95% TFA, 2.5% water, and 2.5% anisole. The product was collected, lyophilized and purified in a C18 reverse phase column in a HPLC system (Shimadzu, Japan) using a linear gradient of 10–80% water–acetonitrile (0.1% TFA). Electron spray mass spectrum (Micromass, Manchester, UK) [*M* + H] calculated for $C_{40}H_{57}N_7O_{10}$ 796.95, found 796.84.

2.6. Spectrofluorimetric determinations

Free parasites (10^7 cells ml^{-1}) were incubated with MOPS buffer in 3 ml cuvettes. The measurements were performed in a Shimadzu RF-5301 PC spectrofluorimeter at 37 °C and fluorescence was measured continuously after the addition of