Analytical Biochemistry 468 (2015) 22–27

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Specific calpain activity evaluation in *Plasmodium* parasites

Mayrim M. Gomes [a,c], Alexandre Budu [b], Priscilla D.S. Ventura [c], Piero Bagnaresi [b], Simone S. Cotrin [b], Rodrigo L.O.R. Cunha [d], Adriana K. Carmona [b], Luiz Juliano [b], Marcos L. Gazarini [c,*]

[a] Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, UNIFESP, São Paulo, SP, Brazil
[b] Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo, SP, Brazil
[c] Departamento de Biociências, Universidade Federal de São Paulo, Santos, SP, Brazil
[d] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André, SP, Brazil

ARTICLE INFO

Article history:
Received 1 July 2014
Received in revised form 13 August 2014
Accepted 6 September 2014
Available online 2 October 2014

Keywords:
Plasmodium
Proteases
Calpain
Calcium signaling
Calmodulin

ABSTRACT

In the intraerythrocytic trophozoite stages of *Plasmodium falciparum*, the calcium-dependent cysteine protease calpain (*Pf*-calpain) has an important role in the parasite calcium modulation and cell development. We established specific conditions to follow by confocal microscopy and spectrofluorimetry measurements the intracellular activity of *Pf*-calpain in live cells. The catalytic activity was measured using the fluorogenic Z-Phe-Arg-MCA (where Z is carbobenzoxy and MCA is 4-methylcoumaryl-7-amide). The calmodulin inhibitor calmidazolium and the sarcoplasmic reticulum calcium ATPase inhibitor thapsigargin were used for modifications in the cytosolic calcium concentrations that persisted in the absence of extracellular calcium. The observed calcium-dependent peptidase activity was greatly inhibited by specific cysteine protease inhibitor E-64 and by the selective calpain inhibitor ALLN (*N*-acetyl-L-leucyl-L-leucyl-L-norleucinal). Taken together, we observed that intracellular *Pf*-calpain can be selectively detected and is the main calcium-dependent protease in the intraerythrocytic stages of the parasite. The method described here can be helpful in cell metabolism studies and antimalarial drug screening.
© 2014 Elsevier Inc. All rights reserved.

Malaria is an aggressive disease that is responsible for millions of deaths annually [1]; therefore, it is urgent to identify new drug targets and develop new treatment strategies. *Plasmodium* proteases participate in essential events for parasite life, including the invasion of and egress from erythrocytes and hemoglobin degradation for the acquisition of amino acids [2–4]. Malaria parasite proteases, such as aspartic, cysteine, and serine proteases and metalloproteases, have been considered potential therapeutic targets [5–7]. Falcipain-2 and falcipain-3, which are the best-studied members of this class, are located in the parasite's food vacuole and are essential for the hydrolysis of hemoglobin [8] and development [9]. In addition, part of *Plasmodium* protease activities are regulated by cellular events with release calcium [10], which is an important second messenger that regulates various functions in eukaryotic cells such as protein secretion, gene expression, and cellular development [11,12]. Calpain, a calcium-dependent cysteine protease expressed in mammals and other organisms, has an ortholog identified in *P. falciparum* that has been associated with the development and invasion and egress of the parasite from the host cell [7,13–18]. The *Plasmodium* genome encodes a unique calpain [15,19] that is expressed during all intraerythrocytic stages and possesses high sequence similarity to *Caenorhabditis elegans* calpain, which is classified as an atypical calpain [19]. However, there is a lack of biochemical data related to this enzyme, most likely due to the unusual gene size (6.147 bp) [15]. Studies performed on rodent malaria parasite species (*Plasmodium chabaudi*) and parasites responsible for human malaria (*Plasmodium falciparum*) have shown that both maintain nanomolar levels of cytosolic calcium during maturation [20] with spontaneous oscillations [21,22] and use specific organelles (endoplasmic reticulum, acidic compartments, and mitochondria) to store this ion [23,24]. In the erythrocyte egress step, the increase in the parasite's cytoplasmic calcium is essential and most likely is related to calpain activation and erythrocyte cytoskeleton destabilization [25]. The growth of *Plasmodium* parasites is inhibited by a variety of Ca^{2+} ionophores, Ca^{2+} channel blockers, calcium chelators, and calmodulin inhibitors [26–28], showing the importance of this ion to parasite survival and development.

Here, we report a selective measurement method to evaluate intracellular calpain activity in *Plasmodium* parasites and the influence of calcium homeostasis disruption by inhibiting calmodulin or endoplasmic calcium pump.

* Corresponding author. Fax: +55 13 32218058.
E-mail addresses: marcos.gazarini@unifesp.br, marcos.gazarini@gmail.com (M.L. Gazarini).

http://dx.doi.org/10.1016/j.ab.2014.09.005
0003-2697/© 2014 Elsevier Inc. All rights reserved.

Materials and methods

Materials

The substrate Z-Phe-Arg-MCA (where Z is carbobenzoxy and MCA is 4-methylcoumaryl-7-amide)[1] and the inhibitors phenylmethylsulfonyl fluoride (PMSF), pepstatin, *ortho*-phenanthroline, E-64 , *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN), thapsigargin (THG), and calmidazolium (CZ) were purchased form Sigma (St. Louis, MO, USA) The peptide Bz-LR-MCA was purchased from AminoTech (São Paulo, Brazil).

Mice and P. chabaudi infection

P. chabaudi parasites (clone AS) were maintained by weekly infection of male Balb/C mice (20–40 g initial body weight, 12 weeks initial age). The animals were housed in a temperature-controlled room (22 °C) with a 12-h dark–light cycle and infected intraperitoneally by injecting 10^5 infected erythrocytes. At the peak of parasitemia, animal euthanasia was performed by cervical dislocation followed by leukocyte/platelet removal with filtration through a powdered cellulose column (Whatman CF11) [29]. The isolation of parasites was performed as described elsewhere [30].

P. falciparum

P. falciparum (3D7 strain) was cultured as described previously [31]. The parasites were isolated from red blood cells when cultures reached approximately 10% parasitemia using a procedure described elsewhere [30].

Spectrofluorimetric assay to assess the proteolytic activity and calcium concentration of isolated parasites

Isolated parasites in suspension (10^6 cells/ml) were transferred to a quartz cuvette or 96-well plate containing Mops buffer and were maintained at 37 °C in a Hitachi F-7000 spectrofluorimeter (Tokyo, Japan). Proteolytic activity was monitored measuring the hydrolysis of the substrate Z-Phe-Arg-MCA. The wavelengths of the fluorimeter were set to λ_{ex} = 380 nm and λ_{em} = 460 nm with a slit of 10/10 nm. The activity observed corresponded to arbitrary fluorescence units (AFU) measured after 10 min of incubation in the presence or absence of the following drugs: endoplasmic reticulum Ca^{2+} ATPase inhibitor THG (10 μM), calmodulin inhibitor CZ (5 μM), calpain inhibitor ALLN (0.05–0.5 μM), reducing agent dithiothreitol (DTT, 2 mM), aspartyl protease inhibitor pepstatin A (1 μM), serine protease inhibitor PMSF (10 μM), metalloprotease inhibitor *ortho*-phenanthroline (1 mM), divalent cation chelator ethylenediaminetetraacetic acid (EDTA, 5 mM), and cysteine protease inhibitor E-64 (10 μM). As a control, dimethyl sulfoxide (DMSO) was added in the same volume as the drugs to test the parasite's viability and the proteolytic activity. Z-Phe-Arg-MCA and THG (10 μM) in a Mops buffer suspension without parasites was also tested.

For calcium measurements, parasites were incubated for 50 min at 37 °C in Mops buffer supplemented with 5 μM of the calcium indicator Fluo-4 AM (Molecular Probes) and 1 mM probenecid, which minimizes indicator extrusion and compartmentalization. Subsequently, the cells were washed twice with the same buffer and transferred to a quartz cuvette. Intracellular calcium was measured using a Hitachi F-7000 spectrofluorimeter by continuous measurement of the fluorescence variation at λ_{ex} = 505 nm and λ_{em} = 530 nm. Maximal fluorescence (F_{max}) was determined after the lysis of parasites with digitonin (33.3 μM), and minimal fluorescence (F_{min}) was determined after adding ethyleneglycoltetraacetic acid (EGTA, 100 μM). The cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) was calculated using K_d = 345 nM: $[Ca^{2+}]_{cyt} = 345 \times [(F - F_{min})/F_{max} - F)]$ [32].

Confocal microscopy

Dynamic imaging was performed using an LSM 510 META laser scanning microscope (Carl Zeiss, Germany) equipped with a 63× water immersion objective. The parasites were added to microscope cover slips (MatTek, USA) pretreated for 1 h with L-polylysine solution and excited at 365 nm. The emitted light was collected through a bandpass filter at 387 to 470 nm. Transmitted light observations were performed during the experiments to assess the integrity of the cells. The measure expressed in AFU was acquired from an average of selected whole-parasite areas. The isolated parasites were resuspended in Mops buffer (pH 7.2), and the catalytic activity was measured using 10 μM Z-Phe-Arg-MCA as the substrate. The results are representative of at least three experiments.

Results and discussion

Modulation of proteolytic activity by calcium in P. falciparum and P. chabaudi

Fig. 1 shows our refined cell assay that allowed exclusive measurements of calcium-dependent peptidase activity by exploring the difference in the activation mechanisms of calpain versus calcium-independent cysteine proteases. The latter enzymes can be fully activated by DTT, whereas calpain requires becoming activated by both DTT and calcium. Furthermore, the specific cysteine

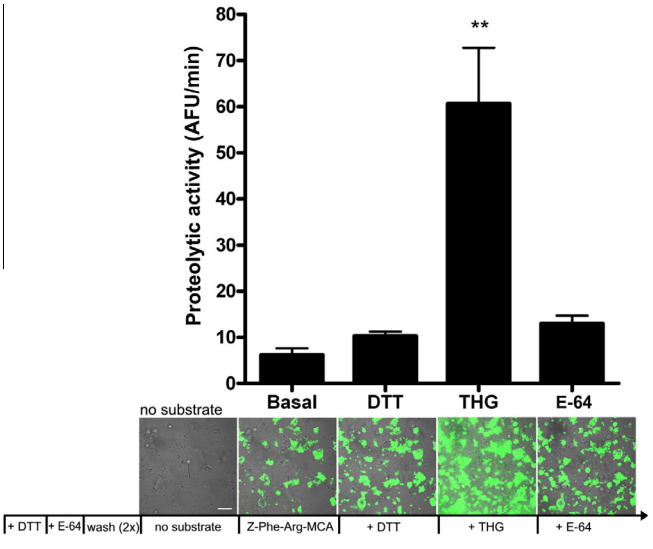

Fig.1. Live cell measurement of calcium-dependent thiol proteolysis (calpain) of *P. chabaudi* parasites using Z-Phe-Arg-MCA as substrate. Isolated *P. chabaudi* trophozoite cells were preincubated with DTT (2 mM, 2 min), followed by inhibition with E-64 (10 μM, 5 min) and by two washes with Mops buffer (pH 6.5) for the removal of free inhibitor. The samples were then transferred to a cuvette. Each addition step was also performed using confocal microscopy and is represented by fluorescence images, as indicated. Additions where performed sequentially on the same preparation as in the order indicated. The data were compared with a one-way analysis of variance and a Newman–Keuls posttest. $^{**}P < 0.001$ ($n = 3$).

[1] *Abbreviations used:* Z, is carbobenzoxy; MCA, 4-methylcoumaryl-7-amide; PMSF, phenylmethylsulfonyl fluoride; ALLN, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; THG, thapsigargin; CZ, calmidazolium; AFU, arbitrary fluorescence units; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycoltetraacetic acid; ER, endoplasmic reticulum.

Download English Version:

https://daneshyari.com/en/article/7558728

Download Persian Version:

https://daneshyari.com/article/7558728

Daneshyari.com