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The effect of kinase, actin, myosin and dynamin inhibitors on host cell egress by *Toxoplasma gondii*

Lucio Ayres Caldas [a,b], Sergio Henrique Seabra [c], Márcia Attias [a,b,*], Wanderley de Souza [a,b,d]

[a] Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil
[b] Instituto Nacional de Ciência e Tecnologia em Biologia Estrutural e Bioimagens, Inbeb, Brazil
[c] Universidade Estadual da Zona Oeste, Rio de Janeiro, Brazil
[d] Instituto Nacional de Metrologia, Qualidade e Tecnologia, Inmetro, Rio de Janeiro, Brazil

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ABSTRACT

Toxoplasma gondii is a protozoan parasite that can infect the nucleated cells of all warm-blooded animals. Despite its medical and veterinary importance, the egress of *T. gondii* from host cells has not been fully elucidated. This process is usually studied with calcium ionophores, which artificially trigger *T. gondii* egress. Among the diverse signaling events that take place during egress, kinases appear to play a crucial role. In this work we employed several kinase inhibitors to examine their role in egress: although parasite egress was only slightly impaired by treatment with the PI3K and PKC inhibitors wortmannin and staurosporine, the addition of the tyrosine kinase-specific inhibitor genistein efficiently blocked the exit of parasites by more than 50%. IPA-3, a non-ATP-competitive inhibitor of p21-activated kinases, which play a role in actin cytoskeleton remodeling inhibited egress of *T. gondii* by only 15%. The myosin motor inhibitor blebbistatin and the actin polymerization inhibitor cytochalasin D also blocked the egress of *T. gondii*. Nevertheless, dynasore, which is known to block the GTPase activity of dynamin, had little or no effect on *T. gondii* egress.

1. Introduction

The protozoan parasite *Toxoplasma gondii* can infect virtually any warm-blooded animal, and it is responsible for toxoplasmosis, an important cause of abortion and malformation in unborn children, life-threatening encephalitis in immunocompromised individuals and other significant effects on the behavior of people and animals [1–3]. Livestock, such as pigs and cattle, play important roles in the transmission process, and they are associated with seasonal outbreaks in certain countries [4–6]. Acute toxoplasmosis results from the rapidly dividing tachyzoite form, which invades host cells and ultimately resides in a parasitophorous vacuole (PV) that increases in size as the parasite divides [7,8]. Upon successive rounds of replication, the parasites leave the PV, eventually carrying remnants of the PV membrane (PVM) and the endoplasmic reticulum membranes towards the extracellular medium [9].

Calcium concentrations within *T. gondii* have been shown to increase prior to both invasion and egress from the host cell [10,11]. It has also been reported that cytoplasmic K^+ and perforins play a role in egress [12,13]. Nevertheless, very little is known about this process, which has predominantly been studied in cells that have been treated with the calcium ionophore A23817 [14–17].

Some reports have pointed to similarities between the processes of host cell invasion and egress by *T. gondii* [10]. Recent studies have shown that some *T. gondii* calcium-dependent protein kinases, such as TgCDPK1 and TgCPDK3, are crucial for intracellular parasite microneme secretion [18], the latter being specially involved in parasite egress [19]. However, the intracellular cycle of *T. gondii* also depends on host cell factors as previously demonstrated and some kinase inhibitors – wortmannin, staurosporine and genistein, which are known to block PI3K, PKC and tyrosine kinase activities, respectively – have been shown to effectively block calcium ionophore A23817-induced invasion [20] and egress at early stages of infection [17]. The effects of the myosin II inhibitor blebbistatin [21] and the actin depolymerizing drug cytochalasin D on host cell egress were also investigated. Certain cytoskeletal proteins, such as actin and myosin, are known to play a role in host cell invasion by *T. gondii* [22]. These components are involved in processes such as plasma membrane rearrangement, which takes place during endocytosis, as well as vesicular and vacuolar trafficking [23,24]. While blebbistatin blocks the ATPase activity of myosin II, an actin-based molecular motor, cytochalasin D interferes both with parasite invasion and egress [25–27]. Actin filaments were labeled with colloidal gold and fluorescent markers to examine their possible role as a substrate for *T. gondii* gliding during egress. The involvement of actin polymerization and cell motility makes use of IPA-3, a highly selective

* Corresponding author at: Instituto de Biofísica Carlos Chagas Filho, Av. Carlos Chagas Filho, 373 Prédio do CCS, Bloco G- sala 19 Cidade Universitária. Ilha do Fundão, Cep 21941-902 Rio de Janeiro, RJ, Brazil. Tel./fax: +55 21 22602364.
E-mail address: mattias@biof.ufrj.br (M. Attias).

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non-ATP-competitive inhibitor of Pak1, prior to *T. gondii* egress attractive [28,29].

Finally, dynamin is known for catalyzing membrane fission during endocytosis. However, studies have raised the possibility that it also plays a role in exocytosis through global changes in the plasma membrane–cytoplasm interface [30–32]. Although *T. gondii* egress does not appear to be an exocytic-like event, the direct participation of dynamin on actin cytoskeleton regulation [33] led us to investigate the importance of dynamin during *T. gondii* egress from host cells. Three dynamin-related proteins have recently been identified in the genome of Apicomplexan parasites. In particular, reductions in *Tg*DrpA and *Tg*DrpB expression reportedly led to deficiencies in apicoplast division and motility, respectively [34,35]. To fully examine the role of dynamin in *T. gondii* egress, we employed dynasore, a compound that interferes with the multidomain GTPase dynamin, to block the activity of dynamin1, dynamin2 and the mitochondrial dynamin Drp1 [36,37].

2. Material and methods

2.1. Chemicals

Wortmannin, staurosporine (antibiotic AM-2282 from *Streptomyces* sp.), genistein (4′,5,7-trihydroxyisoflavone) and cytochalasin D (C30H37NO6) were diluted, to 1 mM, 100 mM, 20 mM and 59 mM stock solutions, respectively, in dimethyl sulfoxide (DMSO). The stock solutions for IPA-3 (2,2′-dihydroxy-1,1′-dinaphthyldisulfide) and blebbistatin (1-phenyl-1,2,3,4-tetrahydro-4-hydroxypyrrolo [2.3-b]-7-methylquinolin-4-one) were diluted to 100 μM and 7 mM in DMSO, respectively. The dynasore was kindly provided by Dr. Tomas Kirchhausen (Department of Cell Biology, Harvard University, Boston, MA and IDI Research Institute, Boston, MA). The compound was dissolved in DMSO to a stock concentration of 200 mM. The aliquots were stored at −40 °C and diluted to their final concentrations in the culture medium immediately prior to use. Wortmannin, staurosporine, genistein and the calcium ionophore A23817 were purchased from Sigma Chemical Company (St. Louis, MO). Blebbistatin and IPA-3 were acquired from Merck KGaA (Darmstadt, Germany) and the IDI Research Institute (Boston, MA), respectively.

For each compound, viability tests with neutral red were performed and no cytotoxic effect was observed with the concentrations and incubation time used.

2.2. Parasites and host cell culture

T. gondii tachyzoites of the RH wild-type strain were maintained in mice by intraperitoneal inoculation and harvested by peritoneal washing of 2–3 day-infected mice. The suspension was then centrifuged at 1000 ×*g* for 10 min to remove the cell debris and the peritoneal leukocytes, and the number of parasites in the supernatant was quantified in a Neubauer chamber. The parasites were resuspended in Dulbecco's modified Eagle's medium (DMEM). Swiss mice were bred at the Universidade Federal do Rio de Janeiro animal facility. The experimental protocol was approved by the Instituto de Biofísica Carlos Chagas Filho (Universidade Federal do Rio de Janeiro) Ethics Committee for animal experimentation (Protocol n. IBCCF 096/097).

Macaca mulatta monkey epithelial kidney cells (LLC-MK2) were maintained in vitro in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 either in 25 cm^2 plastic falcon bottles or on round glass coverslips in 24-well plates. The cellular viability was determined using neutral red, and the absorbance values were determined with an ELISA reader. For light microscopy, the cultures were fixed and stained using a Panotic Solution Kit® (Laborclin Ltda. Pinhais/PR, Brazil).

2.3. In vitro infection

A ratio of 5 parasites per cell was used in each assay. After 40–50 min of incubation at 37 °C and 5% CO_2, the supernatant containing the free parasites was aspirated and replaced by fresh medium, and the infection was allowed to proceed for 24 h. The cells were then rinsed with DMEM, and the appropriate inhibitors were added. After 20 min, the cells were rinsed again in DMEM, and 9 μM of the calcium ionophore A23817 was added. After 5 min, the monolayers were fixed for light or electron microscopy. All of the compounds, including the calcium ionophore, were diluted in serum-free DMEM prior to use. The untreated infected monolayers of LLC-MK2 cells were used as negative controls.

2.4. Light microscopy

For light microscopy, the experiments were carried out on round coverslips in 24-well plates. For the observation by bright field microscopy, the samples were fixed and stained with the Panotic Solution Kit®. Intracellular parasites, corresponding to the total number of parasites, confined inside vacuoles were counted in cells treated with each drug. The number of parasites per vacuole was not taken into account. The inhibition percentages were calculated considering the number of egressed parasites in ionophore treated infected cells (ITIC) as 100% of egress. The observations were carried out on a Zeiss-Axioplan microscope.

2.5. Confocal microscopy

For immunofluorescence microscopy, the cells were seeded onto round coverslips, fixed with 4% formaldehyde in PBS, pH 7.2, for 20 min and then permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. The samples were pre-incubated with 50 mM ammonium chloride and 3% BSA in PBS, pH 8.0, for 45 min to block the free aldehyde groups. The samples were then incubated with primary antibodies at a 1:100 dilution for 1 h, rinsed, and incubated with 1:400 secondary antibodies at room temperature for 1 h. After rinsing in phosphate buffered saline (PBS) and mounting with a prolong antifade (Vector Labs, Burlingame, CA), slides were examined in a Zeiss 510 LSM 510 NLO. Polyclonal primary antibody used was: a mouse anti-tubulin-α (Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary antibody was goat anti-mouse IgG (H + L) conjugated to Alexa Fluor 488 (green). The antibody for the actin marker phalloidin (red) was diluted 1:40 and incubated for 20 min in a dark at room temperature.

2.6. Electron microscopy

For the field emission scanning electron microscopy (FESEM), the host cells were cultivated onto round sterile coverslips in 24-well plates until the pre-confluent monolayers were ready to be infected at a ratio of 10 parasites per cell. After 24 h, 9 μM of the calcium ionophore was added. After 5 min of incubation, the monolayers were washed and fixed in a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2; post-fixed for 1 h with 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.2 and 0.8% potassium ferrocyanide; dehydrated in ethanol; critical point dried in CO_2; sputtered with carbon; and observed in a Jeol 6340 field emission scanning electron microscope. A subset of the samples was dry-cleaved with carbon adhesive tape prior to sputtering to expose the interior of the cell [38].

For immunolabeling, the samples were permeabilized with 0.1% Triton-X-100 for 2 min prior to fixation with 0.1% glutaraldehyde and 4% freshly prepared formaldehyde. The samples were subsequently probed with rabbit anti-actin antibody at a 1:100 dilution, followed by a goat-anti-rabbit antibody conjugated to 10 nm colloidal gold particles, both incubated for 1 h. The samples were then