FULL PAPER *Parasitology*

Calcium ions are involved in egress of *Babesia bovis* merozoites from bovine erythrocytes

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ABSTRACT. Bovine babesiosis is a livestock disease known to cause economic losses in endemic areas. The apicomplexan parasite *Babesia bovis* is able to invade and destroy the host's erythrocytes leading to the serious pathologies of the disease, such as anemia and hemoglobinuria. Understanding the egress mechanisms of this parasite is therefore a key step to develop new therapeutic strategies. In this study, the possible involvement of Ca^{2+} in the egress of *B. bovis* merozoites from infected erythrocytes was investigated. Egress was artificially induced *in vitro* using calcium ionophore A23187 and thapsigargin to increase Ca^{2+} concentration in the cytosol of the parasite cells. The increased intracellular Ca^{2+} concentration following these treatments was confirmed using live cell Ca^{2+} imaging with confocal laser scanning microscopy. Based on our findings, we suggest a Ca^{2+} signalling pathway in the egress of *B. bovis* merozoites.
KEY WORDS: *Babesia bovis*, calcium ionophore, calcium signalling, egress

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Babesia bovis is a tick-borne protozoan parasite of cattle that causes serious economic losses in the livestock industry [19]. Infection with *B. bovis* is characterized by hemolytic anemia, hyperpyrexia, hemoglobinuria, lethargy, anorexia and recumbency. In severe cases, cerebral babesiosis is manifested by several signs of central nervous system involvement, and the outcome is almost invariably fatal [7]. Although vaccine development has been the subject of intense focus, to date, only a live attenuated vaccine, with some restrictions, has been introduced to the field in certain regions of the world [12]. Chemotherapy remains one of the main components of control strategies against babesiosis, and current drugs used against bovine babesiosis include diminazene aceturate and imidocarb. It has been proven that these drugs reduce the risk of severe infection in endemic areas. However, the withdrawal of many anti-babesia drugs from the market for various reasons [21] has made the search for new potent chemotherapeutic agents highly important. Understanding the signalling pathways governing the parasite's growth in the erythrocytic stages may help in strategizing new control measures to combat babesiosis.

Calcium ion (Ca^{2+}) is a ubiquitous intracellular signal messenger that is responsible for controlling a wide range of cellular activities in eukaryotic cells [6]. In protozoan parasites, Ca^{2+}-mediated signalling controls various vital functions, such as protein secretion, motility, cell invasion and differentiation [11, 22, 25, 28]. In contrast to the *Plasmodium* and *Toxoplasma* parasites, little is understood about calcium signalling in *Babesia*, with the exception of a small amount of information on the involvement of Ca^{2+} in the invasion of erythrocytes by the merozoites of *B. divergens* and equine *Babesia* parasites [23, 26]. The inhibitory effect of Ca^{2+} dependent protein kinase inhibitor on the *in vitro* growth of *B. bovis* has also been reported [8]. While egress (release) of *Plasmodium* merozoites and *Toxoplasma* tachyzoites from their host cells has been studied intensively in terms of Ca^{2+} signalling [2, 15, 17, 22], at the present time, there are no available data showing the role of Ca^{2+} in the egress of *Babesia* parasites. Therefore, in the present study, we aimed to investigate the involvement of Ca^{2+} in the egress of *B. bovis* merozoites from infected erythrocytes. We found calcium ionophore A23187 and thapsigargin (Tg; an inhibitor of sarco/endoplasmic reticulum Ca^{2+}–ATPase (SERCA)), which have been used in various studies to artificially increase Ca^{2+} concentration in the cytosol of apicomplexan parasite cells [4, 13, 17], induced egress of *B. bovis* from host erythrocytes. In addition, we observed changes in intracellular Ca^{2+} concentration after these treatments using the live cell Ca^{2+} imaging technique with confocal laser scanning microscopy.

MATERIALS AND METHODS

Parasite culture: *B. bovis* (Texas strain) [18] was maintained in a serum-free GIT medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 10% bovine erythrocytes, 60 U/m*l* of penicillin G, 60 g/m*l* of streptomycin and 0.15 g/m*l* of amphotericin B (Sigma Aldrich Japan Co., Tokyo, Japan) (complete culture medium) using a

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continuous microaerophilic stationary-phase culture system [1]. The animal experiments in this study were carried out in compliance with the Guide for Animal Experimentation at Obihiro University of Agriculture and Veterinary Medicine (Permission number 25–78–3).

In vitro egress assay: The effect of calcium ionophore A23187 (Sigma Aldrich Japan) and Tg (Sigma Aldrich Japan) on the egress of the parasite from infected erythrocytes was examined using a method for measuring drug activity as previously described [9, 10] with some modifications. Briefly, the parasite culture was diluted with a fresh complete culture medium to obtain a parasitemia of 4–7% in a 1.5 m*l* plastic tube. A23187 or Tg, which had been dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich Japan), was added to the culture in the tube at 1 nM to 10 μM or 1.25–5 μM, respectively. The mixture was then incubated in a humidified multi-gas water-jacketed incubator with cap open at 37°C for indicated periods of time. In parallel, normal culture supplemented with the same concentration of DMSO was prepared as a control. All of the experiments were carried out in triplicate for each compound. Parasitemia was monitored by counting approximately 1,000 erythrocytes in a Giemsa-stained thin smear, while the percentage of extracellular merozoites was calculated as the ratio of extracellular merozoites to the entire parasite population (extracellular and intraerythrocytic merozoites) in approximately 500 parasites.

Fluorescence Ca^{2+} imaging: Fluorescence Ca^{2+} imaging was performed as described previously [14]. In brief, a culture of infected erythrocytes was diluted 10-fold with RPMI 1640, phenol red (−), culture medium (Invitrogen Japan Co., Tokyo, Japan), which served as the imaging medium. The infected erythrocytes were collected from the 1 m*l* aliquot by centrifugation (1,000 × *g* for 5 min at room temperature) and resuspended in 350 μl of the imaging medium. Loading solution was prepared by adding 10 μM Fluo-4 AM (Invitrogen Japan) and 100-fold dilution of PowerLoad (Invitrogen Japan) to the imaging medium and was used for the loading of Fluo-4 AM to the parasite cells. A suspension of erythrocytes (350 μl) was mixed with 150 μl of loading solution to give a final concentration of 3 μM of Fluo-4 AM and then shaken at 200 rpm for 15 min at 37°C with a TAITEC bioshaker BR-22UM (TAITEC, Tokyo, Japan). Erythrocytes were then mixed with 10 m*l* of the imaging medium, centrifuged at 1,000 g × for 5 min at room temperature and resuspended in 1.2 m*l* of the imaging medium. A suspension of erythrocytes (200 μl) was applied in a 35 mm glass-bottomed dish (MatTek Co., Ashland, MA, U.S.A.) that had been coated with 1 mg/m*l* poly-L-lysine before use. After 30 min incubation in a humidified multi-gas water-jacketed incubator at 37°C, suspended erythrocytes were removed by gentle washing with the imaging medium. The glass-bottomed dish was then placed in the culture chamber of a Leica confocal microscope (TCSSP5, Leica Microsystems, Wetzlar, Germany). Sequential time-lapse imaging of Fluo-4 AM and transparent images was performed using the Leica confocal microscope system (Leica Microsystems) with a 40 × oil immersion objective lens and excitation at 488 nm (Argon laser) for Fluo-4 AM and transparent images. Emissions were collected using the true spectral detection method developed by Leica Microsystems. Images were captured every 5–15 sec for 200–300 sec. Specific Fluo-4 AM fluorescence in a parasite (F) was calculated by the subtraction of background fluorescence and normalized by the average fluorescence obtained before the tested compound was added (F0).

Perfusion system: A manipulator system (type YOU-4, Narishige Co., Ltd., Tokyo, Japan), Perista pump (SJ-1211h-NO, 483313- ATTO Co, Tokyo, Japan) and Enomoto Micro Pump (model MV-6005VP, Enomoto Micro Pump Mfg. Co., Ltd., Tokyo, Japan) were used to add and remove A23187 and Tg, continuously to and from the parasite preparation during the live cell imaging process.

RESULTS

A23187 induces the egress of B. bovis merozoites from infected erythrocytes: In order to investigate the effect of the increase in cytosolic Ca^{2+} concentration on the egress of *B. bovis* merozoites from bovine erythrocytes, Giemsa-stained smears of the parasite culture were prepared after 10 min incubation *in vitro* with two different concentrations of A23187 (1 and 10 μM). In *Plasmodium*, *Toxoplasma* and *Neospora* parasites, micromolar concentrations of A23187 have induced egress [4, 13, 17], however, these treatments resulted in the emergence of rarely seen degenerated and dot-shaped parasites in the control culture of *B. bovis* (data not shown). The parasite was therefore incubated with low concentrations of A23187 (1, 10 and 100 nM), and Giemsa-stained smears were prepared every 10 min until 30 min after the treatments. In this experiment, we found that 10 min incubation with 1–100 nM A23187 resulted in a significantly lower parasitemia in all concentrations in comparison to control without A23187. Extending the incubation with the A23187 for another 10 min resulted in an increase in parasitemia. After 30 min incubation, parasitemia was significantly higher in all concentrations as compared to control (Fig. 1A). These findings suggest that A23187 induces the parasite's egress from and consequent invasion to erythrocytes. To distinguish the A23187 effect on the egress from the invasion step, cultures incubated with 10 nM A23187 were then monitored for free merozoites (merozoites outside erythrocytes). Giemsa-stained smears were prepared every 1 min from the parasite culture for 10 min after A23187 treatment. The results showed that ratio of free merozoites to total parasites began increasing from 1 min after treatment upto 10 min after treatment (Fig. 1B). To confirm this observation and determine the time point suitable for observing the egress, we compared the culture for free merozoites at 5 and 10 min after A23187 treatment and found that 10 min incubation gave clearer difference in the ratio of free merozoites (Fig. 1C) between test and control parasites. These data suggest that A23187 induces the parasite's egress from infected erythrocytes.

Tg induces the egress of B. bovis merozoites from infected erythrocytes: To further examine whether the parasite's egress can be induced by the increased cytosolic Ca^{2+} concentration in the parasite, we incubated the parasite culture

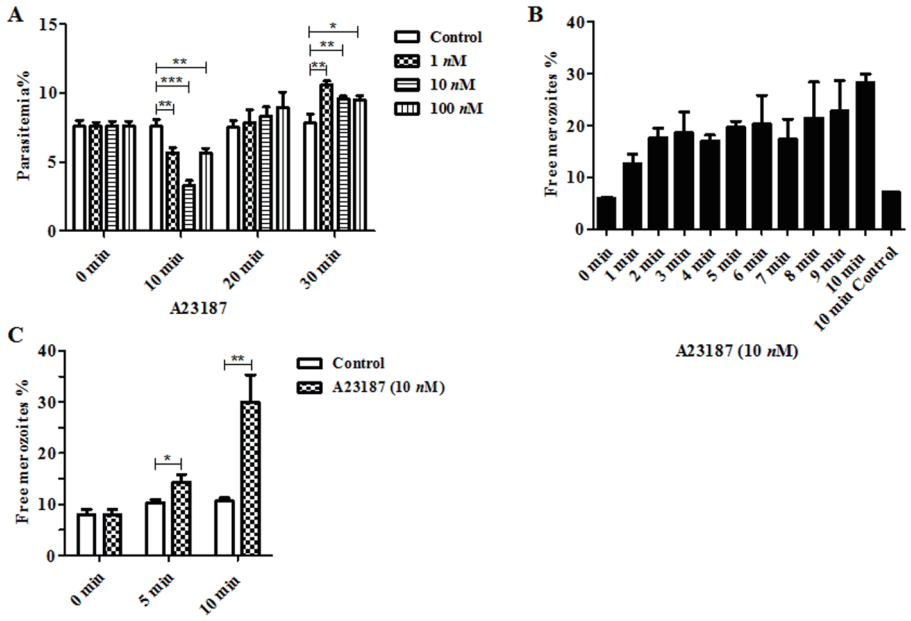

Fig. 1. Effect of A23187 on *B. bovis* culture. The effect was evaluated on parasitemia (A), percentage of extracellular merozoites (number of free merozoites/ number of free merozoites + number of intraerythrocytic parasite × 100) obtained every 1 min until 10 min after treatment (B) and the percentage of extracellular merozoites obtained every 5 min until 10 min after treatment (C). Each value represents mean ± SD in 3 independent experiments. The statistical significance of differences was assessed with Student's *t*-test. Asterisks indicate significant differences (**P*<0.01, ***P*<0.005 and ****P*<0.0002) between A23187-treated groups and solvent (DMSO)-treated control group.

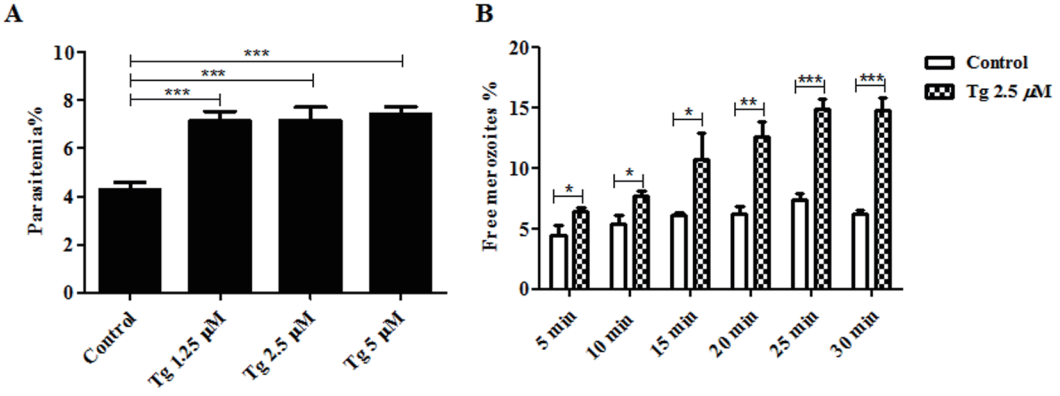

Fig. 2. Effect of thapsigargin (Tg) on *B. bovis* culture. The effect was evaluated on parasitemia after 90 min incubation (A) and the percentage of extracellular merozoites obtained every 5 min until 30 min after treatment (B). Each value represents mean ± SD in 3 independent experiments. The statistical significance of differences was assessed with Student's *t*-test. Asterisks indicate significant differences (**P*<0.02, ***P*<0.001 and ****P*<0.0002) between Tg-treated groups and solvent (DMSO)-treated control group.

with Tg, an inhibitor of the uptake of cytosolic Ca^{2+} to the endoplasmic reticulum by specific inhibition of SERCA. We first incubated the parasite with different concentrations of Tg (1.25, 2.5 and 5 μM), and Giemsa-stained smears were prepared after 90 min incubation. In this experiment, we found that all tested concentrations showed significantly higher parasitemia in comparison to control (Fig. 2A). These data suggest that Tg increases parasitemia as a result of egress acceleration, followed by the reinvasion of the egressed merozoites into new erythrocytes. To investigate whether Tg can induce egress, parasite culture incubated with 2.5 μM of Tg was monitored for free meroziotes for 30 min, and Giemsa-stained smears were prepared every 5 min. The results from this experiment revealed that, in comparison to non-treated

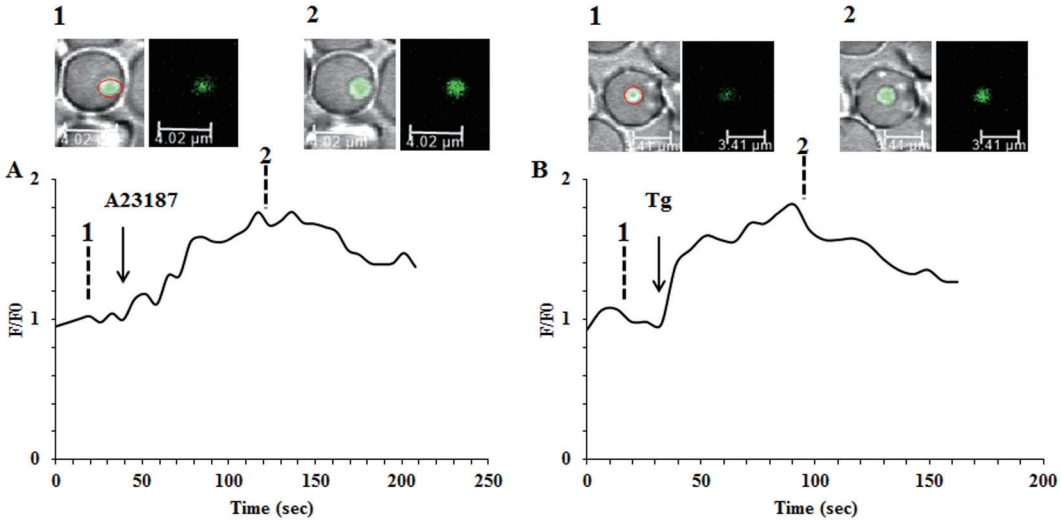

Fig. 3. Ca^{2+} imaging of *B. bovis* merozoites analyzed by confocal microscopy. Parasite cells were loaded with Fluo-4 AM, and fluorescence in the parasite cytoplasm (F/F0) was calculated (see MATERIALS AND METHODS). Treatment with 100 nM A23187 caused an increase in mean fluorescence ratio of 1.01 ± 0.3 (n=7) (A). Treatment with 2 μM thapsigargin (Tg) caused an increase in mean fluorescence ratio of 0.65 ± 0.1 (n=6) (B). Data are representative of seven and six similar experiments for A23187 and Tg, respectively. Images (1, 2) above each graph show the fluorescence time-lapse images in the parasite cytoplasm at the indicated time points (dotted lines). Red circle represents region of interest (ROI) set for data acquisition.

control, Tg-treatment significantly increased the ratio of free merozoites to total parasites at all tested time points and that the increase of the ratio was clearer with 25 and 30 min of treatment (Fig. 2B). These results indicate that the increase in cytosolic Ca^{2+} concentration most probably induces the parasite's egress and suggest a Ca^{2+} signalling pathway in the egress of this parasite.

A23187 and Tg increase cytosolic Ca^{2+} concentration in B. bovis merozoite: To confirm the effects of treatment with A23187 and Tg toward egress of *B. bovis* merozoites, time lapse imaging of live cell Ca^{2+} was applied using Leica confocal laser microscopy by loading the parasite cell with the Ca^{2+} sensitive indicator Fluo-4 AM. The addition of 100 nM of A23187 or 2 μM of Tg to the parasite preparation induced an increase in cytosolic Ca^{2+} concentration of the parasite cells (Fig. 3A and 3B). These findings suggest that the induced egress by these two cytoplasmic Ca^{2+} modulators might be, at least, due to their effect in increasing cytosolic Ca^{2+} concentration. The cause-and-effect link between the increase of cytosolic Ca^{2+} and the merozoite egress needs to be proved in our future studies.

DISCUSSION

The information available on Ca^{2+} signalling components in apicomplexan parasites is still fragmentary and insufficient. Important features of their life cycle, such as motility, host cell invasion and egress from infected cells, are known to be linked with Ca^{2+} [20]. Obligate intracellular parasites like *T. gondii* replicate inside its host cell, but at some point need to exit the cell by rupturing the infected host cell in order to infect other cells. This rapid egress process is still poorly understood. However, it is known that calcium ionophores like A23187 can stimulate the process [13]. *T. gondii* mutants with delayed egress have been isolated and found to have elevated intracellular Ca^{2+} level [3]. In the schizont stage of *P. falciparum*, it has also been observed that intracellular Ca^{2+} level was increased just prior to parasite egress and that A23187 artificially induced the egress [17]. Synchronization of *Plasmodium* and *Toxoplasma* parasites cultures has made the study of egress easier. However, this may not be the case in the *Babesia* parasite, wherein the limitation in tools for obtaining synchronized cultures [26] might hamper the study of egress. To overcome this difficulty, we adopted a criterion for evaluating the egressed parasite in compound-treated culture through parasitemia and by counting the free merozoites to provide direct evidence of the parasite being outside the cell as a result of the treatment. The data obtained here with A23187 treatment were consistent with those obtained in the other apicomplexan parasites, *Plasmodium*, *Toxoplasma* and *Neospora,* suggesting that apicomplexan parasites may share the same Ca^{2+}-dependent machinery of egress. It should be noted, however, that a lower concentration (10 nM) of A23187 was required to induce egress of *B. bovis* merozoite than that of the other characterized apicomplexan parasites. This may be due to the lack of the parasitophorous vacuole membrane in *B. bovis*-infected erythrocytes. We further examined that the egress induced by A23187 treatment was due to the increase in cytosolic Ca^{2+} concentration by incubating the parasite with Tg. Tg has been used in previous studies to increase the cytosolic Ca^{2+} concentration in the mammalian cells [16, 27]

and in protozoan parasites [5, 24]. To confirm our assumption that the increase in cytosolic Ca^{2+} concentration is the reason for A23187 and Tg-induced egress, live cell imaging of Ca^{2+} was carried out using a confocal laser scanning procedure. As expected, both A23187 and Tg were found to have increased cytosolic Ca^{2+} concentration.

This study demonstrated that *B. bovis* egress from erythrocytes could be pharmacologically induced by modulators of cellular Ca^{2+} homeostasis, and thus, these reagents could be used to study the egress pathway in a controlled manner. Our results suggested that *B. bovis* is similar to *Toxoplasma* and *Plasmodium* with respect to the involvement of Ca^{2+} in its egress. However, *B. bovis* is not surrounded by a parasitophorous vacuole membrane in the host cell, and thus, there may be some differences to the other apicomplexan parasites downstream to the increase of cytosolic Ca^{2+} concentration [2].

In summary, our results provide information to better understand the mechanism of the egress pathway and its molecular components in *Babesia* parasites. Further studies would therefore elicit new therapeutic and prevention strategies against babesiosis.

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