Studies on Calcium Signaling in Hemoprotozoan Parasites

（住血原虫におけるカルシウムシグナリングに関する研究）

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Abbreviations

2-APB	2-aminoethyl diphenylborinate
ACT	Artemisinin-based combination therapy
AFV	Acidic Food Vacuole
B. bovis	*Babesia bovis*
B. divergens	*Babesia divergens*
CA	Concanamycin A
Ca^{2+}	Calcium ion
CQ	Chloroquine
CQR	Chloroquine-resistant
DMSO	Dimethyl sulfoxide
EDTA	Ethylendiaminetetraacetic acid
ER	Endoplasmic reticulum
fura 2-AM	Fura 2-acetoxymethylester
H^+	Hydrogen ion
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	IP_3 inositol 1,4,5-trisphosphate receptor
mmol	Millimole
Na^+	Sodium ion
NCX	Sodium calcium exchangers

P. chabaudi	*Plasmodium chabaudi*
P. falciparum	*Plasmodium falciparum*
P. knowlesi	*Plasmodium knowlesi*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PM	Plasma membrane
RFU	Relative fluorescent unit
rpm	Round per minute
RYR	Ryanodine receptor
RT	Room temperature
SERCA	Sarco/endoplasmic reticulum Ca^{2+}–ATPase
SR	Sarcoplasmic reticulum
T. gondii	*Toxoplasma gondii*
Tg	Thapsigargin
U	Unit
vol	Volume
VP	Verapamil
WHO	World Health Organization
Wt	Weight
λ	Lambda

Unit abbreviations

µl	Microliter
µM	Micromolar
µs	Microsecond
g	Gram
Kg	Kilogram
L	Liter
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
ms	Millisecond
nm	Nanometer
nM	Nanomolar
ºC	Degree Celsius
s	Second

General introduction

1. Overview of human malaria

Malaria is a life-threatening disease (7) persisting in more than 90 countries inhabited by 3.4 billion people. WHO estimated 207 million cases of malaria worldwide in 2012 with 627,000 malaria deaths (114). Despite several notable attempts are being made to control and eradicate the disease, it still continues to remain as a major public health problem all in many areas in the world (50). So far, nearly half of the countries in the world have already eleminated malaria. However, the disease is still endemic in 99 countries and of which, 67 are controlling malaria while the other 32 are following elimination strategy (Fig. 2; (41)).

Human malaria is caused by the hemoprotozoan parasites of five species of the genus *Plasmodium* of the phylum Apicomplexa including *Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium ovale, Plasmodium vivax* and *P. falciparum*. Among these species, *P. falcifarum* is known to be often lethal whereas *P. vivax* and *P. falciparum* are the principal agents of morbidity and mortality in malaria because of their vast geographical distribution (48).

The main vectors of malaria transmission to humans are *Anopheles gambiae* and *Anopheles stephensi* (59) which serve as the definitive hosts of the parasite. In the complex life cycle of *Plasmodium* (Fig. 3), the parasite undergoes sporogony inside the female *Anopheles* mosquito producing the motile infective sporozoites. These sporozoites will then be transmitted by the mosquito to the intermediate human host

through blood feeding. The sporozoites travel through the blood vessels to the liver cells where the asexual reproduction known as exoerythrocytic schizogony will occur producing many merozoites. Merozoites then will infect erythrocytes and initiate erythrocytic schizogony (96). All the symptoms and pathology of malaria are attributed to the intraerythrocytic stages of the *Plasmodium* life cycle. Because *Plasmodium* parasites cannot replicate outside the host cells, their ability to recognize and invade erythrocytes is an essential step for both parasite survival and malaria pathogenesis (115).

Case detection and treatment have been important parts of malaria control. However, the development and spread of parasite resistance to a wide range of antimalarial agents has presented a major obstacle to successful disease management in malaria-endemic areas. This has also probably contributed to the resurgence of infection and increase in malaria-related deaths in the recent years (57). Resistance to almost all commonly used antimalarials including chloroquine (CQ), sulphadoxine-pyrimethamine, artemisinin, amodiaquine, mefloquine and quinine, has been observed in *P. falciparum* (57, 34). Besides drug resistance, cases of insecticide resistance in the mosquito vectors were also seen to dramatically increase throughout the last decade (27). In addition, the lack of an effective vaccine (64) is also considered to be a great challenge in malaria control. Effective management of malaria as suggested by the WHO focuses on long-lasting insecticidal nets, indoor residual spraying of insecticide, intermittent preventive therapy in pregnancy and artemisinin-based combination therapy (81).

2. Overview of bovine babesiosis

Bovine babesiosis otherwisely known as ‘redwater fever’, ‘cattle tick fever’, ‘Spanish fever’ or ‘Texas cattle fever’ (42) is caused by the tick-transmitted hemoprotozoan *Babesia bovis*, *Babesia bigemina* and *Babesia divergens*. This parasitic disease commonly results in substantial cattle morbidity and mortality in endemic areas.

Babesia spp. are unicellular eukaryotes which have been defined as piriform, round, or rod-shaped parasites (2). The genus *Babesia* belongs to the phylum Apicomplexa, class Sporozoasida (18). Its life cycle (Fig. 4), involves an invertebrate host, an Ixodidae tick, for sexual reproduction of the parasite and a vertebrate host, wherein the parasite undergoes asexual reproduction exclusively within erythrocytes (43). Inside the tick, sporozoites, the vertebrate-infective life stage, are generated by sporogony in the salivary glands and are later injected with the tick’s saliva into the bloodstream of a cow during a bloodmeal. Sporozoites then invade the erythrocytes, transform into haemoglobin-feeding trophozoites, asexually divide into typically two pear-shaped merozoites, and eventually lyse their host cells to invade new ones repeating it’s asexual propagation cycle (63).

Babesia bovis causes acute disease in a course of 3 to 7 days characterized by fever (>40ºC) usually present for several days before other signs become obvious. This is followed by inappetence, depression, increased respiratory rate, weakness and a reluctance to move. Haemoglobinuria is often present leading to anemia in some cases; hence, the disease is known as redwater. Muscle wasting, tremors and recumbency develop in advanced cases followed terminally by coma (18). The fever during infections may cause pregnant cattle to abort (24) and bulls to show reduced fertility

lasting 6 to 8 weeks (30). Cerebral babesiosis is manifested by a variety of signs of central nervous system involvement and the outcome is almost invariably fatal (18)

The disease causes serious economic losses in the livestock industry (67). Costs due to babesiosis are incurred not only from mortality, ill-thrift, abortions, loss of milk/meat production and from control measures (such as acaricide treatments, purchase of vaccines and therapeutics), but also through its significant impact on international cattle trade (18).

Despite the fact that chemotherapy is still considered the base for treatment and control, the high prevalence of infection worldwide and the emergence of drug resistance (108) have spurred an interest in developing more effective control measures. So far, only live attenuated vaccine is available with a reasonably long-lasting protection. However, the possible spread of silent pathogens such as leukemia virus, difficulties in standardizing the vaccine dose, and the risk of reversion of virulence have restricted the use of this type of vaccine in many regions of the world (103).

3. Overview of calcium signaling

Calcium is the most abundant mineral in the human body with a total approximately 1 kg in an average adult and 99% of which is in the skeleton in the form of calcium phosphate salts. The extracellular fluid contains approximately 22.5 mmol, of which about 9 mmol is in the plasma (70).

Calcium ion (Ca^{2+}) is a highly versatile intracellular signal that regulates various cellular functions (15, 25). Ca^{2+} signaling system works through different ways to regulate cellular processes that function over a wide dynamic range (Fig. 1; (14)). Ca^{2+}

triggers cardiac contraction in microseconds (82), whereas on the other hand, Ca^{2+} has to operate over minutes to hours to control other events such as oocyte activation at fertilization (86) and gene expression (33). One of the great challenges is to understand how these widely different Ca^{2+} signaling systems can be set up to control so many divergent cellular processes.

The level of intracellular Ca^{2+} is determined by a balance between the 'on' reactions that introduce Ca^{2+} into the cytoplasm and the 'off' reactions through which this signal is removed by the combined action of buffers, pumps and exchangers (Fig. 1; (14)). During the 'on' reaction, a small proportion of the Ca^{2+} binds to the effectors that are responsible for stimulating various Ca^{2}-dependent processes (Fig. 1; (14)). Ca^{2+} homeostasis is a very important and crucial feature of Ca^{2+} signaling. It is the mechanism by which the body maintains adequate calcium levels. Cells avoid a net loss or gain of Ca^{2+} by ensuring that the fluxes occurring during the 'on' and 'off' reactions are always balanced (14).

Cells utilize several types of Ca^{2+} influx (entry) channel, which can be categorized on the basis of their activation mechanisms (15). Voltage-operated Ca^{2+} channels are employed by excitable cell types such as muscle and neuronal cells. Other types of channels include receptor-operated Ca^{2+} channels comprised of a range of structurally and functionally diverse channels that are particularly present on secretory cells and at nerve terminals. Mechanically activated Ca^{2+} channels are present on many cell types and respond to cell deformation. Such channels convey information into the cell concerning the stress/shape changes that a cell is experiencing. A typical example of mechanically induced Ca^{2+} signaling was observed in epithelial cells from the trachea,

where deformation of a single cell led to a radial Ca^{2+} wave that synchronized the Ca^{2+}-sensitive beating of cilia on many neighboring cells (19). Another type is store-operated Ca^{2+} channels which are activated in response to depletion of the intracellular Ca^{2+} store, either by physiological Ca^{2+}-mobilizing messengers or pharmacological agents (20).

Ca^{2+} is also released from intracellular stores by several distinct types of messenger-activated channels. The binding of many hormones, and growth factors to specific receptors on the plasma membrane (PM) leads to the activation of phospholipase C (PLC) which catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce the intracellular messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 is highly mobile in the cytoplasm and diffuses into the cell interior where it binds to specific receptors (IP_3R) on the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR). This results in the mechanism known as IP_3-induced Ca^{2+} release (15).

Since Ca^{2+} influx was not enough to explain how electrically non-excitable cells, devoid of voltage-gated Ca^{2+} channels, execute numerous Ca^{2+}-dependent processes, different studies were done for a better understanding of this mechanism. Investigations on muscle cells have identified an intracellular source of Ca^{2+} associated with a specialized intracellular organelle, the SR. This organelle was initially described in 1902 (73), and then rediscovered (87) identifying this organelle as the ER present in a variety of non-muscle cells. The function of the SR as an intracellular Ca^{2+} storage/release site (36) and its role in the initiation of muscle contraction through Ca^{2+}-induced Ca^{2+} release (37, 40) have since then identified. Furthermore, the role of mitochondrion in Ca^{2+} accumulation was also made known in various studies (75, 76).

It was proved that hormones and neurotransmitters can induce release of Ca^{2+} from a non-mitochondrial store, most likely the ER, by a mechanism still unknown at that time (84). The missing connection between the plasma membrane receptors and the intracellular Ca^{2+} release sites was found to be a receptor-controlled PLC acting on PIP_2 (74), resulting in production of diacylglycerol and the water-soluble agent IP_3. Later in 1983, IP_3 was found to release Ca^{2+} from the same non-mitochondrial pool (100). Cloning and sequencing of IP_3 receptor were significantly done in a more recent study (45).

4. Calcium signaling in apicomplexan parasites

Malaria parasites (*Plasmodium* spp.) and babesia (*Babesia* spp.) are hemoprotozoan parasites belonging to the phylum Apicomplexa. Apicomplexa is an ancient phylum of some 5,000 diverse eukaryotic species that are largely parasitic on marine invertebrates, insects, and vertebrates. These parasites are named for their peculiar apical end, which contains a number of unique organelles and structures (78). In vertebrates, they have an important role because they significantly cause animal and human diseases (17). For example, five *Plasmodium* species are responsible for significant mortality and morbidity due to malaria, the most serious form of which is caused by *Plasmodium falciparum* (98). Related organisms such as *Toxoplasma gondii* (62) and *Cryptosporidium* spp. (107) cause opportunistic infections of considerable importance in immunocompromised individuals. Among the livestock animals, *Babesia bovis* is a tick-borne parasite of cattle causing serious economic losses in the livestock industry (67). Moreover, apicomplexans often contain plant-like features, owing to two events: 1) very early branching that likely predates the animal-plant split (10), 2)

acquisition of a secondary endosymbiont derived from engulfment of an algal cell (110). As such, signaling pathways in apicomplexan parasites contain both conserved and unique features as compared to other eukaryotic cells (17).

Ca^{2+} homeostasis and storage have been studied mainly in *T. gondii* and *Plasmodium* spp. using the fluorescent calcium indicator fura 2-AM (fura 2-acetoxymethylester). In *T. gondii*, the cytosolic Ca^{2+} concentration in the tachyzoites was measured at 70 ± 6 nM (79). On the other hand, the concentrations obtained in *Plasmodium chabaudi* and *P. falciparum* using fura 2-AM loaded free parasites were also at nanomolar levels performed in single-cell imaging experiments (49).

In addition to known eukaryotic Ca^{2+} stores including acidocalcisomes, the ER, Golgi apparatus and mitochondria, apicomplexan parasites contain other unique compartments that potentially could contribute to diverse Ca^{2+} transients necessary for crucial functions within the parasites. These include the apicoplast, a remnant plastid derived from a secondary endosymbiotic event, and various acidic organelles such as the recently described plant-like vacuole (78).

Apicomplexans have highly polarized cells that are specialized for regulated secretion and directed entry into their host cells (28, 94). Ca^{2+} controls a number of critical events in their life cycles including secretion of adhesins, gliding motility cell invasion, and egress (79). Ca^{2+} also influences developmental processes that occur at distinct stages in their complex life cycles (5).

Ca^{2+} regulation in parasitic protozoa differs in several aspects from the processes that occur in other eukaryotic cells, providing great opportunities for targeting them for new therapies (78).

5. Objectives of the present study

Therefore, given this general background, the objectives of this study are: 1) To study the involvement of Ca^{2+} signaling in reversing the chloroquine-resistance in the malaria parasites to provide a new therapeutic strategy against the resistant parasites. 2) To study the involvement of Ca^{2+} in egress of *B. bovis* merozoites from bovine erythrocytes as it is considered one of the most important steps in the parasite's life cycle.

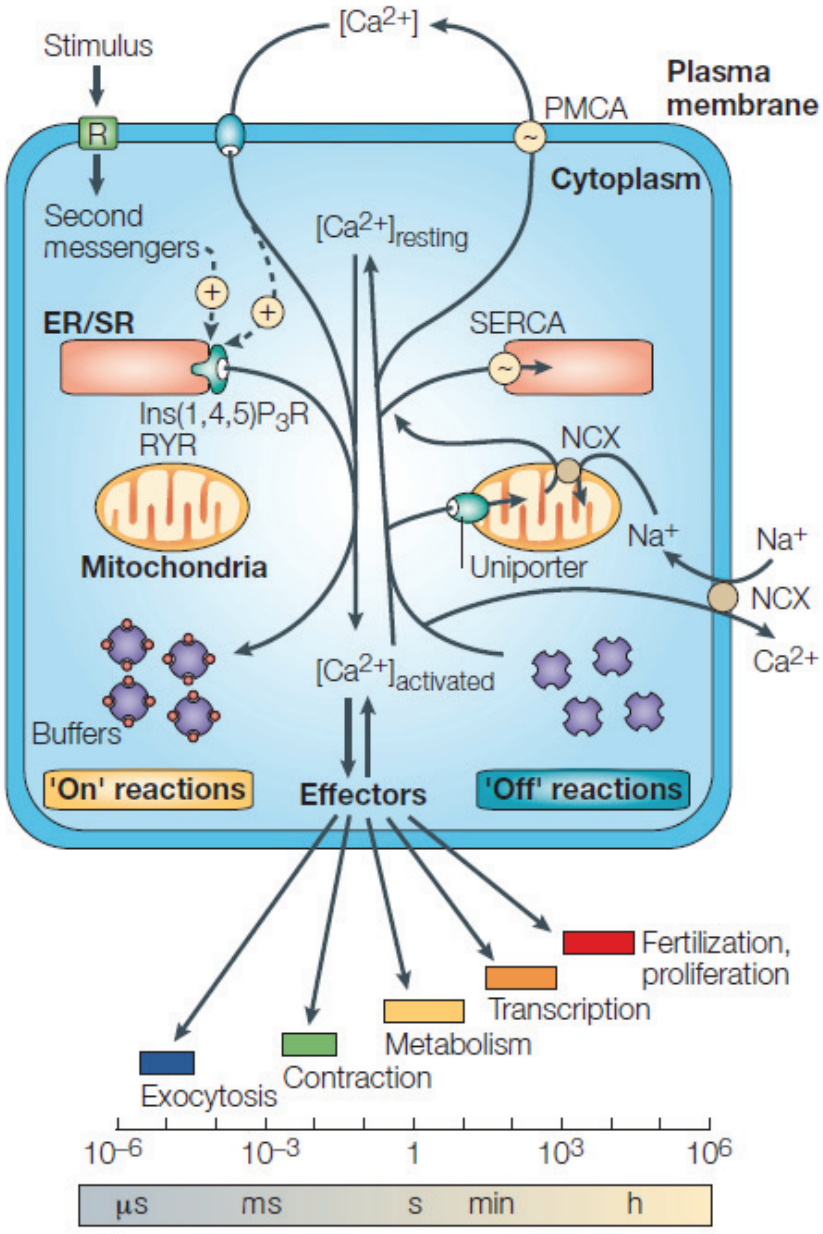

Fig. 1. Ca^{2+} signaling dynamics and homeostasis in eukaryotic cells (14)

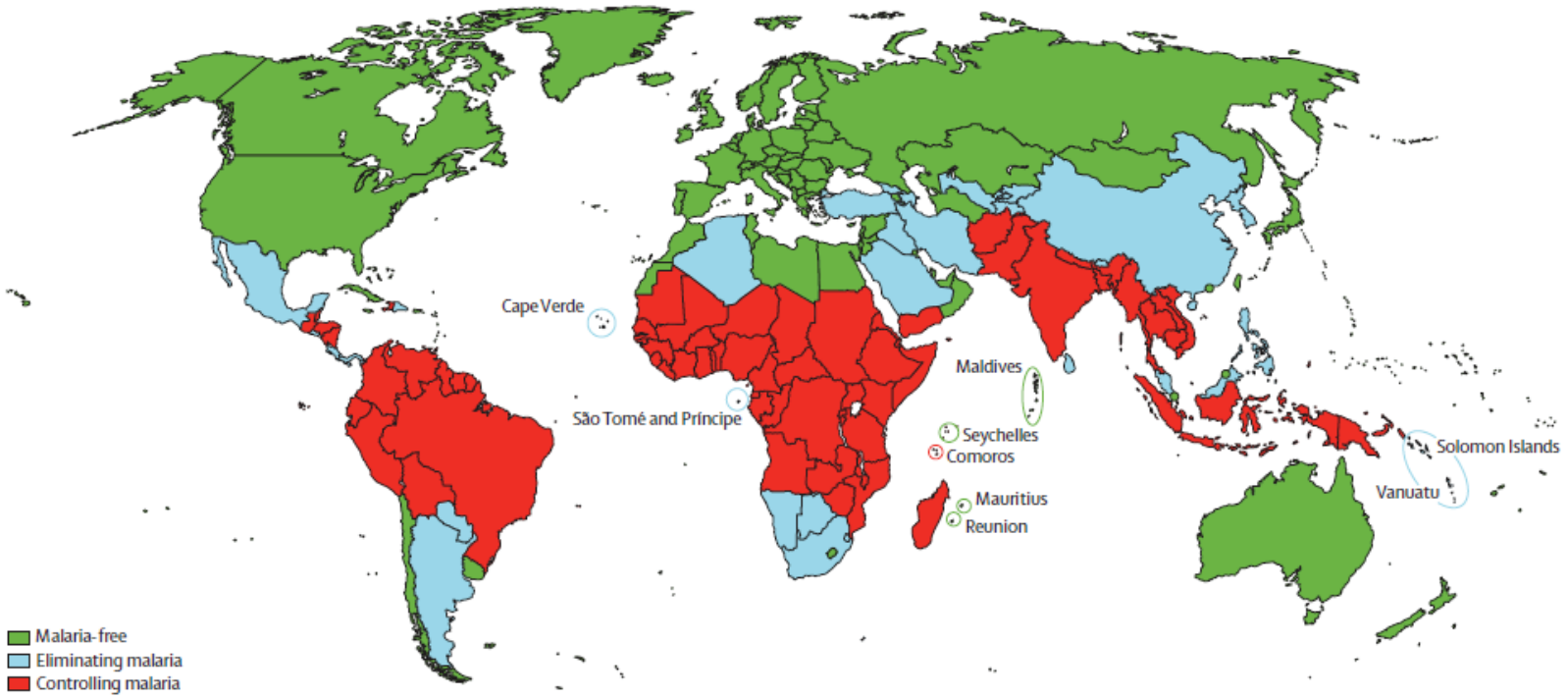

Fig. 2. Categorization of countries as malaria free, eliminating or controlling malaria (41)

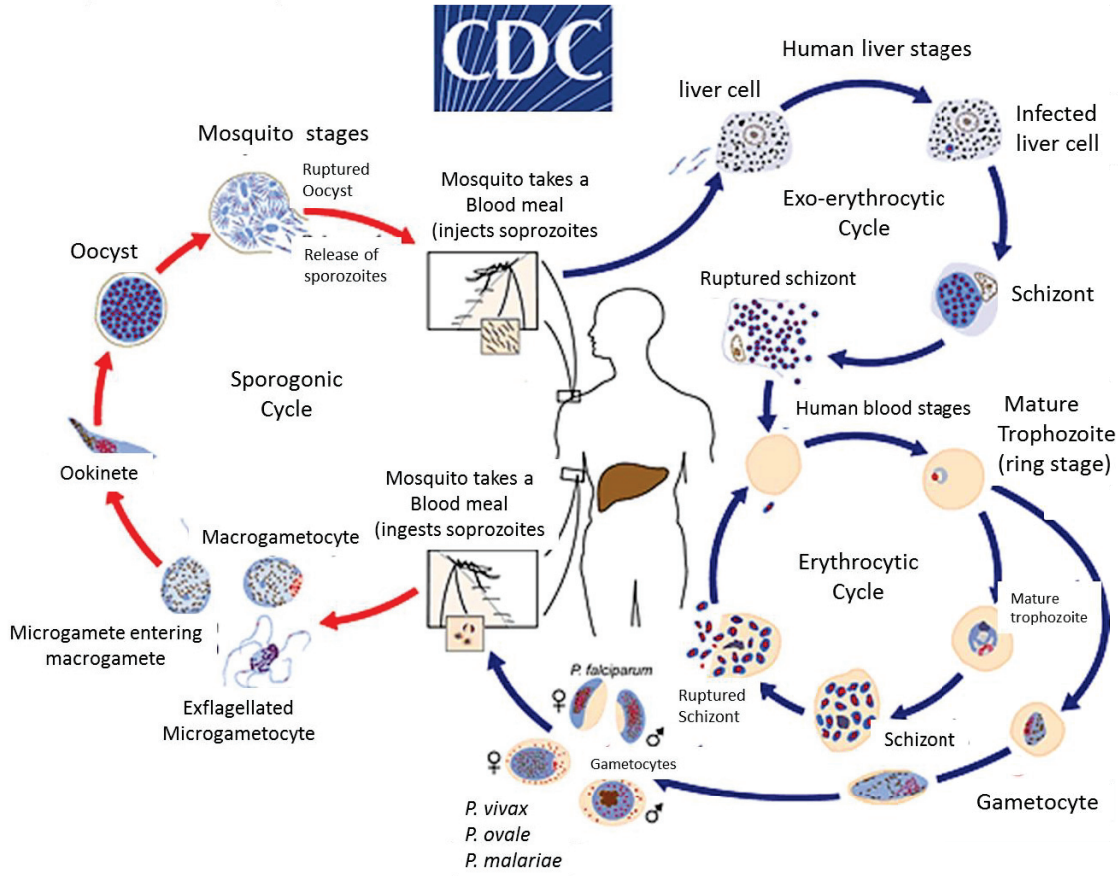

Fig. 3. The life cycle of malaria parasite.

Source: Center for Disease Control and Prevention (modified from the original figure).

http://www.cdc.gov/malaria/about/biology/

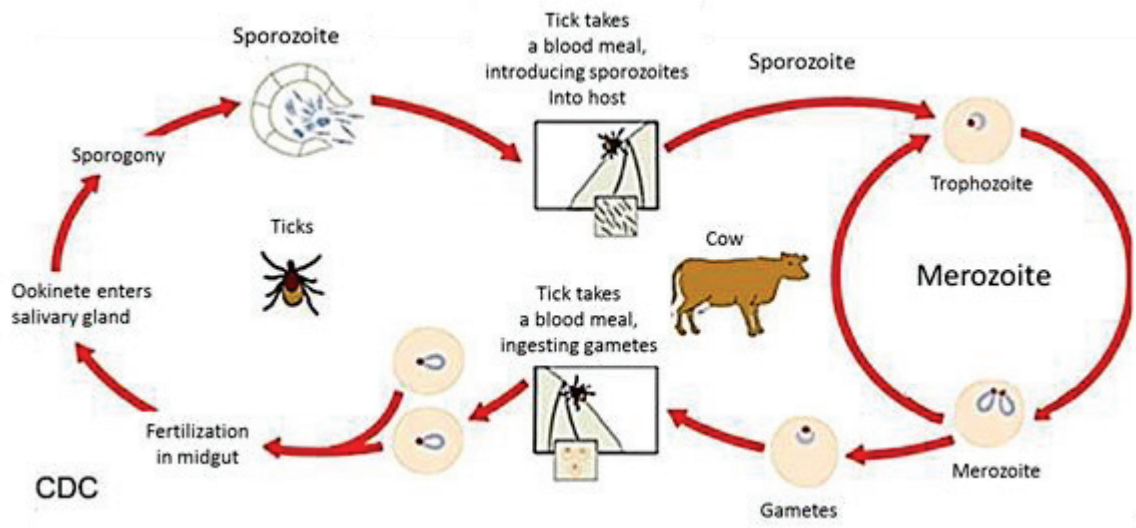

Fig. 4. The life cycle of *Babesia*.

Source: Full-Babesia (modified from the original figure).

http://fullmal.hgc.jp/bb/docs/aboutdb.html

Chapter I

Simultaneous administration of 2-aminoethyl diphenylborinate reverses chloroquine-resistance in *Plasmodium falciparum*

1.1 Introduction

Malaria continues to be a worldwide public health problem causing significant morbidity and mortality and its resistance to existing antimalarial drugs is a growing problem (113). The emergence, followed by the spread throughout most malaria-endemic regions, of *P. falciparum* parasites resistant to the antimalarial drug chloroquine (CQ) has worsened the global malaria situation (106). Malaria is caused by a protozoan parasites of the genus *Plasmodium*, its blood stage replicates inside the host erythrocytes (8) and responsible for most of the clinical symptoms of the disease (97). Understanding the signaling pathways governing the parasite's blood stage growth may aid in discovering new therapeutic targets for antimalarial drugs.

Ca^{2+} is a ubiquitous intracellular signal responsible for controlling a wide range of cellular activities in eukaryotic cells (15). In protozoan parasites, Ca^{2+}-mediated signaling controls various vital functions such as protein secretion, motility, cell invasion and differentiation (83, 95). In the malaria parasite, *Plasmodium*, the intracellular signaling pathways controlling the activity of Ca^{2+} dependant protein kinases has extensively been studied (95, 35). The role of Ca^{2+} signaling underlying modulation of *Plasmodium* cell cycle has also been widely investigated including the effect on protease activity (11, 90). Moreover, some research groups tried to investigate how Ca^{2+} signaling is triggered by demonstrating the important role of the host derived

hormone melatonin and its derivatives that elicit a rise in cytosolic Ca^{2+} in *Plasmodium* (99, 54). It has also been demonstrated recently, for the first time, the spontaneous Ca^{2+} oscillation in *P. falciparum* (39). Furthermore, it has also been shown that the blockage of this oscillation in the trophozoite stage by 2-aminoethyl diphenylborinate (2-APB) which inhibits IP_3-induced Ca^{2+} release (72, 116, 117), caused severe degeneration and breakdown of successive asexual reproduction in the intraerythrocytic parasites, resulting in their death (39).

The antimalarial drug CQ is thought to exert its toxic effect in the intraerythrocytic parasite at the digestive vacuole (44). The compound was also found to induce Ca^{2+} release and disrupt Ca^{2+} and H^+ homeostasis in cytoplasm of *P. chabudai* cells (88, 53). Therefore, with regard to disruption of intracellular Ca^{2+} homeostasis, it was hypothesized that the potentiation of the CQ activity could be achieved in the malaria parasite with the simultaneous administration of 2-APB.

In this chapter the potential of 2-APB in reversing CQ-resistance in *P. falciparum* was examined. This resulted in a complete reversal of CQ-resistance in *P. falciparum* K-1 strain.

1.2 Materials and Methods

Parasite culture. Chloroquine resistant (CQR) K-1 strain of *P. falciparum* was cultured with the modified method of Trager and Jensen (105) using a multi-gas incubator (5% O_2 and 5% CO_2) in RPMI 1640 medium (Life Technologies Japan Co., Tokyo, Japan) supplemented with 0.5% Albumax (Life Technologies Japan), 25 mM HEPES, 24 mM sodium bicarbonate, 0.5 g/L L-glutamine, 50 mg/L hypoxanthine, 25 µg/L gentamycin and human erythrocyte (from healthy Japanese volunteers) at a hematocrit of 2% in 4 ml cultures. Growth synchronization was achieved with 5% D-sorbitol (68). *P. falciparum* K-1 strain was kindly provided by Prof. Shigeyuki Kano.

***In vitro* drug susceptibility test.** The assessment of the outcome of the in vitro drug susceptibility test was done using the SYBR Green I method (61). *P. falciparum*-infected erythrocytes were cultured with the standard method using a multi-gas incubator (5% O_2 and 5% CO_2). After reaching 1.5% ring form parasitemia, the parasites were synchronized with 5% D-sorbitol for 30 min at room temperature (RT) and washed with RPMI 1640 medium twice by centrifugation at 1,000× g for 5 min. Then, the erythrocytes were resuspended in the culture medium at 2% hematocrit. One hundred µl of the erythrocyte suspension was then replaced in each well of a tissue culture plate (96-well flat bottom, Corning Japan Co., Tokyo, Japan) in triplicate. For the CQ sensitivity test, chloroquine diphosphate (Sigma Aldrich Japan Co., Tokyo, Japan) was added to the parasite culture (100 µl in total) in each well to give a series of dilutions from 10,240 to 1.25 nM. The sensitivity tests for 2-APB (obtained from Laboratory for Developmental Neurobiology, RIKEN Brain Science Institute), was mixed with dimethyl sulfoxide (DMSO, Sigma Aldrich Japan) before being added to the

culture to give a series of dilutions from 200 to 25 μM. The simultaneous addition of the two compounds was performed by adding 50 μM 2-APB to the serially diluted CQ. After 72 h of incubation, each test plate was removed from the incubator and 100 μl of lysis buffer [130.1 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.016 (wt/vol) saponin and 1.6 (vol/vol) Triton X-100] containing SYBR green I (Life Technologies Japan) (2× final concentration) was added directly to each well in the plates and gently mixed. The plates were then covered with aluminum foil and incubated for another 24 h at RT in the dark. Relative fluorescent units (RFU) per well were determined using Fluoroskan Ascent (Thermo Fisher Scientific K.K., Yokohama, Japan) with excitation and emission wavelength bands set at 485 and 530 nm, respectively. The concentration of anti-malarial drug inhibiting parasite growth by 50% (IC_{50}) was calculated using the probit method as described previously (102).

Fluorescence Ca^{2+} imaging. Fluorescence Ca^{2+} imaging was performed as described previously (39). A culture of *P. falciparum*-infected erythrocytes was diluted 10-fold with RPMI 1640, without phenol red, culture medium (Invitrogen Japan Co., Tokyo, Japan), which served as the imaging medium. The infected erythrocytes were collected from the 1 ml aliquot by centrifugation (1,000× g for 5 min at RT) 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 30 min at 37°C with a TAITEC bioshaker BR-22UM (TAITEC, Tokyo,

Japan). Erythrocytes were then mixed with 10 ml of the imaging medium, centrifuged (1,000× g for 5 min at RT) and resuspended in 1.2 ml of the imaging medium. A suspension of erythrocytes (200 µl) was applied in a 35 mm glass-bottomed dish (MatTek Co., Ashland, MA, USA) that had been coated with 1 mg/ml poly-L-lysine before use. After 30 min incubation in a humidified multi-gas water-jacketed incubator at 37ºC, 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 microscopy 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 s for 300–600 s. 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).

Fluorescence H^+ imaging. Fluorescence H^+ imaging was performed as described previously (91). Briefly, a culture of *P. falciparum*-infected erythrocytes was diluted 10-fold with RPMI 1640, phenol red (-), culture medium (Invitrogen Japan), which served as the imaging medium. The infected erythrocytes were collected from the 1 ml aliquot by centrifugation (1,000× g for 5 min at RT) and resuspended in 500 µl of the imaging medium. The parasite's cytoplasm was then loaded with 5 µM cSNARF-5F AM (Invitrogen) for 40 min, the stock solution of the dye was prepared in Pluronic® F-

127 (20% (w/v) solution in DMSO . Pluronic® F-127 was used to facilitate AM dye entry and allow for efficient loading while maintaining cell's integrity. The parasite suspension was then shaken at 200 rpm for 30 min at 37°C with a TAITEC bioshaker BR-22UM (TAITEC, Tokyo, Japan). Erythrocytes were then mixed with 10 ml of the imaging medium, centrifuged (1,000× g for 5 min at RT) and resuspended in 1.2 ml of the imaging medium. A suspension of erythrocytes (200 µl) was applied in a 35 mm glass-bottomed dish (MatTek Co., Ashland, MA, USA) that had been coated with 1 mg/ml poly-L-lysine before use. After 40 min incubation in a humidified multi-gas water-jacketed incubator at 37ºC, 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 for cytoplasmic pH ratio determination of cSNARF-5F AM was performed using the Leica confocal microscopy system (Leica Microsystems) with a 40× oil immersion objective lens and excitation at 488 nm (Argon laser). A dual-emissions were collected in two different wave lengths (λ_1= 580 and λ_2 = 640) using the true spectral detection method developed by Leica Microsystems. Images were captured every 5 s for 300–600 s. The specific fluorescence ratio of cSNARF-5F AM in the cytoplasm (R) was calculated by subtraction of background fluorescence separately from each channel and the fluorescence ration (R) was calculated $R\ (\lambda_1/\lambda_2)$.

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 CQ, thapsigargin (Tg) (Sigma Aldrich Japan) and concanamycin A (CA) (Sigma Aldrich Japan) continuously to and from the parasite preparation during the live cell imaging process.

1.3 Results and Discussion

In order to investigate the potential of 2-APB in reversing CQ-resistance in K-1 strain, the IC_{50} values of both 2-APB and CQ were assessed by *in vitro* drug sensitivity test using SYBR Green I method, and were found to be 73.5 ± 3 µM and 1050 ± 95 nM, respectively (Fig. 5A and B). The addition of the suboptimal dose of 2-APB (50 µM) to the series of CQ concentrations, which causes a minimum effect on *in vitro* growth of the parasite (Fig. 5A) potentiated antimalarial effect of CQ against the CQR K-1 strain and resulted in a significant decline in IC_{50} from 1050 ± 95 nM to 14 ± 2 nM (Fig. 5B). This suggested that 2-APB completely reversed the CQ-resistance in CQR *P. falciparum* K-1 strain. This could be due partial blockage of IP_3 pathway for Ca^{2+} release which is critical for the blood stage development of the parasite using 2-APB (39) and the involvement of CQ in Ca^{2+} homeostasis disturbance (88, 53).

To figure out the mechanism, live cell Ca^{2+} imaging with confocal laser scanning microscopy was performed by loading the trophozoite stage with the calcium sensitive indicator Fluo-4 AM. It was previously observed that CQ induces Ca^{2+} release to the cytoplasm of *P. chabaudi* (88) as well as *P. falciparum* (6). Thus, in this study, the fact that CQ-induced Ca^{2+} release causes disturbance in Ca^{2+} homeostasis in the parasite's cytoplasm was considered, and these observations were utilized to investigate the role of CQ in this strategy. In this study, the CQ-induced Ca^{2+} release to the cytoplasm of the CQR K-1 strain was confirmed (Fig. 6). It has also been demonstrated that there are two Ca^{2+} stores in the parasite, namely, the ER (89) and the acidic food vacuole (AFV) (47). Moreover, In this study, the CQ-induced Ca^{2+} release was demonstrated to be from the AFV by using Tg, the specific inhibitor of

sacro/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) (104) and CA, the specific inhibitor of vacuolar-type H^{+}- ATPase (92) (Fig. 6).

To further elucidate this mechanism, a time lapse imaging of live cell cytoplasmic pH was applied using Leica confocal laser microscopy by loading the trophozoite stage with the florescent ratiometric pH indicator, cSNARF-5F AM (5 μM). It has been reported that Ca^{2+} is stored in the AFV of the parasite under acidic condition (16). It has also been found out that the treatment with CQ causes CQ-associated H^{+} leak from the AFV to the cytoplasm of the CQR parasite causing the alkalinisation of the AFV (69). In the present study the decrease in the cytoplasmic pH due to CQ treatment was also demonstrated (Fig.7). These data suggest that CQ induced Ca^{2+} release could be as a result of alkalinisation of the AFV since it is known that Ca^{2+} is trapped under acidic condition at the AFV of the parasite (16).

In this chapter, a novel strategy to kill the CQR parasites was presented. The strategy based on the partial blockage of IP_3 pathway and the involvement of CQ in Ca^{2+} homeostasis disturbance, may help resolve the longstanding problem of CQ-resistance. These results suggest that 2-APB, and other functionally-related compounds that block the IP_3 pathway, could be promising candidates as leads for searching for novel resistance reversing agents. It is anticipated that future studies will be undertaken to develop a 2-APB analogue that selectively affects Ca^{2+} homeostasis in the parasite cell.

1.4 Summary

Plasmodium spp. have developed resistance to most of the existing antimalarials reflected as measurable rise in malaria morbidity and mortality in African children. This occurs most especially in CQ known to be cheap and non-toxic causing the drug to loss its efficacy. Thus, there is an urgent need to develop new antimalarial drugs and find novel agents to reverse the antimalarial resistance. The antimalarial drug CQ is thought to accumulate in the acidic compartment, although its mechanisms of action are controversial. Recently, some researchers observed that addition of CQ to the parasite preparations caused an increase in cytosolic Ca^{2+} concentrations in these parasites. CQ-induced Ca^{2+} release causes disturbance in Ca^{2+} homeostasis in the parasite's cytoplasm. In this respect, it was hypothesized that the potentiation of CQ activity could be achieved in the malaria parasite with the simultaneous administration of 2-APB which inhibits 1,4,5-trisphosphate (IP_3) -induced Ca^{2+} release. This resulted in a complete reversal of CQ-resistance in the CQR *P. falciparum* K-1 strain. Live cell Ca^{2+} imaging confirmed the CQ-induced Ca^{2+} release. The CQ-induced Ca^{2+} release was further found to be from the AFV of the parasite. Live cell imaging of cytoplasmic pH showed that CQ causes the cytoplasmic pH to decrease as a result of the CQ-associated H^+ leak from the AFV causing the alkalinisation of the organelle, which in turn might be the reason of the CQ-induced Ca^{2+} release since it is known that Ca^{2+} is trapped under acidic condition at the AFV of the parasite (16). These results suggest that 2-APB, and other functionally-related compounds that block the IP_3 pathway, could be promising candidates as leads for searching for novel resistance reversing agents.

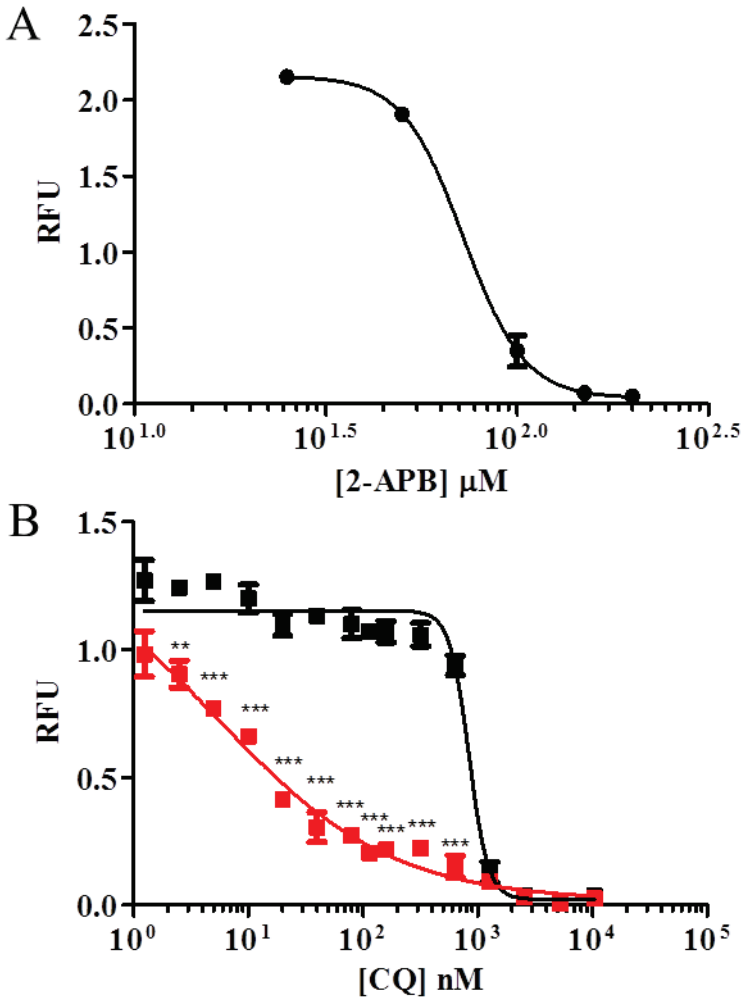

Fig. 5. Dose-dependent activities of 2-APB and chloroquine (CQ) on *P. falciparum* synchronized cultures of CQR K-1 strain. Various concentrations of 2-APB (A). Various concentrations of CQ plus DMSO as solvent control (black line) and CQ plus 50 µM of 2-APB (red line) (B). The statistical significance of differences between treatments was assessed with student's *t*-test. **P<0.004 and ***P<0.0005. Results are presented as the mean ± SD of three independent experiments. RFU denotes relative fluorescence units in SYBR Green I assay (See Materials and Methods).

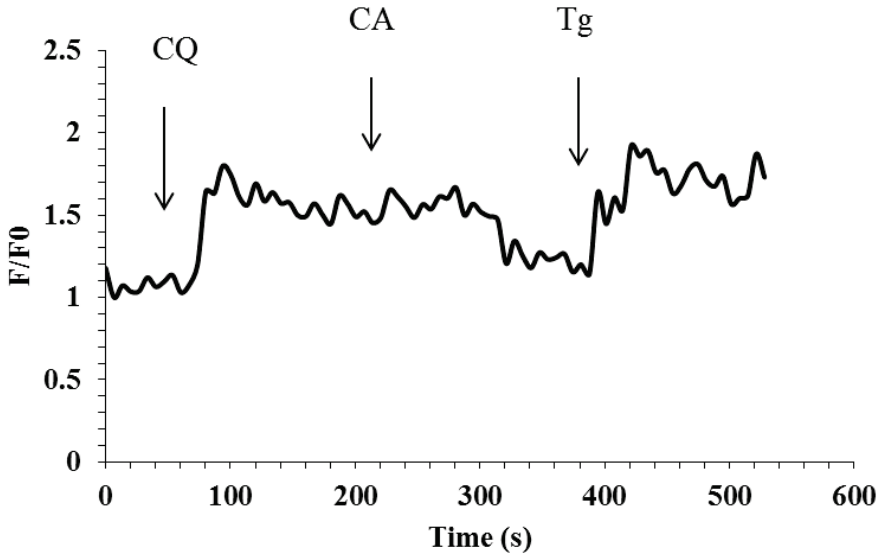

Fig. 6. Ca^{2+} imaging of *P. falciparum* analyzed by confocal microscopy. Trophozoite stages were loaded with Fluo-4 AM, and fluorescence in the parasite cytoplasm (F/F0) was calculated (see Materials and Methods). Treatment with chloroquine (CQ) (10 μM) caused an increase in mean fluorescence ratio of 0.8 ± 0.3 (n = 10). Treatment with concanamycin A (CA) (100 nM) didn't show any change in the fluorescence ratio suggesting that CQ and CA induce Ca^{2+} release from the same compartment (AFV). Treatment with thapsigargin (Tg) (2 μM) caused an increase in the mean fluorescence ratio of 0.9 ± 0.2 (n = 6). Data are representative of eight similar experiments.

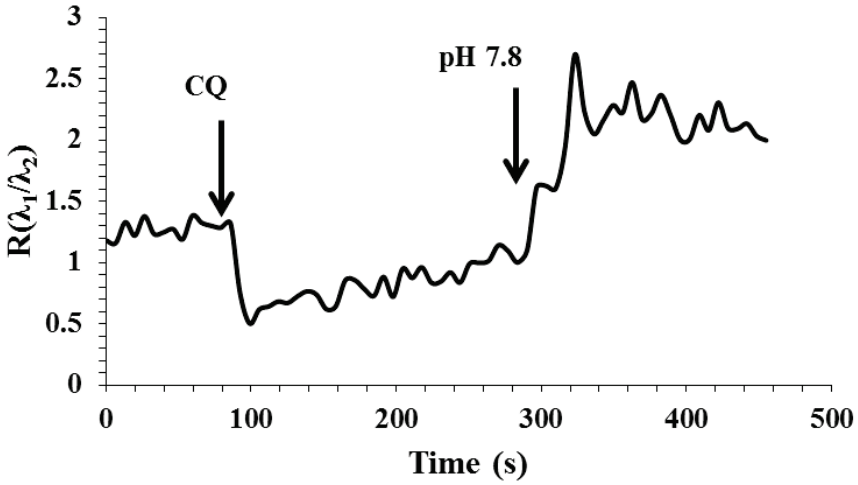

Fig. 7. H^+ imaging of *P. falciparum* analyzed by confocal microscopy. Trophozoite stages were loaded with cSNARF-5F AM (5 μM), and fluorescence ration (R) was calculated $R\ (\lambda_1/\lambda_2)$ (see Materials and Methods). Treatment with 10 μM chloroquine (CQ) caused a decrease in the fluorescence ratio of 0.9 ± 0.2 (n = 9). Treatment with imaging medium (pH 7.8) (served as control) caused an increase in fluorescence ratio of 1.7 ± 0.5 (n = 9) suggesting that CQ causes cytoplasmic pH to decrease. Data are representative of 6 similar experiments.

Chapter II

An *in vivo* application of the IP_3 receptor blocker 2-APB as an effective resistance reverser to chloroquine-resistant *Plasmodium chabaudi*

2.1 Introduction

Malaria is a major cause of illness and death in children and adults in tropical countries. The spread of resistance to available antimalarial drugs such as chloroquine is a major threat to malaria control. The discovery of artemisinin as an antimalarial drug which was isolated from the leaves of the sweet wormwood, *Artemisia annua*, had a great impact on treatment of malaria and more potent derivatives of artemisinin were subsequently developed. Presently, artemisinin-based combination therapy (ACT) is recommended by the WHO as the first-line therapy for *P. falciparum* malaria (113). However, the cost of artemisinins limits their use in the developing world (112) and the emergence of artemisinin-resistant malaria has also been recently reported (34). The development of new, efficacious, affordable drugs remains crucial. Extensive searches for novel compounds have met with only limited success (29). Therefore, to combat malaria, there is an urgent need to develop new antimalarial drugs (65, 56, 46) and find novel agents to reverse antimalarial resistance.

Following the discovery that the Ca^{2+} channel-blocker verapamil (VP) restores chloroquine-sensitivity in CQR strains (66, 71), there has been considerable interest in such so-called resistance-reversing agents (93). In this connection, it is worth to mention that there are a number of reports of attempts to reverse CQ-resistance *in vitro* through the use of Ca^{2+} channel antagonists such as VP (71, 32, 3). However, all of these studies

focused on reversing CQ-resistance through the blockage of CQ efflux from the acidic food vacuole of resistant parasites.

In chapter I of this study, CQ-resistance was successfully reversed in *P. falciparum* by a novel strategy which mainly targets Ca^{2+} homeostasis through the partial blockage of IP_3 pathway for Ca^{2+} release which is critical for the blood stage development of the parasite (39). That was achieved by using 2-APB which inhibits IP_3 -induced Ca^{2+} release. In this study, the strategy was applied *in vivo* to the rodent malaria mouse infection model, CQR *P. chabaudi* AS (30 CQ) strain, to confirm the universality of my finding.

2.2 Materials and Methods

Mice and parasite. 6 week-old female ICR mice (CLEA Japan, Tokyo, Japan; 3 mice/group), were infected with 5×10^6 parasitized erythrocytes of the CQR *P. chabaudi* AS (30 CQ) strain by the intraperitoneal injection. (*Plasmodium chabaudi* chabaudi AS (30 CQ) was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH:, MRA-744, deposited by D Walliker).

***In vivo* drug susceptibility test.** The *in vivo* simultaneous administration of CQ and 2-APB was performed as previously described (77) with some modifications. Briefly, mice were infected by the intraperitoneal injection of 5×10^6 parasitized erythrocytes of the CQR *P. chabaudi* AS (30 CQ) strain. To first evaluate the antimalarial activity of CQ and 2-APB, CQ (3 mg/kg), 2-APB (0.1 and 1 mg/kg) and DMSO (as solvent control) were administered separately via intraperitoneal injection to different groups of mice at days 0, 1 and 2. Thin blood films were prepared at days 1-4 and stained with Giemsa. The number of parasitized erythrocytes per 10,000 erythrocytes in each stained preparation was counted with the mean values obtained from 3 preparations used as an index of parasitemia (%). Antimalarial activity was evaluated at day 4 as follows: Normalized parasitemia= (Parasitemia in the compound-treated group) / (Parasitemia in DMSO control group). To evaluate the CQ-resistance reversing activity with 2-APB, CQ (3 mg/kg) and 2-APB (0.1 mg/kg) were simultaneously administered to a group of mice. A group receiving CQ (3 mg/kg) and VP, a Ca^{2+} channel antagonists, (Wako Chemical Co, Osaka, Japan; (20 mg/kg) served as a positive control, while a group receiving CQ (3 mg/kg) alone served as negative control. The three treatments were administered via intraperitoneal injection at days 0, 1

and 2 after the infection. The potential of 2-APB in reversing CQ-resistance was evaluated at day 4 as follows: Normalized parasitemia= (Parasitemia in the two compound-treated group) / (Parasitemia in the CQ-treated group). 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 25-71).

2.3 Results and Discussion

The antimalarial activity of CQ (3 mg/kg) and 2 different doses of 2-APB (1 mg/kg and 0.1 mg/kg) was initially tested against the CQR *P. chabaudi* AS (30 CQ) strain by intraperitoneal injection. Results showed that 1 mg/kg of 2-APB exhibited antimalarial effect but not the lower concentration of 2-APB (0.1 mg/kg) (Fig. 8A). Furthermore, the simultaneous intraperitoneal injection of a lower concentration of 2-APB (0.1 mg/kg), which presented minimal antimalarial activity, produced a CQ-resistance reversing effect in the mice (Fig. 8B). It is noteworthy that the potency of 2-APB as a CQ-resistance reverser was equivalent to that of verapamil (20 mg/kg, intraperitoneal injection) (Fig. 8B). Verapamil is the first Ca^{2+} channel antagonist that is reported to exhibit a resistance reversing effect in CQ-resistant *P. falciparum* (71). These outcomes suggest the complete reversal of CQ-resistance *in vivo* with 2-APB. These data has strengthened the *in vitro* outcomes confirming the universality the strategy.

2.4 Summary

Drug resistance against most of the available antimalarial drugs has worsened the global malaria situation. In search for novel agents to reverse CQ-resistance *in vitro*, 2-APB was introduced as an effective compound through a novel strategy in chapter I. In this chapter, the same strategy was applied *in vivo* to the rodent malaria mouse infection model, CQR *P. chabaudi* AS (30 CQ) strain, to confirm the universality of my finding in chapter I. Firstly, CQ (3mg/kg) and (2-APB 0.1 mg/kg) were separately intraperitoneal-injected to different groups of mice to check their antimalarial activity. Both two compounds didn't show significant antimalarial activity. Simultaneous intraperitoneal administration of lower concentration of 2-APB (0.1 mg/kg) which presented minimal antimalarial activity produced CQ-resistance reversing effect in the mice. This is probably due to the disturbance of Ca^{2+} homeostasis in the parasite cell. These results strengthened the *in vitro* finding that 2-APB, and other functionally related compounds that block IP_3 pathway, could be promising candidates as leads for searching for novel resistance reversing agents. To the best of our knowledge, this is the first observation of a CQ-resistance reversing effect induced by an IP_3 receptor inhibitor in the malaria parasite.

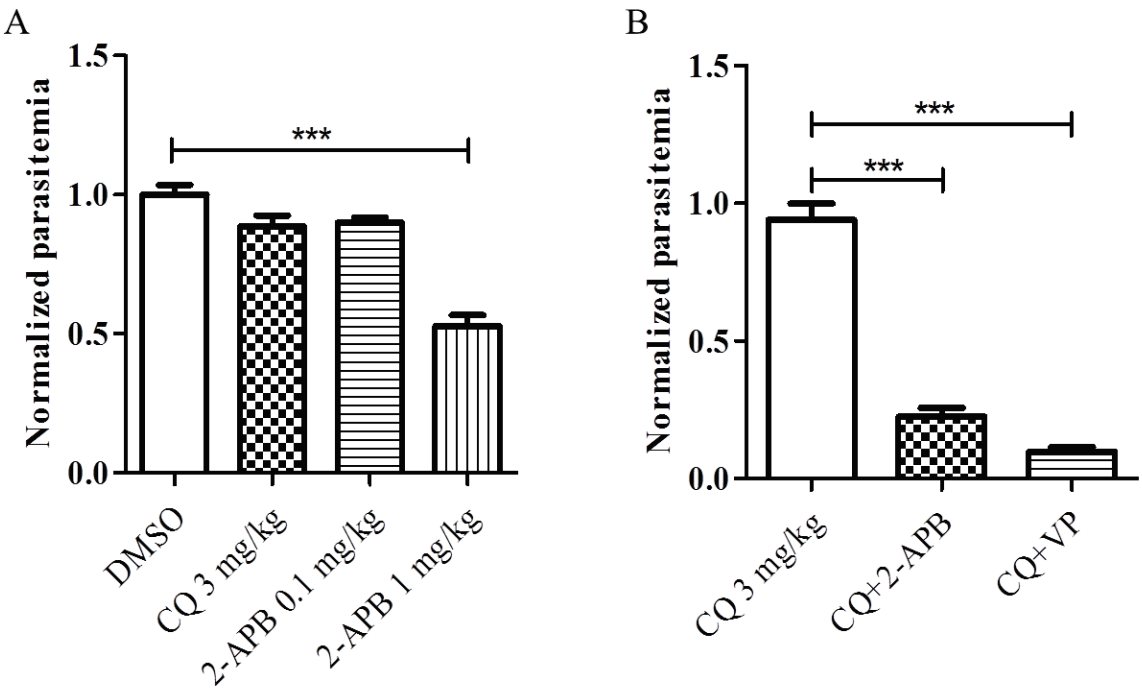

Fig. 8. *In vivo* activities of chloroquine (CQ) and 2-APB on CQR *P. chabaudi* AS (30 CQ) strain (A), and the potential of 2-APB in reversing CQ-resistance (B). Antimalarial activity of each experimental group is shown as the ratio of parasitemia relative to the control (DMSO in A and CQ 3 mg/kg in B). CQ + 2-APB and CQ + verapamil (VP) in B represent CQ (3 mg/kg) plus 2-APB (0.1 mg/kg) and CQ (3 mg/kg) plus VP (20 mg/kg), respectively. The statistical significance of differences between the groups (n = 3) was assessed with one-way ANOVA followed by Dunnett's test. $^{***}P<0.0001$versus control group.

Chapter III

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

3.1 Introduction

As explained in the General Introduction, *B. bovis* is a tick-borne hemoprotozoan parasite of cattle that causes serious economic losses in the livestock industry (67). 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 (31). 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 (80) has made the search for new potent chemotherapeutic agents highly important. Understanding the signaling pathways governing the parasite's growth in the erythrocytic stages may help in strategizing new control measures to combat babesiosis.

Ca^{2+} is a ubiquitous intracellular signal messenger that is responsible for controlling a wide range of cellular activities in eukaryotic cells (15). In protozoan parasites, Ca^{2+}-mediated signaling controls various vital functions, such as protein secretion, motility, cell invasion and differentiation (26, 83, 94, 111). In contrast to the *Plasmodium* and *Toxoplasma* parasites, little is understood about Ca^{2+} signaling 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 (85, 101). The inhibitory effect of Ca^{2+} dependent protein kinase inhibitor on the *in vitro* growth of *B. bovis* has also been reported (21). While egress (release) of *Plasmodium* merozoites and *Toxoplasma* tachyzoites from their host cells has been studied intensively in terms of Ca^{2+} signaling (4, 51, 58, 83), at the present time, there are no available data showing the role of Ca^{2+} in the egress of *Babesia* parasites. Therefore, in this chapter, the involvement of Ca^{2+} in the egress of *B. bovis* merozoites from infected erythrocytes was investigated. Calcium ionophore A23187 and Tg, an inhibitor of SERCA, which have been used in various studies to artificially increase Ca^{2+} concentration in the cytosol of apicomplexan parasite cells (12, 38, 58), were found to induced egress of *B. bovis* from host erythrocytes. In addition, changes in intracellular Ca^{2+} concentration after these treatments were also observed using the live cell Ca^{2+} imaging technique with confocal laser scanning microscopy.

3.2 Materials and Methods

Parasite culture. *B. bovis* (Texas strain) (60) was maintained in a serum-free GIT medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 10% bovine erythrocytes, 60 U/ml of penicillin G, 60 g/ml of streptomycin and 0.15 g/ml of amphotericin B (Sigma Aldrich Japan Co., Tokyo, Japan) (complete culture medium) using a 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 thapsigargin (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 (22, 23) 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 ml plastic tube. A23187 or Tg, which had been dissolved in DMSO, 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 humidified multi-gas water-jacketed incubator with cap open at 37ºC for indicated periods of time. In parallel, normal culture supplemented with the same concentration of DMSO was prepared as 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 in chapter I with some modifications. In brief, a culture of *B. bovis*-infected erythrocytes was diluted 20-fold with RPMI 1640, phenol red (-), culture medium (Invitrogen Japan), which served as the imaging medium. The infected erythrocytes were collected from the 1 ml aliquot by centrifugation (1,000× g for 5 min at RT) 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). Erythrocytes were then mixed with 10 ml of the imaging medium, centrifuged (1,000× g for 5 min at RT) and resuspended in 1.2 ml of the imaging medium. A suspension of erythrocytes (200 µl) was applied in a 35 mm glass-bottomed dish (MatTek) that had been coated with 1 mg/ml poly-L-lysine before use. After 30 min incubation in a humidified multi-gas water-jacketed incubator at 37ºC, 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). 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 s for 200–300 s. 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. A23187 and Tg were added and removed continuously to and from the parasite preparation during the live cell imaging process using the same perfusion system descried in chapter I (Materials and Methods).

3.3 Results and Discussion

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 (12, 38, 58), however, these treatments resulted in the emergence of rarely seen degenerated and dot-shaped parasites (Fig. 9B and C) in the control culture of *B. bovis* (Fig. 9A). 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, it was 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. 10A). 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. 10B). To confirm this observation and determine the time point suitable for observing the egress, I 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. 10C) between test and control parasites. These data suggest that A23187 induces the parasite's egress from infected erythrocytes.

To further examine whether the parasite's egress can be induced by the increased cytosolic Ca^{2+} concentration in the parasite, the parasite culture was incubated with Tg, an inhibitor of the uptake of cytosolic Ca^{2+} to the ER by specific inhibition of SERCA. The parasite was first incubated 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, It was found that all tested concentrations showed significantly higher parasitemia in comparison to control (Fig. 11A). 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 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. 11B). These results indicate that the increase in cytosolic Ca^{2+} concentration most probably induces the parasite's egress and suggest a Ca^{2+} signaling pathway in the egress of this parasite.

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. 12A and B). 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 the future studies.

The information available on Ca^{2+} signaling 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+} (78). 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 (38). *T. gondii* mutants with delayed egress have been isolated and found to have elevated intracellular Ca^{2+} level (9). 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 (58). 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 (101) 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. I 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 (55, 104) and in protozoan parasites (13, 88). To confirm my 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. These 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 (4).

3.4 Summary

Egress is an important feature of the life cycle of *B. bovis*. At some point the parasite needs to exit the cell by rupturing the infected host cell in order to infect other cells to continue its life cycle. In spite of the importance of egress as a crucial step in the parasite's life cycle, to date, there is no available information in the mechanism of egress of *B. bovis* merozoites. In this chapter, the involvement of Ca^{2+} in egress of *B. bovis* merozoites from infected bovine erythrocytes was investigated. The increase in cytosolic Ca^{2+} concentration induced by A23187 and Tg was found to accelerate the parasite's egress. Time lapse imaging of live cell Ca^{2+} revealed that these treatments induced an increase in cytosolic Ca^{2+} concentration of the parasite cells. The data suggest the involvement of Ca^{2+} and a Ca^{2+} signaling pathway in the egress of this parasite. The data provided here is the first report on the parasite egress and therefore, 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.

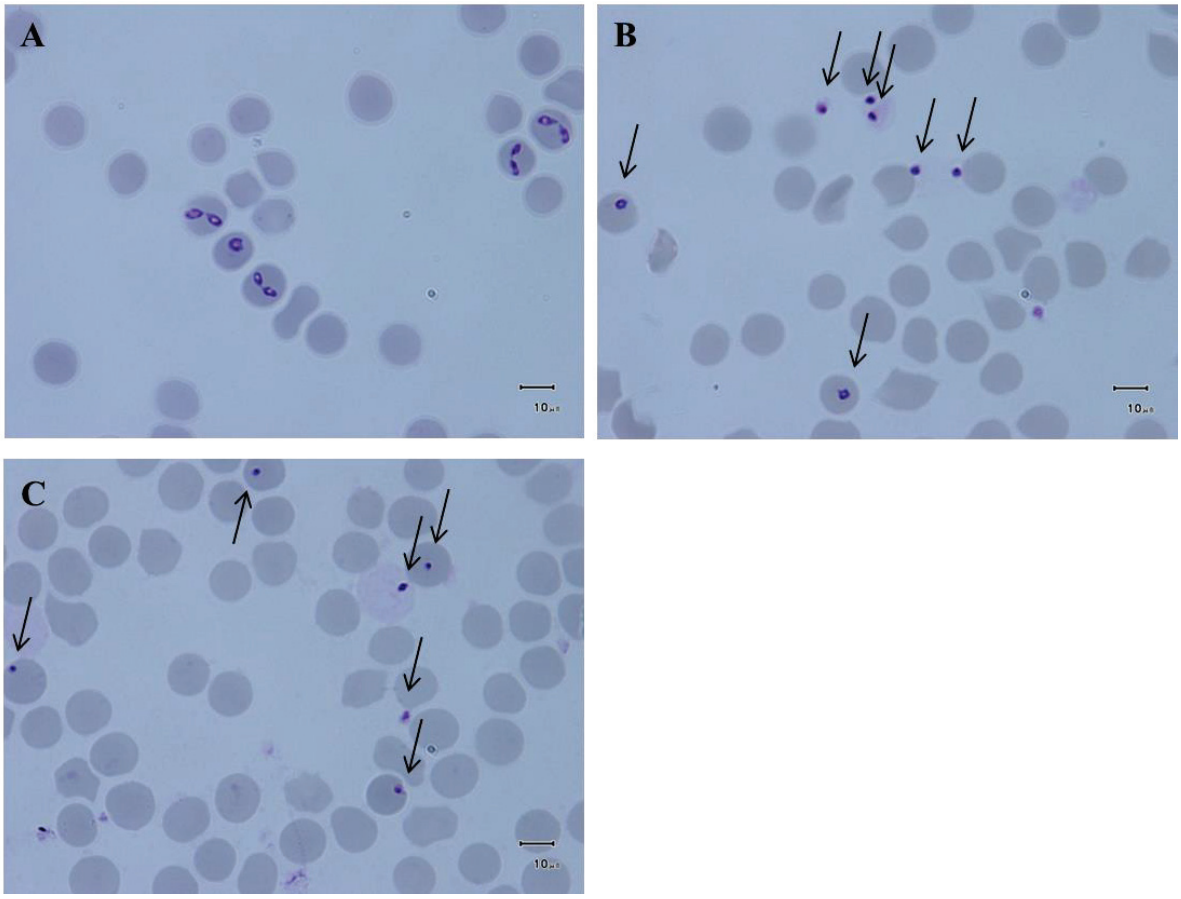

Fig. 9. Light microscopic observation of A23187-treated *B. bovis* in an *in vitro* culture. Micrographs were taken after 10 min incubation with DMSO solvent-control (A), 1 µM A23187 (B) and 10 µM A23187 (C). The A23187 treatments showed a higher number of degenerated and dot shaped parasite (black arrows) than the control. Scale bars indicate 10 µm.

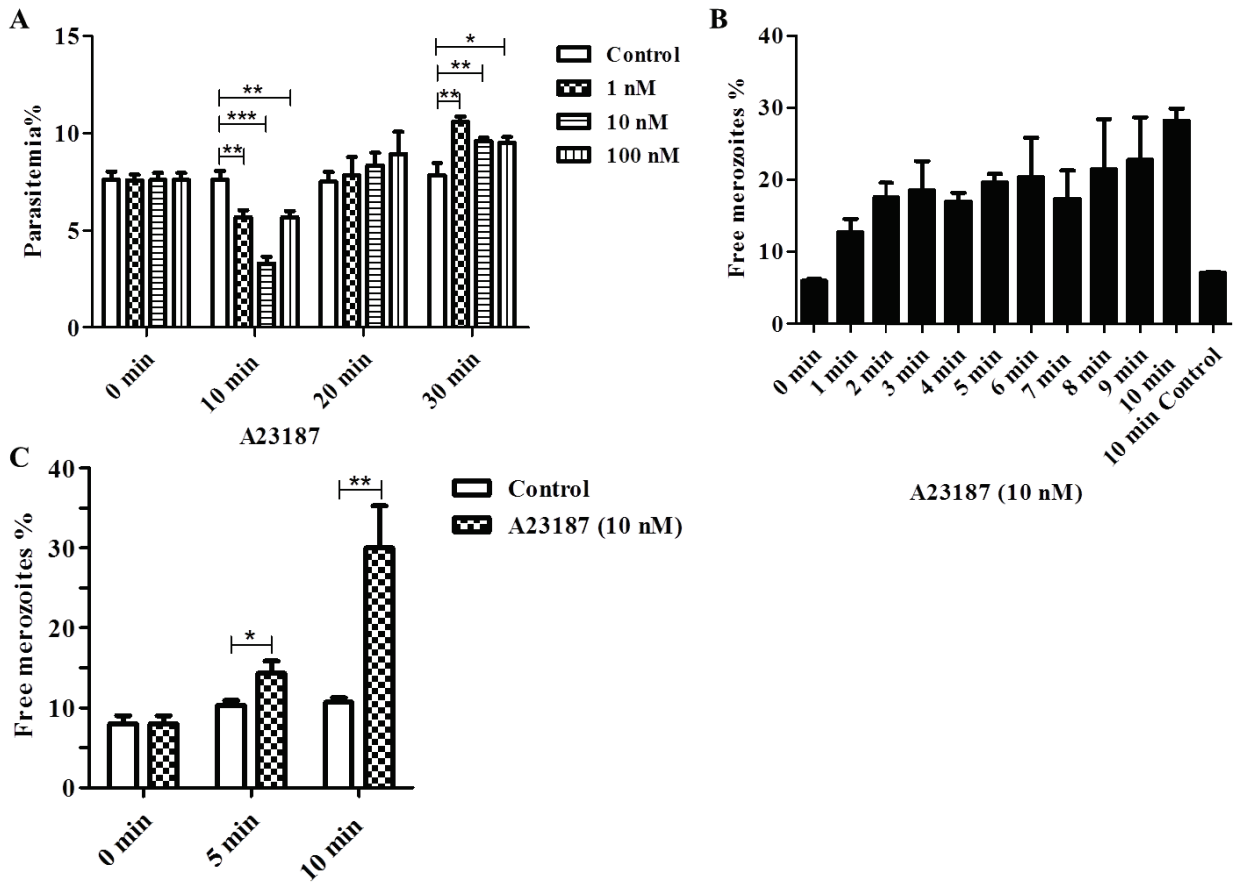

Fig. 10. Effect of A23187 on *B. bovis* culture. The effect was evaluated on parasitemia (A), percentage of extracellular merozoites (number of free merozoites/ number of free merozoites + number of intraerythrocytic parasite × 100) obtained every 1 min until 10 min after treatment (B) and the percentage 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.

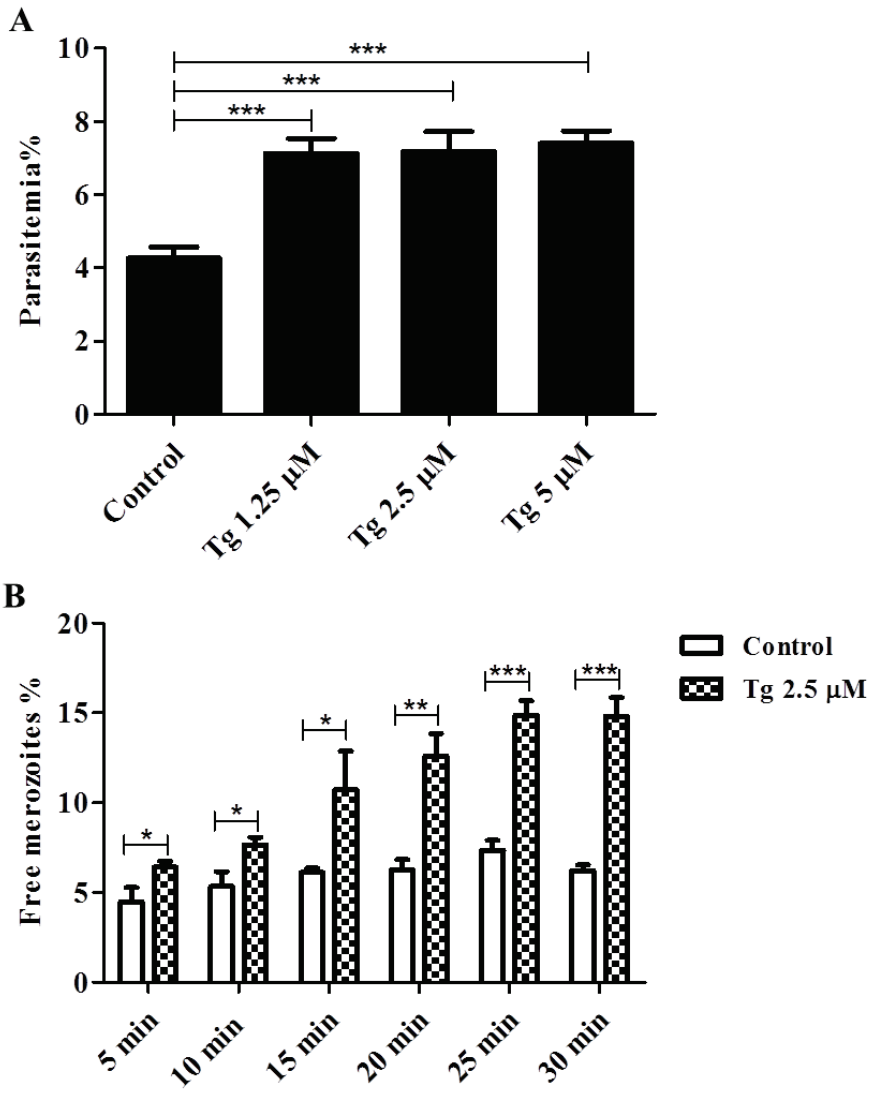

Fig. 11. Effect of thapsigargin (Tg) on *B. bovis* culture. The effect was evaluated on parasitemia after 90 min incubation (A) and the percentage of extracellular merozoites obtained every 5 min until 30 min after treatment (B). Each value represents mean ± SD in 3 independent experiments. The statistical significance of differences was assessed with Student's *t*-test. Asterisks indicate significant differences (*P < 0.02, **P < 0.001and ***P < 0.0002) between Tg-treated groups and solvent (DMSO)-treated control group.

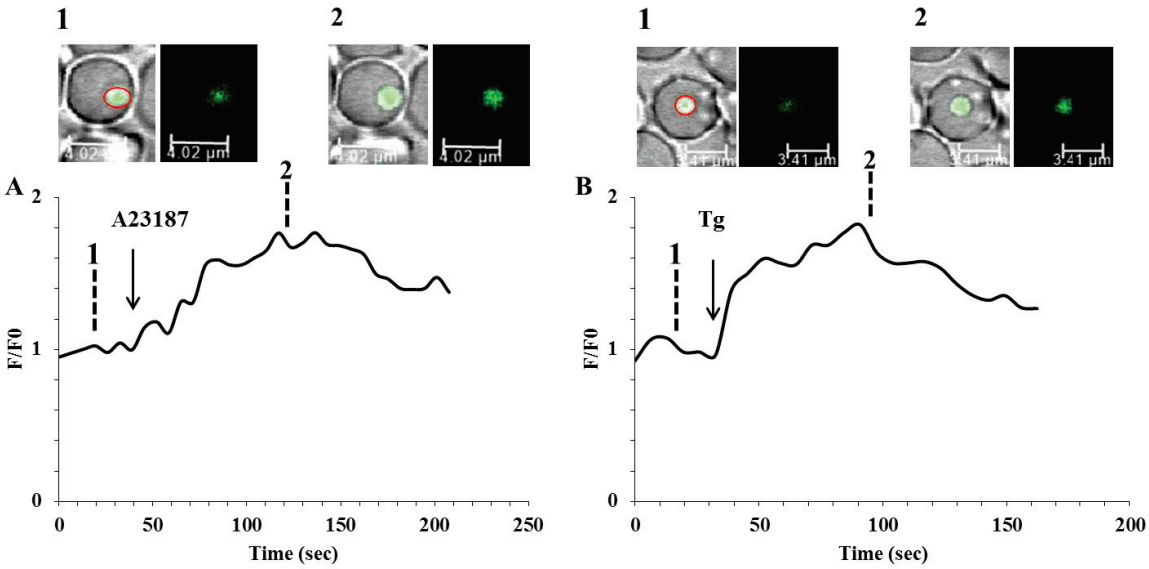

Fig. 12. Ca^{2+} imaging of *B. bovis* merozoites analyzed by confocal microscopy. Parasite cells were loaded with Fluo-4 AM, and fluorescence in the parasite cytoplasm (F/F0) was calculated (see Materials and Methods). Treatment with 100 nM A23187 caused an increase in mean fluorescence ratio of 1.01 ± 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.

General Discussion

Although hemoprotozoan parasites of the phylum Apicomplexa like most eukaryotes, utilize second messenger signaling cascades including Ca^{2+} to coordinate cell functions (6), and Ca^{2+} is known to be relevant for several vital functions in these parasites, the information available on Ca^{2+} signaling components in these parasites is still fragmentary and insufficient (78). The current study's main thrust was to further understand the Ca^{2+} signaling in two hemoprotozoan parasites from the phylum Apicomplexa namely *Plasmodium* and *Babesia*. This study consists of two parts: 1) Reversing of CQ-resistance by potentiation of the antimalarial activity of CQ *in vitro* against CQR *P. falciparum* K-1 strain and *in vivo* against CQR *P. chabaudi* AS (30 CQ) strain by using 2-APB (Chapters I and II); and 2) the involvement of Ca^{2+} in egress of *B. bovis* merozoites from bovine erythrocytes (Chapter III).

In the first chapter, the potential of 2-APB in reversing CQ-resistance in CQR *P. falciparum* K-1 strain was examined. This resulted in a complete reversal of CQ-resistance in the parasite. There are already a number of reports of attempts to reverse CQ-resistance *in vitro* through the use of Ca^{2+} channel antagonists such as verapamil (71, 32, 3). However, all of the studies focused on reversing CQ-resistance through the blockage of CQ efflux from the acidic food vacuole of resistant parasites wherein the compound is thought to exert its effect. In this study, a novel strategy was presented wherein, the possible involvement of CQ itself in the disruption of Ca^{2+} and H^{+} homeostasis in the cytoplasm of *P. chabaudi* cell reported in previous studies (88, 53), might be potentiated with 2-APB which blocks the IP_3 pathway for Ca^{2+} release. This

probably resulted in the disturbance of Ca^{2+} homeostasis in the cytoplasm of the resistant parasite, and consequently reversal of CQ-resistance in the CQR human malaria parasite *P. falciparum* K-1 strain. Attempts have then been made to investigate the mechanism using live cell Ca^{2+} imaging technique. The CQ-induced Ca^{2+} release (88, 53) was demonstrated. It was then found that CQ induces Ca^{2+} release from the AFV of the parasite. Further investigation was done using live cell imaging of cytoplasmic pH confirming that CQ causes the decrease of cytoplasmic pH as a result of the CQ-associated H^+ leak from the AFV. This leads to the alkalinisation of the organelle (69), and therefore it might be the reason behind the CQ-induced Ca^{2+} release since it is known that Ca^{2+} is trapped under acidic condition at the AFV of the parasite (16). Since the strategy depends mainly on the blockage of the IP_3 pathway for Ca^{2+} release and the drawback of CQR parasite that CQ induces Ca^{2+} release, the parasite might not further develop resistance. IP_3 receptor has yet to be identified in malaria parasites, however pharmacological data clearly demonstrate that these parasites maintain intracellular Ca^{2+} stores (13, 52, 109). Moreover, the IP_3 pathway has also been demonstrated in *P. chabaudi* (88). This available information on Ca^{2+} signaling in the malaria parasites leads to the outcome of this study. However, further studies are still required to elucidate the relationship between Ca^{2+} homeostasis and CQ-resistance in malaria parasites as well as developing 2-APB analogue that selectively affect Ca^{2+} homeostasis in the parasite cell.

In the second chapter, the strategy was applied, *in vivo*, to the mouse malaria infection model, CQR *P. chabaudi* AS (30 CQ) strain, to confirm the universality of the obtained result in chapter I. Interestingly, the minimum concentration of 2-APB that

affects the parasite growth, significantly reversed the CQ-resistance in CQR *P. chabaudi*. The potency of 2-APB as CQ-resistance reverser was equivalent to that of verapamil, the first drug reported to exhibit the resistance reversing effect in CQR *P. falciparum* (71). These results strengthened the *in vitro* finding that 2-APB, and other functionally-related compounds that block the IP_3 pathway, could be promising candidates as leads for searching for novel resistance reversing agents. To the best of my knowledge, this is the first observation of a CQ-resistance reversing effect induced by an IP_3 receptor inhibitor in the malaria parasites.

In the third and last chapter, a first report on egress of *B. bovis* merozoites from infected bovine erythrocytes was presented. This was achieved by using tow Ca^{2+} modulators; A23187 and Tg. Both two compounds were found to increase cytosolic Ca^{2+} concentration in a wide range of eukaryotic cells including apicomplexan parasites (12, 38, 58). The Ca^{2+} -dependent induced egress obtained by A23187 and Tg treatments was consistent with that obtained in the other apicomplexan parasites, *Plasmodium*, *Toxoplasma* and *Neospora,* suggesting that apicomplexan parasites may share the same Ca^{2+}-dependent machinery of egress.

Results of this study showed that *B. bovis* egress from erythrocytes could be pharmacologically induced by modulators of cytosolic Ca^{2+} concentration and thus, these reagents could be used to study the egress pathway in a controlled manner. Further studies to investigate the egress pathway downstream to the increase of cytosolic Ca^{2+} concentration might therefore elicit new therapeutic and prevention strategies targeting the molecular components of the egress pathway.

In general, this study demonstrated the involvement of Ca^{2+} in tow important features of two hemoprotozoan parasites of the phylum Apicomplexa. The study demonstrated that the disturbance in Ca^{2+} homeostasis obtained by the simultaneous administration of 2-APB and CQ to the CQ-resistant malaria parasites has resulted in a complete reversal of CQ-resistance in these parasites. This may help resolve the longstanding problem of CQ-resistance. On the other hand, Ca^{2+} was also demonstrated, for the first time, to be involved in egress of *B. bovis* merozoites from infected erythrocytes. This could help in developing new strategies targeting the components of the egress pathway. This may lead to the blockage of the egress of the parasite and consequently the erythrocytic life cycle of the parasite.

Conclusion

In this study, the potential of 2-APB, which inhibits IP_3 pathway for Ca^{2+} release, in reversing CQ-resistance in CQR malaria parasites was examined. This probably resulted in the disturbance of Ca^{2+} homeostasis in the cytoplasm of the resistant parasite, and consequently reversal of CQ-resistance in *P. falciparum* and *P. chabaudi*. In addition, the CQ-induced Ca^{2+} release was demonstrated using live cell Ca^{2+} imaging and it was found that CQ induces Ca^{2+} release from the AFV of the parasite. Also, live cell imaging of cytoplasmic pH showed that CQ causes the cytoplasmic pH to decrease as a result of the CQ-associated H^+ leak from the AFV. This leads to the alkalinisation of the organelle which might be the reason of the CQ-induced Ca^{2+} release since it is known that Ca^{2+} is stored under acidic condition at the AFV of the parasite. These data suggest that 2-APB, and other functionally related compounds that block IP_3 pathway, could be promising candidates as leads for searching for novel resistance reversing agents.

With regards to the role of Ca^{2+} in egress of *B. bovis* merozoites from the infected bovine erythrocytes, the increase in cytosolic Ca^{2+} concentration by Ca^{2+} ionophore A23187 and Tg, which inhibits SERCA, was found to induce the parasite's egress. This suggests the involvement of Ca^{2+} in this crucial step in the life cycle of the parasite, giving a good opportunity for future studies to target the components of the egress pathway for new therapies and prevention strategies.

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