RGS2-Mediated Intracellular \( \text{Ca}^{2+} \) Level Plays a Key Role in the Intracellular Replication of \textit{Brucella abortus} Within Phagocytes

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**Background.** \textit{Brucella abortus} can proliferate within professional and nonprofessional phagocytic host cells and thereby successfully bypass the bacteriocidal effects of phagocytes. However, the intracellular survival mechanism and factors of virulence are not fully understood.

**Methods.** We have investigated the role of the regulator of G protein signaling 2 (RGS2), an intracellular calcium ([\( \text{Ca}^{2+} \)]_i) regulator of the host cell, in the intracellular survival of \textit{B. abortus} within phagocytes.

**Results.** \textit{B. abortus} infection markedly induced RGS2 messenger RNA expression in early phase and increased the [\( \text{Ca}^{2+} \)]_i level up to 24 hours postinfection within macrophages from wild-type mice. The [\( \text{Ca}^{2+} \)]_i level, however, was not influenced by \textit{B. abortus} infection within macrophages from RGS2-deficient mice. Furthermore, \textit{B. abortus} survival was reduced within RGS2-deficient macrophages, and hence bacterial proliferation was inhibited in RGS2-deficient mice. Moreover, treatment with the \( \text{Ca}^{2+} \) chelator ethylenediaminetetraacetic acid (EDTA) or 1,2-bis-(2-amino-phenoxy)ethane-N,N,N',N'-tetraacetic acid acetylomethyl ester (BAPTA-AM) and the L-type \( \text{Ca}^{2+} \) channel-blocking agent nifedipine or genistein also showed a reduced intracellular replication of \textit{B. abortus} within macrophages.

**Conclusion.** These results indicate that \textit{B. abortus} infection induces host RGS2 expression and that up-regulation of [\( \text{Ca}^{2+} \)]_i levels is an essential factor for the intracellular survival of \textit{B. abortus} within phagocytes.

Brucellosis is a bacterial zoonosis that causes chronic, debilitating disease in humans, and abortion and sterility in livestock. \textit{Brucella abortus}, the causative agent of brucellosis, is considered a facultatively extracellular/intracellular pathogen that survives and replicates inside both phagocytic and nonphagocytic host cells [1, 2]. The virulence of \textit{B. abortus} is thought to be due to its ability to avoid the bactericidal effects of professional and nonprofessional phagocytic host cells [3]. The macrophage response to infection has important consequences for both the survival of phagocytosed bacteria and the further development of host immunity. However, very little is known about the macrophage cell signaling pathways initiated upon infection and the virulence strategy that \textit{Brucella} uses to counteract these responses and secure its survival. Some studies with phagocytes have shown that Rho1, Rac1, Cdc42, Sar1, and phosphoinositide 3-kinase activities of host cells have been shown to be important for \textit{Brucella} infection [4, 5]. Despite these advances, the factors that mediate \textit{Brucella} infection of host cells remain obscure.

Many extracellular stimuli elicit physiological responses in target tissues by activating receptors that couple to G proteins. G proteins behave as molecular switches, which coordinate the transfer of information from environmental signals to the cell interior [6]. Upon activation of the G protein, \( \alpha \) is stimulated to exchange bound guanine diphosphates for cytosolic guanine triphosphates (GTP). \( \alpha \) is then capable of interacting
with cellular effectors for a period of time that is limited by the intrinsic GTPase activity, and Go activates specific downstream effectors such as adenyl cyclase, phospholipase Cb, and GTPases, mitogen-activated protein kinases, and ion channels [6]. These effectors, in turn, produce a number of cellular responses, including proliferation, morphological changes, and gene transcription [7]. Regulator of G protein signaling (RGS) proteins bind to activated Go subunits, increasing the rate of GTP hydrolysis and attenuating the interactions between Go and its target enzymes [8, 9]. RGS2, one of the RGS protein families, is widely expressed in mouse tissues that interact with Go and Go within G protein–coupled receptor (GPCR) pathways [6, 10]. RGS2 might bind to a receptor G protein effector signaling complex to regulate Go-dependent cyclic adenosine monophosphate (cAMP) production. RGS2 regulates cAMP production and appears to interact with both adenyl cyclase and its stimulatory Go protein [11]. Stimulation of GoGo which is regulated by RGS2, promotes the activity of phospholipase Cb that hydrolyzes phosphatidylinositol bisphosphate to generate inositol 1,4,5-trisphosphate (IP3) [12]. IP3 releases calcium from endoplasmic reticulum (ER), and there is an influx of calcium, which is a consequence of the activation of store-operated calcium channels in the plasma membrane [13, 14].

In the present study, intracellular replication and survival of B. abortus in RGS2−/− phagocytes and knockout (KO) mice was assessed. Moreover, intracellular cAMP and [Ca2+]i levels in B. abortus–infected phagocytes from RGS2−/− and wild-type (WT) strains were evaluated. Finally, we determined the effect of L-type calcium channel inhibitors and calcium chelators on intracellular survival of B. abortus.

**MATERIALS AND METHODS**

**Animals**

Male C57BL/6 RGS−/−, RGS−/+ , and RGS+/+ were obtained from the Department of Cell Biology and Physiology, Washington University School of Medicine (St. Louis, MO) [15]. All mice in this study were provided ad libitum with normal food and water and subjected to a standard light/dark cycle. The in vivo experiments were conducted in accordance with internationally accepted guidelines on the use of laboratory animals, and the protocols were approved by the Animal Ethical Committee of Gyeongsang National University.

**Bacterial Culture and Media**

B. abortus 544 (American Type Culture Collection 23448) was maintained as frozen glycerol stock and was cultured in Brucella broth (BD), Brucella broth containing 1.5% agar, or Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone).

**Cell Culture**

Bone marrow–derived macrophages (BMMs) from RGS2+/+ and RGS2−/− mice were prepared by the method described previously [16]. After culture in L-cell–conditioned medium, BMMs were resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS). The BMMs were seeded (1 × 10^5 or 1 × 10^6 per well) in 96- or 12-well tissue culture plates 1 day before infection for all assays.

**Determination of Bacterial Intracellular Growth Within BMMs**

To conduct the bacterial intracellular survival assay, B. abortus was deposited onto BMMs grown on 96-well tissue culture plates with Dulbecco’s modified Eagle’s medium (DMEM) or RPMI 1640 containing 10% FBS at a multiplicity of infection of 20 and centrifuged at 150g for 10 minutes at room temperature. For analysis of intracellular growth, cells were washed once with medium after 1 hour of incubation at 37°C, incubated with DMEM or RPMI 1640 with gentamicin (30 μg/mL), and then incubated for 2, 24, or 48 hours. Cells were washed with PBS and lysed with distilled water. Colony-forming units (CFUs) were measured on Brucella agar plate.

For Ca2+ chelators and channel inhibitors, cytotoxic effects of 1,2-bis-(2-amino-phenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA-AM; Calbiochem), ethylene diaminetetraacetic acid (EDTA), genistein, and nifedipine (Sigma) were dose-dependently evaluated using a tetrazolium (MTT)–based colorimetric assay, and optimal doses were selected for subsequent examination; dimethyl sulfoxide was used as a vehicle (0.1%). Accordingly, cells were incubated with vehicle, BAPTA-AM (10 μmol/L), EDTA (1 mmol/L), genistein (100 μmol/L), or nifedipine (50 μmol/L) for 30 minutes before bacterial infection for 48 hours. Bacterial survival or proliferation was then assayed.

**Virulence Determination in Mice**

Two groups of 10 mice each were infected intraperitoneally with 2 × 10^8 CFU/mL B. abortus. In addition, 2 groups of 10 mice each were used as an uninfected control. The spleen weight and bacterial CFU in the spleen of infected mice were examined 10 days postinfection as described by previous methods [17].

**RNA Preparation and mRNA Analysis by Reverse Transcription–Polymerase Chain Reaction**

BMMs were cultured in a 12-well plate; bacterial infection and gentamicin treatment were performed as described above. After 0, 1, 4, 8, or 24 hours of incubation, the total RNA was isolated by using a RNA purification kit (Intron). For preparation of the complementary DNA (cDNA), each RNA sample was incubated with a cDNA synthesis kit at 45°C for 60 minutes, and the cDNAs were denatured at 95°C for 5 minutes. The polymerase chain reactions (PCRs) were performed using primers for RGS2, 5’-CAA AAC TCC TCT ACT CCT GGG AA-3’ (sense), and 5’-GT TGT CTC CAT CAG GCT GTA CA-3’ (antisense); and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5’-CAA TGC CAA GTA TGA TGA CAT-3’ (sense), and 5’-CCT GTT ATT ATG GGG GTC TG-3’ (antisense) [18, 19]. The PCR
conditions for RGS2 were 35 cycles (95°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds) and 72°C for 10 minutes. For GAPDH, the process involved amplifying by 30 cycles (95°C for 40 seconds, 55°C for 40 seconds, 72°C for 40 seconds) and 75°C for 5 minutes.

cAMP Assay
Bacterial infection and gentamicin treatment were performed as described above. After 0, 2, 4, 8, or 24 hours of incubation, the BMMs were rapidly washed, lysed, and then the intracellular cAMP level was determined with a Biotrak cAMP enzyme immunoassay system (Amersham).

Determination of Intracellular Calcium
RGS2+/+ and RGS2−/− BMMs were infected with B. abortus and incubated for 0, 4, 8, and 24 hours at 37°C. After incubation, the BMMs were reacted with 10 mmol/L of Fura2-AM in Hank’s balanced salt solution (HBSS) for 30 minutes in the dark at 37°C. Fura-2AM fluorescence was measured in a spectrofluorometer (F-2500, Hitachi) with an excitation wavelength that ranged between 340 and 380 nm; the emission wavelength was 510 nm. Calibration was performed with the addition of 0.1% Triton X-100 to determine the maximum fluorescence, and an additional 25 mmol/L of ethyleneglycoltetraacetic acid (EGTA) was sequentially added to determine the minimum fluorescence. The ratios of the corrected fluorescence intensities (R) were then converted to the actual calcium concentration using the formula $[Ca^{2+}] = \frac{K_d (R - R_{min})}{(R_{max} - R)}$ [20].

For the microscopic investigation of intracellular calcium, the BMMs were reacted with 15 mmol/L of Calcium Orange-acetoxyethylester and 0.01% Pluronic F-127 (Molecular Probes) in HBSS. The cells were then washed 3 times with HBSS, and the intracellular calcium level was determined immediately [21]. Calcium Orange fluorescence emission was collected by using a confocal laser scanner (FV-1000, Olympus) in response to a 543 nm excitation wavelength.

Lysosomal-Associated Membrane Protein 1 Staining
Lysosomal-associated membrane protein 1 (LAMP-1) staining was performed as described previously [16]. Macrophages were infected with B. abortus and then incubated for 30 minutes and fixed in 4% periodate-lysine-paraformaldehyde-sucrose. The samples were washed 3 times in PBS for 5 minutes and then permeabilized at −20°C in methanol for 10 seconds. After 3 cycles of 5-minute incubations with a blocking buffer (2% goat serum in PBS), the samples were washed and then incubated with 1% mouse monoclonal antibody (Santa Cruz) followed by 3 cycles of washing before staining with Texas red-goat antirabbit immunoglobulin G (Santa Cruz). The samples were stained with anti-B. abortus polyclonal rabbit serum and fluorescein isothiocyanate-conjugated goat antirabbit immunoglobulin G (Sigma) in blocking buffer to identify the bacteria using fluorescence microscopy. One hundred bacteria within macrophages were selected randomly, and the extent of LAMP-1 acquisition of bacteria was determined.

Statistical Analysis
The results obtained were expressed as mean ± standard deviation for each of the experiments. A 2-tailed Student t test was used to make a statistical comparison between the groups. Results with $P < .05$ were considered statistically significant.

RESULTS

Bacterial Intracellular Growth Within RGS2+/+ and RGS2−/− BMMs
To determine whether RGS2 regulates B. abortus replication in BMMs, the bacteria were infected in RGS2+/+ or RGS2−/− BMMs, and the intracellular replications were investigated. As shown in Figure 1A, the bacterial replication was significantly inhibited in RGS2−/− BMMs at 24 and 48 hours of incubation. To prove whether the extent of RGS2 expression is one of the mechanisms for intracellular survival of B. abortus in BMMs, the level of RGS2 expression in WT strain was evaluated at different time points in B. abortus–infected BMMs. An increase in RGS2 expression was observed after 1 hour of bacterial infection with a marked induction at 8 hours after infection, which was restored to basal level at 24 hours (Figure 1B).

Bacterial Proliferation in Mice
To further confirm whether the positive correlation between bacterial survival and enhanced RGS2 expression in BMMs cells is translated into biological systems, RGS2−/− and RGS2+/+ mice were infected with B. abortus. As shown in Figure 2A, bacterial proliferation (Figure 2B), were dramatically reduced in RGS2−/− mice compared with their RGS2+/+ counterparts. These results suggest that RGS2 may play a key role in the intracellular survival of B. abortus in macrophages and mice.

Role of RGS2 in cAMP Activation Within B. abortus–Infected Macrophages
To elucidate the RGS2 signaling pathways for intracellular replication of B. abortus within macrophages, the intracellular cAMP level was investigated on B. abortus–infected BMMs. The results showed that cytosolic cAMP levels were increased from 1 to 4 hours of bacterial infection in both RGS2+/+ and RGS2−/− BMMs. However, there was no significant differences in cAMP levels between RGS2+/+ and RGS2−/− BMMs (Figure 3).

RGS2 Regulates the Intracellular Calcium Level in B. abortus–Infected BMMs
To further examine whether the observed link between bacterial survival rate and RGS expression is regulated by $[Ca^{2+}]$, oscillation, the $[Ca^{2+}]$ level was assayed in B. abortus–infected RGS2+/+ macrophages. As shown in Figure 2, the bacterial replication was significantly inhibited in RGS2−/− BMMs at 24 and 48 hours of incubation. To prove whether the extent of RGS2 expression is one of the mechanisms for intracellular survival of B. abortus in BMMs, the level of RGS2 expression in WT strain was evaluated at different time points in B. abortus–infected BMMs. An increase in RGS2 expression was observed after 1 hour of bacterial infection with a marked induction at 8 hours after infection, which was restored to basal level at 24 hours (Figure 1B).
and RGS2−/− BMMs. Interestingly, increase in \([\text{Ca}^{2+}]\) was evident between 4 and 24 hours postinfection in RGS2+/+ BMMs. However, bacterial infection at the indicated incubation time had no effect on \([\text{Ca}^{2+}]\) levels in RGS2−/− counterparts (Figure 4A). Confocal microscopy further revealed that the level of \([\text{Ca}^{2+}]\) in \(B.\) abortus–infected RGS2+/+ BMMs was increased at 24 hours postinfection compared with uninfected RGS2+/+ BMMs. However, the intracellular calcium level of \(B.\) abortus–infected RGS2−/− BMMs was not different from uninfected RGS2−/− BMMs (Figure 4B), suggesting that RGS2 may regulate the \([\text{Ca}^{2+}]\) in \(B.\) abortus–infected BMMs such that the elevated \([\text{Ca}^{2+}]\), in turn, plays a key role in the intracellular replication of \(B.\) abortus in RGS2+/+ BMMs.

Since RGS2 protein is reported to regulate \(\text{Ca}^{2+}\) influx through L-type calcium channels and its up-regulation has been reversed by calcium channel inhibitors [22], we determined whether the link between increased RGS2 expression and \([\text{Ca}^{2+}]\), elevation in the enhanced survival of \(B.\) abortus within BMMs is mediated by these channels. Pretreatment of BMMs with BAPTA-AM (intracellular calcium chelator) or EDTA (extracellular calcium chelator) inhibited the replication of \(B.\) abortus (Figure 5A). Further, treatment of cells with genistein and nifedipine (L-type calcium channels inhibitors) reduced proliferation of \(B.\) abortus compared with the untreated and bacteria-infected groups (Figure 5B) [23]. These results suggest that RGS2 plays an important role for the intracellular proliferation of \(B.\) abortus and that the regulation of \(\text{Ca}^{2+}\) influx in \(B.\) abortus–infected BMMs may be through the L-type calcium channels from an extracellular calcium source.

Survival of \(B.\) abortus Under Nutrient-deprived Conditions

Our result showed that \(B.\) abortus was not able to survive in RGS2−/− and calcium-depleted conditions in BMMs. To further
explore the difference in the survival factor of the bacteria between RGS2
1/
1 and RGS2
2/
2 BMMs, we examined the colocalization of phagosomes (B. abortus–containing vacuoles [BCV]) and lysosomes using LAMP-1 staining [24]. The LAMP-1 co-localization rates of BCV within RGS2
1/
1 and RGS2
2/
2 BMMs were 18.6 ± 3.2% and 19.8 ± 2.4%, respectively. This suggests that RGS2 does not affect the colocalization between BCV and either late endosomes or lysosomes. To test the bacterial survival under calcium-depleted conditions in vitro, EDTA was added to RPMI 1640, known as minimum essential medium, and B. abortus was incubated at 37°C in a shaking condition for the indicated time period. The CFUs were then measured in a serial dilution of sample inoculated onto Brucella agar. As shown in Figure 6, bacterial growth in the EDTA-untreated control was not detectable until 8 hours of incubation, after which a marked elevation was observed until 24 hours with a more pronounced increase until 48 hours. In contrast, B. abortus did not replicate in Ca2+−depleted conditions across the range of treatment time course (Figure 6). This result suggests a delicate balance in [Ca2+]i, may exist for the survival of the bacteria under nutrient-deprived conditions, such as within the intracellular space of macrophages.

DISCUSSION

Brucella proliferate within professional and nonprofessional phagocytic host cells and successfully bypass the bactericidal effects of phagocytes [3, 25]. In this study, we showed that

B. abortus infection induced the RGS2 messenger RNA (mRNA) in phagocytes and the bacteria did not survive within RGS2−/− phagocytes.

RGS2 proteins bind to activated Gα subunits, increasing the rate of GTP hydrolysis, and block the interactions between Gα and its target enzymes [8, 9]. RGS2 mRNA was independently upregulated by activators of the Gαs- and Gαq-coupled pathways.
in cells [19]. Activated Gαs stimulates adenylyl cyclase to generate intracellular cAMP [26]. Because RGS2 inhibits Gαs signaling to certain adenylyl cyclases, in the absence of RGS2 cAMP levels elevate within the cell [10]. Intracellular cAMP-induced protein kinase A activation is an essential factor for intracellular replication and phagocytic pathways in brucellosis [27]. When the cAMP level is induced by B. suis in macrophages, phagolysosome fusion is inhibited. In contrast, decreasing cAMP levels stimulate actin assembly within phagocytes and induced phagolysosome fusion and acidification in Brucella-infected cells [27]. However, there were not differences in the intracellular cAMP level between B. abortus–infected RGS2+/+ and RGS2−/− BMMs. Therefore, RGS2 would not be activated by increased intracellular cAMP in B. abortus–infected BMMs.

RGS2 potently interferes with signaling through receptors that couple to Gαq. Following receptor activation, the activation of phospholipase C, intracellular Ca2+ mobilization, and ion channel activity are reported to be regulated by RGS2. RGSs as regulating proteins capable of modifying G protein activity may play an important role in regulating Ca2+ influx through L-type calcium channels [22]. As such, RGS2 can interact with a G protein that is known to inhibit L-type calcium channels [26, 28]. In this concept, overexpressed RGS2 mRNA on B. abortus–infected phagocytes should lead to decreased Ca2+ entry via L-type calcium channels. In addition, the [Ca2+]i level of RGS2−/− phagocytes with B. abortus infection would be increased. However, the results of this study showed a different and opposite result from this hypothesis. RGS2-mediated reduction of G protein pathways that target L-type calcium channels may have an important role for Ca2+ homeostasis. Raising the RGS2 intracellular concentration leads to decreased channel inhibition and to increased Ca2+ entry [22, 29]. Therefore, [Ca2+]i levels were elevated in RGS2+/+ BMMs with B. abortus infection. This suggests that the virulence of bacteria may perturb the host cell physiology to the increased frequency of [Ca2+]i oscillation to increase [Ca2+]i. However, RGS2−/− cells in this study may adapt to the increased [Ca2+]i oscillation and reduce excitability of cells via adaptation of all transporters to the reduced Ca2+ influx. Such adaptation mechanisms have been reported to occur in RGS2 KO cells for the reduced

Figure 5. Influence of a calcium chelator on bacterial intracellular survival within bone marrow–derived macrophages (BMMs). BMMs were preincubated for 30 minutes with intracellular (BAPTA-AM, 10 μmol/L) or extracellular (EDTA, 1 mmol/L) calcium chelators or L-type calcium channel inhibitors (genistein, 100 μmol/L; or nifedipine, 50 μmol/L). Then BMMs were infected with Brucella abortus and incubated at 37°C for the periods of time indicated and depicted for BAPTA-AM and EDTA (A) and genistein and nifedipine (B). Values are mean ± SD of colony-forming units (CFUs) in triplicate samples. Statistically significant differences between the bacterial growth of the inhibitor-untreated control (control) and inhibitor-treated BMMs are indicated by an asterisk (*P < .01). Abbreviations: BAPTA-AM, 1,2-bis-(2-amino-phenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester; EDTA, ethylenediaminetetraacetic acid.

Figure 6. Bacterial survival under calcium-depleted conditions. Brucella abortus was grown in RPMI 1640 medium with or without EDTA (1 mmol/L) at 37°C by the shake condition for the periods of time indicated. Values are mean ± SD of colony-forming units (CFUs) in triplicate samples. Statistically significant differences between the bacterial CFU of the EDTA-untreated control (control) and the calcium-depleted conditions are indicated by asterisks (*P < .01; **P < .001). Abbreviations: RPMI, Roswell Park Memorial Institute; EDTA, ethylenediaminetetraacetic acid.
excitability of Ca$^{2+}$ signaling to changes in the ER Ca$^{2+}$ load and a decrease in the [Ca$^{2+}$]$\_i$ level [12, 30]. The observed difference in [Ca$^{2+}$]$\_i$ between RGS2$^{-/-}$ and RGS2$^{+/+}$ BMMs in this study may be a result of such adaptation mechanisms to maintain [Ca$^{2+}$]$\_i$, homeostasis under *Brucella* infection.

Cyclic AMP activity was not different between RGS KO and WT cells. Interestingly, however, our study revealed that increased [Ca$^{2+}$]$\_i$, oscillation and RGS2 up-regulation positively influenced the survival of bacteria, whereas in RGS KO cells, both [Ca$^{2+}$]$\_i$ oscillation and bacterial growth were arrested. A similar effect was also observed in calcium-deprived conditions using chelators and L-type channel inhibitors. These results suggest that up-regulation of RGS2 may be regulated by increased [Ca$^{2+}$]$\_i$, oscillation under *Brucella* infection as a negative feedback mechanism. Alternatively, [Ca$^{2+}$]$\_i$ reduction and bacterial growth are suppressed in RGS KO or under Ca$^{2+}$-depleted conditions, suggesting that Ca$^{2+}$ influx through L-type channels is inhibited in both conditions [12, 30].

The [Ca$^{2+}$]$\_i$ level is an important factor for the survival of intracellular pathogens, and a rise in [Ca$^{2+}$]$\_i$ levels is a common phenomenon upon ingestion of *Brucella* by macrophages. Calcium concentrations in macrophage lysosomes are much higher than cytosolic calcium levels [31, 32]. Most bacteria containing ingestion vacuoles acidify rapidly and enter the classic endosomal pathway, which results in degradation of the vacuole content. [Ca$^{2+}$]$\_i$, which is involved in the progression of the phagosomal maturation process, has been demonstrated to increase upon ingestion of *Escherichia coli* and *Mycobacterium tuberculosis* by macrophages [31, 33]. In *M. tuberculosis* infection, intracellular *M. tuberculosis* inhibits the rise in [Ca$^{2+}$]$\_i$ [33]. In addition, a rise in [Ca$^{2+}$]$\_i$ was indispensable for the efficient killing of *Staphylococcus aureus*, *Chlamydia pneumonia*, and *M. bovis* [34–36]. However, several bacteria, such as *Legionella pneumophila*, have different responses to changes in [Ca$^{2+}$]$\_i$. Reducing [Ca$^{2+}$]$\_i$ in infected human monocytes reduces the intracellular replication of *L. pneumophila* [21], and phagosome-lysosome fusion is calcium-independent in *Staphylococci* and *M. bovis*-infected macrophages [37]. Although phagosome-lysosome fusion, which is mediated by [Ca$^{2+}$]$\_i$, is an essential factor for the intracellular killing of several bacteria within macrophages, BCV and LAMP-1 colocalization was not influenced by Ca$^{2+}$ depletion in RGS2$^{-/-}$ BMMs in these experiments, suggesting that RGS2 may not be involved in the regulation of phagosome-lysosome fusion.

After cell infection, intracellular *Brucella* resides within a vacuole that interacts with early endosomes. These early BCV avoid further interactions with the endocytic pathway, yet they acquire LAMP-1 and are found surrounded by, or in close contact with, the ER within the first hours after infection [38–40]. Further maturation of BCV into replicable organelles is characterized by the progressive exclusion of LAMP-1 from the vacuolar membrane, a process that is likely initiated when these vacuoles intercept the secretory pathway at ER exit sites, subsequently undergoing fusion with membranes that contain ER resident proteins, including calreticulin and calnexin [5, 41]. Calreticulin and calnexin are members of a family of ER chaperones that fold newly synthesized polypeptides. Aside from their role as foldases in the ER, all members of this family of proteins modulate Ca$^{2+}$ oscillations. ER-derived organelles support *Brucella* replication in individual vacuoles through continual ER membrane accretion to provide the new input of membrane required during bacterial growth and division [36]. In addition, treatment with an intracellular calcium chelator that depleted [Ca$^{2+}$]$\_i$, reduced survival of *B. abortus* in this study. Therefore, providing sufficient Ca$^{2+}$ to BCV may determine bacterial survival.

A previous report showed that *Bacillus subtilis* and *E. coli* did not replicate with EGTA in nutrient medium [42]. Considering the bacterial growth deficiency observed in calcium-deprived minimum essential medium in this study, calcium would be an essential factor for bacterial survival in nutrient-starved conditions, such as intracellular conditions.

In conclusion, the results of our study suggest that the fate of intracellular *B. abortus* depends strictly on the presence of elevated levels of free intracellular calcium in infected host cells. In particular, the intracellular calcium level mediated by RGS2 is an essential factor for the intracellular survival of *B. abortus* within macrophages. Further study is needed to clarify the mechanism of L-type calcium channels modulated by RGS2 and the calcium-modulating genes of *B. abortus*.

**Notes**

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